Oncolytic Vaccinia Virus for the Treatment of Peritoneal Carcinomatosis: Combination with Chemotherapy or Targeted Radiotherapy

by

Kathryn Ottolino-Perry

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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Doctorate of Philosophy
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Abstract

Oncolytic viruses (OVs) represent a novel class of cancer biotherapeutics currently under clinical development for treatment of a range of malignancies. OVs are ideal candidates for treatment of peritoneal carcinomatosis (PC) due to their high tumour specificity, excellent safety profile and suitability for peritoneal delivery. OVs are unlikely to be delivered alone but rather as part of a combination therapy regimen. The research presented in this thesis provides two examples of novel combination treatment strategies for improving oncolytic VV treatment of colorectal PC. Delivered as a monotherapy, vvDD efficacy was associated with mice that had well-vascularized and highly proliferative tumours. When combined with first-line colorectal cancer chemotherapy drugs, oxaliplatin and irinotecan (CPT-11), we observed synergistic improvements in in vitro tumour cell death and improved in vivo survival in tumour-bearing mice (with CPT-11). Synergy was attributed to sensitization of cells to drug treatment due to VV-induced S-phase arrest. In addition to combinations with chemotherapy, we examined the use of VV-mediated somatostatin receptor (SSTR) gene transfer using the radiolabeled somatostatin analogue $^{177}$Lu-DOTATOC. vvDD-SSTR led to tumour-specific accumulation of $^{177}$Lu-DOTATOC with minimal effects on normal tissue biodistribution in a xenograft model of colorectal PC. Radiovirotherapy was well tolerated and significantly improved survival over monotherapy. Our findings could have important implications for the design of future VV clinical trials and the treatment of colorectal PC. The proposed mechanism of synergy between vvDD and CPT-11 suggests that this combination therapy could represent a treatment option for patients with chemoresistant disease.
Radiovirotherapy has wider potential applicability; any VV susceptible tumour could be made a target of SSTR-directed therapy. Continued investigation of the mechanisms of interaction between these treatment modalities will provide a better understanding of potential improvements to therapy delivery as well as the clinical setting in which they have the greatest impact.
Acknowledgments

The work contained in this dissertation would not have been possible if not for the effort and support of many people. I would like to begin by thanking my supervisor Dr. Andrea McCart, to whom I am immensely grateful. With your continued support and encouragement I have evolved into a confident scientist and more independent thinker. You provided me an environment in which I felt empowered to develop and pursue my own ideas, knowing that your guidance was available whenever I encountered a hurdle I was not yet able to traverse on my own. To my Committee Members, Dr. Raymond Reilly and Dr. Jeffrey Medin, I thank you for always pushing me to think more critically about my research and providing invaluable feedback at critical stages throughout my studies. I wish also to acknowledge my many collaborators and colleagues who contributed their time and expertise generously in support of my research and professional development. To Dr. Ralph DaCosta, I thank you for the use of your PRODIGI™ imaging system (developed by Drs. RS DaCosta, BC Wilson and K Zhang, Ontario Cancer Institute), it was exciting to be a part of some of the first imaging sessions with this innovative device. Furthermore, I will be forever grateful to you for our numerous chats about science and life in general that often extended well beyond the anticipated ‘just a couple minutes of your time’. Your enthusiasm for your work and your endless curiosity is contagious and I always left your office inspired to continue searching for the answers to whatever question I was tackling. I would also like to thank Dr. Mihaela Ginj at the University Health Network and Deborah Scollard at STTARR for their guidance and technical assistance in performing all the radiotherapy experiments. More thanks go to Dr. Hampson and all the faculty and trainees that participated in the University of Toronto CIHR Training Programme in Biotherapeutics. I believe strongly in the mission of the programme and was grateful to have been able to contribute to it. Lastly, I am grateful to the many graduate students, lab technicians and summer students who have helped me along the way, including: Nan Tang, Dr. Fernando Angarita, Dr. Sergio Açuna, Dr. Siham Zerhouni, Calvin Ng, Besmira Cako and Clara Sellers. You made the lab a place I looked forward to coming into (almost) everyday.

I would also like to briefly thank the many wonderful teachers throughout my life who saw in me a curious mind with a love for math and science. As early as Ms. Fonck’s grade 1-3 class at Ossington Old Orchard Public School I declared I wanted to be scientist and cure AIDS
(or be a ballerina) – replace AIDs with cancer and I can say I am in the ballpark of achieving my childhood dream. For stimulating my scientific interests and always pushing me to excel I would like to thank Ms. Fonck, Ms. Midanick, Ms. Tseng, Mr. Payne and Mr. Kobashi As well, to Dr. Matlashewski, my 4th year undergraduate research project supervisor and Dr. Coulton at McGill University, thank you for making my first experiences in a research lab so positive.

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<tr>
<td>5-FU</td>
<td>5-fluouracil</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>ADP</td>
<td>adenovirus death protein</td>
</tr>
<tr>
<td>AE</td>
<td>adverse event</td>
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<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AML</td>
<td>acute myelocytic leukemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>APC</td>
<td>- allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>- antigen-presenting cell</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Att</td>
<td>attenuated</td>
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<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BB2</td>
<td>bombesin receptor 2</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca</td>
<td>cancer</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackievirus and adenovirus receptor</td>
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<tr>
<td>CA-125</td>
<td>carcinoma antigen-125</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CD</td>
<td>cytosine deaminase</td>
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<td>CDC</td>
<td>complement-dependent cytotoxicity</td>
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<tr>
<td>CES</td>
<td>carboxylesterase</td>
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<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
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<tr>
<td>CEV</td>
<td>cell associated-enveloped virion</td>
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<tr>
<td>CGAS</td>
<td>cGAMP synthase</td>
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<td>cGAMP</td>
<td>cyclic guanosine monophosphate-adenosine monophosphate</td>
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<td>CI</td>
<td>combination index</td>
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<td>CIK</td>
<td>cytokine-induced killer</td>
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<td>CPA</td>
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<td>CPK</td>
<td>creatine phosphokinase</td>
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<td>CPT-11</td>
<td>irinotecan</td>
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<tr>
<td>CR</td>
<td>complete response</td>
</tr>
<tr>
<td>CRAd</td>
<td>conditionally replicative adenovirus</td>
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<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CSC</td>
<td>cancer stem cell</td>
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<tr>
<td>CSF-1</td>
<td>colony stimulating factor 1</td>
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CT – computed tomography
CTL – cytotoxic T lymphocytes
CTL – cytotoxic T-cell
Cx – connexin
CYP3A – cytochrome P450A
d - day
DACH – diaminocyclohexane
DAMP – danger associated molecular pattern
DC – dendritic cell
DLT – dose limiting toxicity
DMEM – Dulbecco’s modified Eagles medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DOD – dead of disease
DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTATATE – [DOTA\(^0\),Tyr\(^3\)]octreotate
DOTATOC - [DOTA\(^0\),Tyr\(^3\)]octreotide
DRD2 – dopamine receptor 2
DSB – double strand break
dsDNA – double strand deoxyribonucleic acid
dTMP – deoxythymidine monophosphate
DTPA – diethylene triamine pentaacetic acid
DTR – diphtheria toxin receptor
EBRT – external beam radiation therapy
ECM – extracellular matrix
EEV – extracellular enveloped virion
EGF – epidermal growth factor
EGFP – enhanced green fluorescent protein
EGFR – epidermal growth factor receptor
EMT – epithelial-mesenchymal transition
ERK – extracellular-signal-regulated kinase
FBS – fetal bovine serum
FDA – Food and Drug Administration
FGF – fibroblast growth factor
FGFR – fibroblast growth factor receptor
FOLFIRI – Folinic acid (leucovorin), 5-FU, irinotecan
FOLFOX – Folinic acid (leucovorin), 5-FU, oxaliplatin
G3BP – ras-GTPase activating protein SH3 domain binding protein
GADD34 – growth arrest and DNA damage-inducible protein 34
GAG – glycosaminoglycan
GDEPT – gene-directed enzyme prodrug therapy
GEP – gastroenteropancreatic
(e)GFP – (enhanced) green fluorescent protein
GGT – gamma-glutamyl transferase
GI – gastrointestinal
GM-CSF – granulocyte macrophage- colony stimulating factor
GPCR – G-protein coupled receptor
GRP – gastrin releasing peptide
GRPR – gastrin releasing peptide receptor
GTP – guanosine-5’-triphosphate
h – hour(s)
H&E – hematoxylin and eosin
HBSS – Hank’s buffered saline solution
HCC – hepatocellular carcinoma
hCES – human carboxyesterase
HPLC-ICP-MS – high-performance liquid chromatography with inductively coupled plasma mass spectrometry
HDR – high dose rate
HIPEC – hyperthermic intraperitoneal chemotherapy
HMBG1 – high-mobility group protein 1
HNSCC – head and neck squamous cell carcinoma
hpi – hours post-infection
HRP – horseradish peroxidase
HS – heparin sulfate
HSV – herpes simplex virus
hTERT – human telomerase reverse transcriptase
IC – intracranial
ICAM-1 – intercellular adhesion molecule 1
iCES – intestinal carboxylesterase
ID – injected dose
IEV – intracellular enveloped virus
IFN – interferon
IFNR – interferon receptor
IHC – immunohistochemistry
IL – interleukin
IMV – intracellular mature virion
iNOS – inducible nitric oxide synthase
INR – international normalized ratio
Int – intermediate
IP - intraperitoneal
IP-10 – interferon-gamma-inducible protein 10 (aka CXCL10)
IPEC – intraperitoneal chemotherapy
IRF – interferon response factor
IT - intratumoural
IV – intravenous
LDH – lactate dehydrogenase
LDR – low dose rate
LET – linear energy transfer
LV- leocovorin
M-CSF – macrophage colony-stimulating factor
mAB – monoclonal antibody
MAP - methotrexate, cisplatin and doxorubicin
MAPK – mitogen-activated protein kinase
mCHOI – modified CHOI
mCRC – metastatic colorectal cancer
MDS – multiply damages site
MDS – myelodysplastic syndrome
MHC – major histocompatibiliy complex
mHED – m-hyrdroxyephedrine
MIBG – metaiodobenylguanidine
MIG – monokine induced by gamma interferon (aka CXCL9)
MMC – mitomycin C
MMP – matrix metalloproteinase
MMR – mismatch repair
mo – month
MOA - mechanism of action
MOI – multiplicity of infection
MR – minor response
MRD – minimal residual disease
MRI – magnetic resonace imaging
mRNA – messenger ribonucleic acid
MTD – maximum tolerated dose
mTOR – mammalian target of rapamycin
MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MUC1 – mucin 1
MV – measles virus
MVA – modified vaccinia virus Ankara
MYXV – myxoma virus
NAT – norepinephrine transporter (a.k.a NET)
NDV – Newcastle disease virus
Neg – negative
NET – neuroendocrine tumour
NIS – sodium iodide symporter
NK – natural killer
NOD/SCID – nonobese diabetic/severe combined immunodeficient
OATP – organic anion-transporting polypeptide
ORF – open reading frame
ORR – overall response rate
OS – overall survival
OV – oncolytic virus
OX - oxaliplatin
PAMP – pathogen-associated molecular pattern
PBMC – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PC – peritoneal carcinomatosis
PD – progressive disease
PEG – polyethylene glycol
PET – positron emission tomography
PF4 – platelet factor 4
PFS – progression free survival
pfu – plaque forming units
PI3K – phosphoinositide -3-kinase
PRR – pathogen recognition receptors
PRRT – peptide receptor radiotherapy
PSA – prostate specific antigen
RCC – renal cell carcinoma
(m)RECIST – (modified) Response Evaluation Criteria in Solid Tumours
RFP – red fluorescent protein
RIT – radioimmunotherapy
RNA – ribonucleic acid
RP – radiopetide
RT – radiotherapy
RV – revovirus
SC – subcutaneous
SD – stable disease
SD – standard deviation
SEC – smallpox eradication
SEM – standard error of the mean
SGOT – serum glutamic oxaloacetic transaminase
SPECT – single photon emission computed tomography
SPGT – serum glutamic pyruvic transaminase
SRS – somatostatin receptor scintigraphy
SS – somatostatin
SSB – single strand break
SSTR – somatostatin receptor
STING – stimulator of interferon genes
SWOG – Southwestern Oncology Group
TAM – tumour associated macrophages
TGF – transforming growth factor
TK – thymidine kinase
TLR – toll-like receptor
TNF – tumour necrosis factor
TOP-1 – topoisomerase-1
TOPcc – topoisomerase cleavable complex
TRAIL – tumour necrosis factor-related apoptosis inducing ligand
TTP – time to progression
UDP – uridine diphosphate
UGT – uridine diphosphate glucuronosyltransferase
UK – United Kingdom
USA – United States of America
VCAM-1 – vascular cell adhesion protein 1
VDI – vessel distribution index
VEGF – vascular endothelial growth factor
VEGFR – vascular endothelial growth factor receptor
VGF – vaccinia growth factor
VSV – vesicular stomatitis virus
VT – virotherapy
VV – vaccinia virus
VV-Cop – vaccinia virus Copenhagen strain
vvDD – double-deleted vaccinia virus
vWF – von Willbrand factor
WHO – World Health Organization
wk – week
WR – Western Reserve
WT – wild-type
y – year
YFP – yellow fluorescent protein
Chapter 1  Introduction

1.1  Colorectal Cancer

1.1.1  Overview

Colorectal cancer (CRC) is the third-leading cause of cancer-related deaths in Canada (1) and the fourth worldwide (2). Significant efforts to improve the quality of screening as well as early interventions have reduced the incidence and risk of death due to CRC (3). Unfortunately, declines in the mortality rates have been modest (1) and the disease still represents a significant clinical challenge.

CRC is the third-most common cancer among both men and women, with incidence and mortality rates being slightly higher in men (1, 4). The median age at diagnosis is approximately 70 years and the risk of developing the disease increases significantly after age 50 (5). For the majority of CRC cases the exact etiology is unknown; there is, however, a strong environmental component to disease development, largely related to differences in diet (6). CRC incidence is significantly higher in Western industrialized countries and individuals who migrate into these countries take on the higher risk of developing the disease (7). Several gastrointestinal (GI) inflammatory diseases as well as genetic disorders are also associated with increased risk of developing CRC (5), although together they account for only a small fraction of total CRC cases.

Sporadic CRC (unknown etiology) occurs as a result of somatic mutations that accumulate throughout an individual’s lifetime. Colorectal carcinogenesis is a multistep process that requires loss of function in multiple genes. Normal colonic epithelium progresses through several stages prior to development of adenocarcinoma. The phenotypic progression begins with hyperproliferation of the colonic epithelium followed by the development of adenoma (early, intermediate and late) and finally carcinoma and metastatic spread (Figure 1.1).

The exact genetic alterations associated with each transition are not fully understood and vary within the patient population. However, studies have determined when certain mutations are likely to occur. Inactivating mutations of the gene encoding adenomatous polyposis coli (APC) is the single most common genetic alteration in CRC and likely represents the initiating event in the carcinogenesis pathway (8). APC is a component of the Wnt signaling pathways, the
activation of which is implicated as an initiating event in carcinogenesis (9). Extracellular Wnt ligands activate the Frizzled membrane receptor leading to downstream accumulation of the transcription factor β-catenin, which is responsible for activation of variety of target genes including several proto-oncogenes and genes associated with CRC metastasis (10). APC is a critical component of the β-catenin degradation complex, which regulates β-catenin-mediated transcription. Inactivating mutations of APC lead to constitutive activation of β-catenin target genes and adenoma formation. Mutations in ras oncogenes are reported in approximately 50% of carcinomas and adenomas of a size >1 cm but in only 9% of adenomas <1 cm (11) suggesting they play a role in the early to late adenoma transition. Other common mutations occurring later in disease progression include inactivation of the tumour suppressor gene p53 and transforming growth factor (TGF)-β signaling pathways, which are associated with development of high-grade dysplasia and carcinoma (12). Overall, many of these mutations are driven by loss of chromosomal stability and alterations resulting from DNA-repair mechanisms.

**Figure 1.1. Colorectal cancer disease progression.** The above graphic depicts the genetic events that occur in the development of CRC from normal epithelium to invasive adenocarcinoma. CIN, chromosomal instability; MMR, mismatch repair. Adapted from (13).

Surgical resection of the primary tumour with or without adjuvant chemotherapy results in 5-year (y) survival rates between 70-90%, if detected prior to metastatic spread; unfortunately only 34% of cases are diagnosed at this stage (14). After invasion of the primary tumour into the
submucosa and muscularis propria, CRC cells can further metastasize locally leading to peritoneal carcinomatosis (discussed below) or hematogenously to distant sites. Current reports from The Canadian Cancer Society indicate the 5-y survival rates for patients with stage III and IV disease are 44-80% and 8%, respectively (15). The greatest need and room for improvement in medical intervention exists at each end of the disease spectrum. Further efforts are required to improve the quality and uptake of screening programs that increase the rate of early detection. Additionally, there is significant demand for the development of novel treatments to improve outcomes in patients with late-stage disease. The latter represents the primary objective addressed in this dissertation.

1.1.2 Colorectal peritoneal carcinomatosis

1.1.2.1 Disease development and incidence

Peritoneal carcinomatosis (PC) is a late-stage disseminated disease characterized by a heavy tumour burden in the peritoneal cavity. PC, a manifestation of metastatic CRC (mCRC), arises from the non-haematogenous spread of cells from a primary tumour to distant sites on the peritoneal lining of the abdominal and pelvic cavity. In 2012, there were an estimated 23 300 new cases of CRC and 9 200 deaths (14% of all cancers) reported in Canada (1). PC is present in approximately 7% (16) of CRC patients at the time of primary surgery and between 40-80% of patients who die from CRC (17).

PC develops in a subset of CRC patients following a sequence of defined events. Studies have suggested that there may be a genetic component that determines which CRCs are likely to spread into the peritoneum compared to distant sites of metastases (e.g. liver) (18, 19). The initial release of cells from the primary tumour may occur naturally by sloughing or spontaneous perforation following invasion of the primary tumour through the serosa of the colon. Alternatively, tumour cell shedding may also occur inadvertently during surgery due to perforation of the primary tumour or spillage from resected lymph and blood vessels. Following dissemination, tumour cells must adhere to the mesothelium (the serous membrane lining the peritoneal cavity and covering most peritoneal organs) in a process facilitated by the constitutive and activated expression of adhesion molecules, such as intercellular adhesion molecular-1 (ICAM-1) (20) and vascular adhesion molecule-1 (VCAM-1) (21), on mesothelial cells. Following adhesion, tumour cells invade the mesothelial membrane and adhere to the
submesothelial stroma. Expression of proinflammatory cytokines such as epidermal growth factor (EGF), tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6 have been shown to increase mesothelial expression of ICAM-1 (22). This type of proinflammatory microenvironment, which is thought to favour metastatic tumour cell implantation and proliferation, is typical of postoperative wound healing and is a contributing factor to the increased risk of recurrence for patients with residual disease following surgical resection.

1.1.2.2 Clinical presentation and treatment

Early detection of CRC is a major prognostic factor. Unfortunately, if the primary tumour goes undetected it can progress to PC without displaying any overt signs or symptoms. PC is often accompanied by the development of ascites (abnormal accumulation of fluid in the peritoneal cavity) and it is the ascites that is responsible for most of the signs/symptoms of the disease. Patients may present with painful abdominal distention, difficulty breathing, nausea, constipation, loss of appetite, early satiety and extreme fatigue. Imaging by computed tomography (CT) scan and/or ultrasound is performed to visualize any abnormal masses or signs of disease and a biopsy is taken to confirm the tissue is malignant and classify/stage the cancer.

Up until the past few decades, PC was viewed as a terminal end-stage disease and was treated as such. Palliative chemotherapy was provided to alleviate symptoms and improve quality of life; median survival was around 7 months (mo) (16). Significant advances in surgical techniques (23) and the recognition that PC is a locoregional disease that could benefit from locoregional chemotherapy (24) has led to significantly improved treatment options and patient outcomes.

1.1.3 Current treatment of CRC

1.1.3.1 Introduction of advanced surgical techniques

Complete surgical resection remains one of the best prognostic indicators in patients with colorectal PC (17). In 1995, Sugarbaker published a paper outlining a novel surgical technique (complete perinectomy) to achieve maximal cytoreduction and prepare the abdomen for intraoperative chemotherapy (23). Complete surgical resection (no macroscopic residual disease) is associated with a median survival of 17.8 to 39 mo whereas incomplete resection (macroscopic disease > 2.5mm to 5mm in diameter) is associated with a median survival of 5 to
Today, surgical resection can be combined with intraoperative chemotherapy to achieve improved outcomes in CRC patients (25) (discussed in Section 1.1.3.5).

1.1.3.2 Evolution of chemotherapy regimens

For over 40 years, 5-fluorouracil (5-FU) was the only drug available for the treatment of mCRC. 5-FU is a pyrimidine analogue, which exerts its effect through irreversible inhibition of thymidylate synthase and therefore DNA replication. In the 1990s leucovorin (LV, a.k.a folinic acid) was found to improve the efficacy of systemic 5-FU (26) through stabilization of 5-FU/thymidylate synthase binding and was added to the standard of care (27). Publication of two phase II clinical trails (28, 29) in 2000 on the efficacy of irinotecan (CPT-11; discussed in Section 1.1.3.3) in patients with mCRC resulted in its widespread use as a first-line therapy in combination with 5-FU and LV, or as a second-line treatment for patients with 5-FU refractory tumours. In 2004, the USA Food and Drug Administration (FDA) approved oxaliplatin (OX; discussed in 1.1.3.4) for use in combination with 5-FU/LV as an adjuvant to surgical resection or as a first-line treatment in patients with mCRC (30). Since the introduction of CPT-11 and OX, a variety of drug combinations and treatment schedules have been studied with mixed results. In a phase III clinical trial, FOLFOX (OX with bolus and continuous infusion of 5-FU plus LV) was found to significantly improve the overall response rate, time to progression, overall survival and 5-y response rate relative to irinotecan and bolus 5-FU plus LV (IFL) (31, 32). First-line FOLFIRI (CPT-11 with bolus and continuous infusion of 5-FU plus LV) was found to be similar to FOLFOX in overall survival (33). Bevacizumab, a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody (mAb), was approved by the FDA for use in combination with first-line systemic 5-FU in 2004 and shortly thereafter began to be combined with more effective treatment regimens (e.g. FOLFOX and FOLFIRI) with modest results (34, 35). FOLFOXIRI (OX in combination with CPT-11, 5-FU and LV) has also been investigated in a clinical trial, which reported one of the highest median overall survival rates (21.5 mo) although it was not significantly better than FOLFIRI (19.5 mo)(36).

1.1.3.3 Irinotecan

Irinotecan (CPT-11) is a water soluble camptothecin-derived drug that functions as a nuclear topoisomerase-1 (TOP-1) inhibitor. TOP-1 is a critical enzyme required during DNA replication and mRNA transcription. TOP-1 is responsible for relieving torsional stresses in the
unwinding DNA-helix by making single-strand DNA cuts, allowing controlled rotation of the DNA and re-annealing of the nicked strand.

1.1.3.3.1 Drug class, structure and physical properties

Camptothecin-based drugs are classified as plant alkaloid topoisomerase inhibitors. Camptothecin is composed of a 5-ring heterocyclic alkaloid with an α-hydroxylactone in its E-ring. All camptothecin derivatives contain this 5-ring structure, which is reversibly hydrolysed from its active lactone to inactive carboxylate form. In addition to the camptothecin moiety, CPT-11 contains a carboxybispiperidine side group that is hydrolysed by carboxylesterases (CES) to produce SN-38, the drug’s active metabolite. In the 1970s, clinical development of camptothecin was stopped because of its unpredictable toxic side effects, which were later attributed to its relative insolubility (37, 38). CPT-11 has improved aqueous solubility, making it a much safer drug for clinical use.

1.1.3.3.2 Mechanism of action

Unlike most other chemotherapy drugs, CPT-11 is unique in that it has only one known molecular target. Knock-out of TOP1 in yeast cells leads to complete drug-resistance (39). Nevertheless, the downstream effects of CPT-11 binding to TOP1 can vary depending on the status of numerous genes involved in DNA repair pathways.

Given the essential role of TOP1 in DNA metabolism (replication, transcription, recombination and repair), inhibition by CPT-11 can affect all these processes. While CPT-11 may have detrimental effects during mRNA transcription (40), it is the replication-mediated double-strand breaks (DSB) that are thought to be the main mechanism of cancer cell death. During DNA unwinding, TOP1 cleaves dsDNA and forms a covalent bond with the free 3’ DNA end in an energy cofactor independent manner. The TOP1-DNA intermediate is called the ‘cleavable complex’ (TOPcc). After torsional forces have been relaxed, the covalent bond is reversed and TOP1 religates the DNA. CPT-11 binds simultaneously to TOP1 and the DNA of the cleavable complex in a base- and stereo-specific manner. This stabilizes the TOPcc, thereby slowing the process of religation. The interaction between CPT-11 and the TOPcc is reversible and the removal of the drug leads to rapid religation without damage to the DNA. In order for CPT-11 to exert its effect, the drug-bound TOPcc must come into contact with the DNA.
replication or mRNA transcription machinery. Interaction of the replication machinery with the stabilized TOPcc results in misalignment of the nicked DNA with its substrate and the formation of irreversible TOPccs and double-strand (ds)DNA breaks by a process known as ‘replication run-off’ (41). Misalignment due to existing DNA lesions (e.g. base mismatch, base oxidation and DNA breaks) can also prevent the religation step. Given that it is the replication machinery that ultimately leads to the formation of dsDNA breaks, CPT-11 cytotoxicity is cell-cycle-specific and requires cells to be in S-phase (42).

Transcription-associated responses to CPT-11 are less well understood and thought only to become a determinant of drug-induced cytotoxicity at high drug concentrations (43). TOP1 has several transcription-related functions, including: repressing basal transcription initiation by binding to TFIID; relieving DNA-supercoiling during mRNA elongation; and, phosphorylating serine (S)- and arginine (R)-rich SR-proteins which are involved in RNA splicing. Inhibition of TOP1 with camptothecins thus results in an overall inhibition of mRNA elongation. Interestingly, camptothecins appear to affect certain genes differentially, in some cases as a result of a direct role of TOP1 in gene transcription. For example, TOP1 interacts with transcription factor c-Jun and camptothecin inhibition leads to specific downregulation of c-Jun target gene EGFR and decreased cell proliferation (44).

1.1.3.3.3 Pharmacokinetics and toxicity

The recommended dose range of CPT-11 in patients is 50-350 mg/m² administered as a 90 minute (min) infusion. CPT-11 has a mean terminal half-life of approximately 12 h, while that of SN-38 is approximately 21 h (45). SN-38 plasma concentrations peak approximately 1 h after infusion, with drug bound predominately (approximately 95%) to plasma proteins. This binding appears to stabilize approximately 60-70% of the metabolite in its active lactone form (46). CPT-11 exhibits much lower plasma protein binding (approximately 30-68%) compared to SN-38 and is mainly found associated with erythrocytes.

Relative to other camptothecin derivatives, CPT-11 has a more complex pharmacological profile due to its enzymatic conversion into a variety of different metabolites, of which only SN-38 is active (Figure 1.2). Additionally, CPT-11 and SN-38 can exist in both lactone and carboxylate forms, which have different uptake kinetics and activities. The lactone forms of CPT-11 and SN-38 are favoured at lower pH and their uptake into cells occurs by passive
transport while the carboxylate form is taken up by active absorption (47). Metabolic conversion of CPT-11 into SN-38 is performed primarily by CES as well as other esterases found in the plasma, hepatocytes, intestines, lung, kidneys and tumour cells (48-50).

Metabolism of CPT-11 differs in the tissues of humans and other animals, including mice. In humans the CES isozyme hCES-2, which is expressed primarily in the intestines (iCES), is the most efficient CPT-11-activating enzyme (51). hCES-1, however, remains the main mediator of CPT-11 hydrolysis in the liver due to its much higher expression levels relative to hCES-2 (48). In humans, serum conversion of CPT-11 is performed by esterases other than CES, while in mice several esterases including CES are capable of CPT-11 activation (52). Comparison of human and animal CES enzymes has shown that human CES enzymes are 100-1000 times less efficient than those of other species (46). While hepatobiliary metabolism represents the major pathway of CPT-11 conversion and elimination, levels of CES activity in tumour cells also contribute to chemosensitivity.

UDP-glucuronosyl transferase 1A1 (UGT1A1), one of several isozymes derived from the UTG1 gene, is largely responsible for glucuronidation of SN-38 into the water-soluble inactive metabolite SN-38G. This conversion occurs mostly in hepatic and intestinal tissues, though it may also take place within the tumour. Evidence from in vitro tumour cell lines suggests that high UGT activity within the tumour may lead to drug resistance and that this may be controlled through epigenetic modifications. Alternatively, genetic polymorphisms in the UGT1 gene that lead to decreased or absent enzyme function are associated with increased risk of severe CPT-11 induced toxicity, as the drug cannot be sufficiently inactivated. Indeed, several phase I studies have concluded there are significant benefits to genotype-directed dosing of CPT-11 (53). Glucuronidation of SN-38 in the intestines is reversible due to the presence of microflora expression of β-glucuronidase (46). Not surprisingly, histological damage in human colons was found to correlate with β-glucuronidase – but not CES activity – in the intestinal lumen. As a result, antibiotics have been used to decrease gastrointestinal toxicity by decreasing levels of SN-38 in the intestines while not affecting concentrations of CPT-11, SN-38 or SN-38G in the plasma (46).
Figure 1.2. Irinotecan metabolism, activity and toxicity. Irinotecan (CPT-11) is metabolized by several different enzymes in different tissue compartments to form various inactive and active metabolites. CPT-11 and metabolites are transported into and out of cells primarily by ABC family of transporters although hepatocytes use OATP transporters to remove SN-38 from the circulation. UGT, UDP-glucuronosyl transferase; CES, carboxylesterases; Top1, topoisomerase 1. Adapted with permission from (54).

Other detectable metabolites include APC and NPC, which are formed through oxidation of CPT-11 by cytochrome P450A (CYP3A)-4. CYP3A shows heterogenous intra- and inter-patient expression and activity in the livers and intestines of adults (55, 56). As with CPT-11, APC and NPC show very little anti-tumour activity. APC and CPT-11 also act as inhibitors of
acetylcholinesterase, which is thought to be responsible for the development of acute cholinergic syndrome in some patients following CPT-11 therapy (57).

The primary toxicities of CPT-11 therapy, which include gastrointestinal (GI) and hematological adverse events, have been relatively well-characterized and are generally manageable. Diarrhea and neutropenia have been identified as the major dose-limiting toxicities (DLTs) in several clinical trials, including a prospective single-agent phase III trial where up to 65% of patients experienced grade 3/4 adverse events, at least half of which were either diarrhea and/or neutropenia (58). CPT-11-induced diarrhea may occur as early onset (within 24 h) or late onset (> 24 h post-treatment). The acute side effect, which is dose-dependent and related to other cholinergic symptoms, is typically well controlled with atropine. Alternatively, late-onset diarrhea is much more problematic; it is dose-independent and one of the main reasons for treatment course modifications (59). The frequency of severe diarrhea increases in combination chemotherapy regimens that include fluoropyrimidines (e.g. 5-FU). Up to 22% of patients treated with single agent CPT-11 experienced grade 3/4 late-onset diarrhea compared to 28% in patients treated with IFL (60). Late-onset diarrhea should first be managed with loperamide, however further intervention is required if it is persistant. The other most frequently reported GI adverse events following single agent therapy are vomiting, nausea, abdominal pain, constipation and anorexia.

Neutropenia is the main form of myelosuppression attributed to CPT-11 therapy (58). As with diarrhea, neutropenia becomes more frequent when combined with 5-FU (29), particularly when administered as a bolus compared to infusion (61). Severe neutropenia is associated with increased risk of infection and possibly treatment-related deaths due to sepsis, which although extremely rare have been reported (62, 63).

Cessation of chemotherapy is recommended for patients experiencing grade ≥ 2 diarrhea or neutropenia until resolution of symptoms (45). In addition, dose reductions are advised for patients with grade ≥ 3 diarrhea or neutropenia (45). The presentation of either febrile neutropenia, severe neutropenia without fever or persistent diarrhea should be managed with antibiotic treatment to decrease the risk of sepsis.

Recently, consideration of patients’ genotypic profile has been shown to be predictive of therapeutic efficacy and toxicity. Over 20 gene variants across 12 genes have been identified as
affecting the outcome of CPT-11 therapy and as being potentially useful in determining optimal dosing and schedule (64). These genes encode proteins involved in CPT-11 metabolism (eg. CES1/2, CYP3A4, UGT1A1) and transport (eg. ABCB1, ABCG2, ABCC2). Toxicity-associated polymorphisms in the UGT1A1 gene occur at relatively high frequencies in the general population (15-45%). As such, administration of an FDA-approved genetic test for homozygosity in the UGT1A1*28 allele is recommended prior to commencement of CPT-11 therapy.

1.1.3.4 Oxaliplatin

Oxaliplatin (OX) is a third-generation platinum-based compound that was selected for development based on its good water-solubility and activity in cisplatin- and carboplatin-resistant cells (common in CRC). OX has shown activity in a range of tumour types in both preclinical (65) and clinical settings (66) and is currently approved for use in advanced CRC in North America, Europe, Latin America and Asia.

1.1.3.4.1 Drug class, structure and physical properties

Oxaliplatin (oxalate(trans-l,2-diaminocyclohexane)platinum) contains a platinum atom bound to a diaminocyclohexane (DACH) carrier and oxalate leaving group (Figure 1.3). In plasma, non-enzymatic displacement of the leaving group converts OX into a variety of compounds, the majority of which are pharmacologically inactive. Dichloro(DACH)-platinum compounds, the only active derivatives of OX, can exist in 3 isomeric conformations each of which interacts differently with DNA.

1.1.3.4.2 Mechanism of action

Platinum-based drugs exert their effects through the formation of DNA-DNA and DNA-protein crosslinks. Significant data exists describing the mechanism of first generation platinum-based compounds (e.g. cisplatin) and it was generally assumed that OX behaved in much the same manner. Given its activity in cisplatin-resistant cells (65) and evidence showing it forms many fewer DNA lesions yet is generally more cytotoxic than cisplatin (67), it is hypothesized that the large DACH group enhances the lethality of the adducts. OX exerts its cytotoxicity predominantly through induction of DNA damage secondary to the formation of inter- and intra-
Intra-strand adducts between adjacent guanine (G)-G or G-adenosine (A) bases represent the primary DNA lesions while inter-strand crosslinks comprise only a small portion of all adducts formed (68). DNA platination occurs in 2 steps: firstly, the dichloro(DACH)-Pt complexes lose their Cl\(^{-}\) ions and bind to guanine-N(7) to form monoadducts which are generally non-lethal; secondly, biadducts are formed following binding to an adjacent or distant DNA base or cellular protein. If these lesions are not properly repaired they can lead to apoptosis (69). OX and cisplatin do not differ in the site or type of adduct formation (68), however several of the repair pathways involved in recognizing and correcting DNA lesions have been shown to differentiate between cisplatin and OX adducts (reviewed in (70)). Defects in mismatch repair and damage-recognition proteins significantly increase cisplatin resistance but have little effect on OX cytotoxicity (71) suggesting that these pathways are less effective at repairing OX-induced DNA damage. These differences are attributed to the conformational difference in DNA distortion caused by both these agents (72).

![Chemical structure of oxaliplatin.](image)

Although less well-understood and perhaps less influential, several other mechanisms have been suggested to contribute to OX-induced cytotoxicity. OX can significantly decrease thymidylate synthase activity (73) thereby directly impacting DNA synthesis. Cisplatin has been shown to affect transcription by the following mechanisms and it is thought OX may do the same: (1) sequestering transcription factors through binding to DNA-platinum adducts; (2) preventing the movement of RNA polymerases over the bulky adduct; and (3) forming
nucleosome-DNA adducts that prevent chromatin remodeling. Lastly, OX may also be capable of stimulating toll-like receptor 4 (TLR4)-dependent immunogenic cell death in CRC cells (74).

1.1.3.4.3 Pharmacokinetics and toxicity

Investigation of the clinical pharmacokinetic properties of OX has been limited by an incomplete understanding of which biotransformation products are responsible for in vivo cytotoxicity, as well as the complex nature of OX biotransformation (non-enzymatic), which rapidly produces numerous intermediates (reviewed in (75)). Furthermore, prior to the description of high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) techniques for evaluating OX pharmacokinetics (76) the majority of studies relied on analytical techniques which quantified platinum containing products instead of the parent compound (reviewed in (75)). Many of the reactive intermediates bind irreversibly to components of the blood/plasma and cellular macromolecules and become pharmacologically inactive (77). Plasma ultrafiltrate contains the unbound active platinum-species responsible for both OX anti-tumour and toxic properties (77). Due to the speed and transient nature of the biotransformation reactions, most pharmacokinetic studies have examined the levels of platinum in the plasma and blood rather than the parent compound or particular intermediate. Unfortunately, this has resulted in PKs that may not truly reflect the properties of the main cytotoxic compounds. Terminal half-life estimates based on platinum concentrations can vary between 10-400 h (depending on the analytical method used); the only studies looking at intact OX in patients, however, report a terminal half-life between 14-20 min (76, 78, 79). The redistribution ($t_{1/2 \alpha}$) and elimination ($t_{1/2 \beta}$) half-lives of platinum in plasma ultrafiltrate are 0.28 and 16.3 h, respectively (80). The loss of platinum over this period is attributed to distribution of reactive intermediates in tissues as well as renal elimination (80). In contrast, the longer terminal half-life ($t_{1/2 \gamma}$) estimates likely results from slow release of inactive platinum-bound amino acids following degradation of cellular proteins.

In patients treated with a 2-h infusion (the recommended time to achieve maximum area under the curve (AUC)) of 130mg/m² OX once every 3 weeks (wk) for 5 cycles, approximately 65% of platinum is bound to plasma proteins at 2 h after cycle 5 and 98% is bound by 3 wk (80). The plasma platinum AUC$_{0-24}$ is proportional to the dose (20-180 mg/m²) though there is no
significant platinum accumulation in plasma (< 1-fold; final cycle/first cycle) or blood cells (≤ 2-fold; final cycle/first cycle) over multiple cycles.

Platinum is eliminated primarily through the urine (approximately 30-60%) with minimal fecal elimination (approximately 2-4%) (80). There is a significant correlation between the rates of ultrafilterable plasma platinum and renal platinum clearance with the glomerular filtration rate. Kidney function is correlated with platinum elimination, but hepatic function, sex and age have no significant effect on clearance.

Despite its similarity to cisplatin, OX does not display the same nephrotoxicity and ototoxicity commonly associated with the former. Instead, OX primarily affects the hematopoietic, peripheral nervous and GI systems. In early dose-finding phase I trials the major DLT was neurotoxicity. In a randomized study of patients with CRC, reports of grade 3 neurotoxicity (30.9%) and grade 3/4 neutropenia (40%) and thrombocytopenia (4.5%) were significantly higher in patients receiving FOLFOX6 (n=110) than FOLFIRI (n=110) (33). In this same study the incidence of neutopenic fever was low (4%), though there was one OX-related death reported. The mechanism of hematologic toxicity is not clear, but platinum adducts are detected in leukocytes after OX treatment (81) and the number of platinum adducts has been shown to correlate with the severity of leucopenia and thrombocytopenia in patients treated with cisplatin (82).

OX is associated with both acute-reversible neuropathy as well as late-onset persistent neuropathy. Acute sensory neuropathy, which has been reported in approximately 56% of patients treated with FOLFOX, occurs within hours to days of treatment, and is resolved within 14 d (83). The persistent form of neuropathy occurred in the absence of previous acute neuropathy in approximately 48% of patients treated with FOLFOX. Symptoms can be alleviated by cessation of treatment in some patients, though at 18-mo of follow-up 21% of patients continued to experience symptoms (all grades) (83). Some rare but serious (and potentially fatal) side effects of OX therapy include: pulmonary fibrosis (<1%), anaphylactic/anaphylactoid reactions (2-3%) and secondary acute leukemia (case reports) (84-89).
1.1.3.5 Intraperitoneal chemotherapy

In addition to his contribution to surgical oncology, Sugarbaker also transformed the clinical use of chemotherapy for the treatment of colorectal PC. Prior to the introduction of new chemotherapy drugs, Sugarbaker et al. undertook studies to improve the efficacy of 5-FU through intraperitoneal (IP) administration (90). A previous case study had shown the feasibility of hyperthermic peritoneal perfusion and radical cytoreductive surgery in a patient with pseudomyxoma peritonei (91). The rationale behind loco-regional infusion of 5-FU was two-fold: higher doses were better tolerated when delivered loco-regionally compared to intravenously (IV)(90) and the hematological toxicity of IP 5-FU was significantly decreased (92). The application of heating (40-44°C) to intraperitoneal chemotherapy (IPEC) further improved drug cytotoxicity and tissue penetration (by increasing cell membrane permeability) (93, 94). The use of hyperthermic IP chemotherapy (HIPEC), delivered early postoperative via a catheter placed in the abdomen during surgery, significantly improved survival compared to systemic chemotherapy and palliative surgery in patients with colorectal PC (95).

Clinical studies of cytoreductive surgery and IPEC (with and without hyperthermia) have included intraoperative and/or early postoperative administration; given the considerable variability in study design (e.g. inclusion/exclusion criteria, cytostatic agents used, randomization, etc.) it remains unclear whether intraoperative chemotherapy offers any significant advantage. Patients who receive (H)IPEC with cytoreductive surgery also receive systemic chemotherapy. To increase the likelihood of response and mitigate the risk of chemoresistance, different agents are used for each. Since systemic therapy typically involves 5-FU/LV, and more recently OX or CPT-11, (H)IPEC has primarily been performed with mitomycin C (MMC) (25), an older chemotherapy drug that shows activity in CRC. More recent studies have begun to use either OX or CPT-11 as the (H)IPEC agent. Overall, cytoreductive surgery with IPEC has resulted in median survival rates of 12-34 mo, however it has also been associated with serious complications (14-55%) and mortality (0-19%) (17). In the only randomized controlled study performed to date, 105 patients were stratified to receive either best supportive chemotherapy with or without palliative surgery or complete surgical resection with HIPEC (MMC) and systemic chemotherapy (as in control arm) (95). In the published 8-y follow-up, the median disease-specific survival was 12.5 mo in the control arm and 22.2 mo in the complete resection/HIPEC arm (p = 0.028) (96). When data was stratified for completeness of
resection, those that received a complete resection (41% of experimental arm) had a median survival of 48 mo. It must be noted, though, that the experimental arm was associated with significant toxicity including 4 treatment-related deaths.

1.1.4 Defining the clinical need

Until recently, clinical perception of PC was that it was a preterminal disease for which only palliative treatment was appropriate. Improved surgical techniques as well as novel chemotherapy drugs and delivery methods have drastically altered the way treatment is approached in patients with PC. Today, patients with peritoneally-disseminated disease have the option to undergo potentially life-prolonging aggressive treatment; this is contingent, however, upon their fitting restrictive eligibility criteria and having access to a treatment centre equipped to deliver this advanced treatment. Eligibility criteria vary between centres, and are evolving alongside mounting clinical experience and a better understanding of which tumour and patient characteristics are associated with better outcomes. One retrospective study estimated that approximately 20% of patients with colorectal PC would likely derive benefit from cytoreductive surgery with IPEC (16). For patients undergoing aggressive treatment, median survival ranges from approximately 13-60 mo while for those excluded from aggressive treatment the median survival is approximately 10 mo. There is therefore a clear and salient need for novel therapies with proven activity in CRC that can be used safely in patients at any stage of peritoneal dissemination. Ideally, such novel agents could be delivered perioperatively to reduce tumour burden and (1) convert non-surgical candidates into surgical candidates and/or (2) increase the potential for a successful complete resection. Additionally, benefit could theoretically be achieved through the use of novel agents as adjuvants to cytoreductive surgery for the treatment of minimal residual disease (MRD). In this way, such agents would have the potential to improve outcomes in patients with colorectal PC regardless of their eligibility for current aggressive treatment regimens.

1.2 Oncolytic viruses

Chemo- and radiation therapy form the core of non-surgical first-line treatments for a majority of cancers. These therapies are largely non-specific, with low therapeutic indices and pronounced deleterious side effects. Oncolytic virotherapy (VT) harnesses the cytotoxic
properties of viruses to specifically target and kill tumour cells while causing minimal toxicity in normal cells. Oncolytic viruses (OVs) come from a diverse group of virus genera including human and nonhuman vectors with both RNA and DNA genomes. Many OVs show robust replication in rapidly dividing cancer cells and exhibit high therapeutic indices, due to their intrinsic and genetically engineered tumour specificity. Whereas some OVs are naturally oncotropic (e.g. Newcastle disease virus, reovirus and parvovirus), others require genetic attenuation in order to improve their tumour selectivity (e.g. adenovirus, herpesvirus, vaccinia virus, measles virus and vesicular stomatitis virus). Viruses are dependent on host cells in order to replicate their genomes and produce new viral progeny. Numerous host cell characteristics – including receptor expression, proliferation rate, antiviral defenses and response to cell death signals – determine host permissivity to virus infection. Malignant cells typically show deregulation in some or all of these features thereby making them excellent hosts for virus replication and oncolysis. This section will provide a historical overview and summary of the current state of OV therapy with a focus on oncolytic vaccinia virus.

1.2.1 Discovery and development

The notion of using viruses to treat cancer stemmed from observations first published over a century ago. Clinical case reports began to accumulate describing various incidents of cancer patients undergoing remission following a viral infection (97-100) or vaccination (101). It wasn’t until the 1950s, however, that reliable cell and tissue culture techniques emerged and formal investigations into the potential of viruses as cancer therapies began in earnest. Viruses were selected based both on their oncolytic potency as well as low pathogenicity in humans. Early clinical studies using virus-containing sera were promising (102, 103), but by the late 1970s/early 1980s interest waned due to complications in immunosuppressed individuals and the absence of molecular techniques required to genetically manipulate the vectors (104). In the past several decades, advances in molecular techniques, genomics, proteomics and a better understanding of virus and tumour biology have fueled renewed interest in the development of OVs. Preclinical investigation of genetically-engineered tumour-selective second- and third-generation OVs has proven that they can be potent yet safe anti-tumour agents in a range of cancers. Aided by the expansion and refinement of good manufacturing practices, OVs are increasingly being moved into clinical trials with several having even entered phase III to date (105) (discussed in 1.2.6).
1.2.2 Mechanisms of tumour specificity

1.2.2.1 Intrinsic tumour specificity of OVs

Many OVs take advantage of the many deregulated pathways commonly found in transformed cells. Aberrant expression of cell surface viral receptors as well as defects in cell-cycle control and cellular immune responses (e.g. interferon response pathways) make tumour cells the ideal hosts for OV infection and replication. All first-generation OVs display some degree of natural anti-tumour activity and tumour-specificity, and genetic manipulation of the vectors has led to the development of second-generation OVs with markedly improved specificity and efficacy.

For some viruses, entry is dependent on specific and unique cellular receptors. Tumours with upregulated expression of a specific cell-surface protein are excellent targets for OVs that rely on that protein for viral entry. For example, upregulation of CD46, the predominate viral-entry receptor for Edmonston strain measles virus (MV) (106, 107) on the surface of a range of cancer cell types (108) enables this virus to target a variety of tumours (109).

Unregulated progression through the cell-cycle is a hallmark of cancer. Defects in cell-cycle checkpoint proteins as well as increased mitogenic signaling lead to rapid proliferation of tumour cells and aberrant upregulation of cellular DNA replication, transcription and translation machinery. This serves as the perfect environment for active virus replication. The prototypic tumour suppressor protein, p53, plays a critical role in cell-cycle regulation by mediating arrest or apoptosis through interactions with other cell-cycle proteins. Loss-of-function mutations in p53 are common in many cancer types including approximately 50% of CRCs (110). This discrepancy between normal and tumour cell p53 status has been shown to be a natural determinant of OV cytotoxicity (111) as well as a means to improve the tumour tropism of OVs (112). Cell-cycle-dependent proteins have also been shown to play a direct role in virus replication. For example, ras-GTPase activating protein SH3 domain binding protein (G3BP1), which peaks during S-phase, is involved in cell-cycle regulation and is upregulated in many cancers (113). This is a critical component in the transcription of intermediate VV genes (114). Aberrant activation of the Ras pathway, which is common to many cancer types (115), is also the
main determinant of reovirus (RV) (116, 117) and Newcastle disease virus (NDV) permissivity (118).

Many tumours also have defects in one or more components of critical cellular immune signaling pathways. Activation of type 1 interferon (IFN \(\alpha/\beta\)) responses is one of the primary mechanisms cells have to defend against viral infection. Defects in components of IFN signaling pathways are common in colorectal tumours (119), thus increasing their susceptibility to infection relative to normal cells. NDV (120) and VSV (121, 122) are both inherently IFN-sensitive and have been shown to discriminate in infect and kill tumour cells based on the cells’ lack of IFN-responsiveness.

1.2.2.2 Engineered tumour specificity in OVs

Building upon their natural tumour tropism, investigators have applied an increased understanding of cancer biology, virology, and recombinant technologies to create second-generation OVs with improved specificity and efficacy. Several strategies have been employed to enhance the specificity of OVs including: (1) deleting viral genes necessary for replication in normal cells but redundant in tumour cells; (2) placing essential virus genes under the control of tumour-specific promoters; (3) modifying viral entry proteins to target tumour-specific receptors; and (4) deleting immune-blocking viral proteins. Depending on the characteristics of a particular OV, multiple strategies may be used.

The first strategy can be applied to almost any OV, assuming the virus is complex enough to encode redundant genes. The large double-stranded DNA viruses including VV (discussed in Section 1.2.4), herpes simplex virus (HSV) and adenovirus (Ad) have all been modified using this strategy (123-125). HSV is commonly deleted of its \(\gamma_1 34.5\) and \(ICP6\) (ribonuclease reductase) genes, which decrease its neurotoxicity and limit replication in normal cells with low ribonuclease reductase activity, respectively (124). Ad vectors are commonly deleted of the E1B 55KDa fragment, which is thought to either restrict virus replication to cells with defective p53 (125) or to those that can complement the mRNA export function performed by E1B (126).

Creating a transcriptionally-regulated virus is only feasible with viruses that replicate in the nucleus and in cancers associated with abnormal activation of a specific promoter. Conditionally replicating Ads are an excellent example of this strategy. The viral E1A gene,
which is required for virus replication, has been put under the control of various tumour-specific promoters including those for hTERT (human telomerase), PSA (prostate-specific antigen), α-fetoprotein, CEA (carcinoembryonic antigen) and MUC1 (127). Recently, a dual-regulated HSV was generated with both transcriptional and translational-regulation of essential viral genes (128).

The third method, virus retargeting, is only possible in viruses with known and unique cellular and viral entry receptors. This strategy has been particularly useful for improving specificity of MVs (129), Ads (reviewed in (130)) and VSVs (131). MV has been successfully retargeted towards receptors other than CD46, such as CD20 (132) and CD38 (133), through insertion of specific antibody-encoding genes and disruption of the viral protein involved in binding of the natural entry receptor (132). Ad fiber knob modifications have also been performed to successfully retarget the virus to specific tumour receptors (134-143). Ad entry is dependent on interactions between the viral fiber knob and cellular coxsackie-adenovirus receptor (CAR), which is widely expressed on normal cells and heterogeneously expressed in primary human tumours (130, 136, 143). Modification of the fiber knob using one of a variety of strategies (130, 138, 141, 144) is necessary to create a virus with sufficient tumour-specificity. Viruses, such as VV, without a known cellular receptor and multiple viral proteins involved in entry are not amenable to this type of tumour targeting. Virus entry retargeting has also been investigated for HSV (145-147). Unlike Ad and MV, which require only 2 viral proteins to mediate entry, HSV entry is more complex and involves the interactions of 5 viral glycoproteins (148) with a variety of cellular receptors (149-153). As a result, most retargeting efforts for HSV and other viruses with complicated or undefined entry mechanisms, such as VV, have focused on events in the virus lifecycle downstream of entry.

OVs are naturally sensitive to antiviral immune response; this sensitivity contributes to their natural tumour-specificity. Deletion of viral genes encoding proteins designed to inhibit cellular anti-viral immune responses can improve the therapeutic index of an OV by promoting its clearance from normal cells with functional anti-viral immune responses. Deletion of VV B18R, which is responsible for binding and sequestering type-1 IFNs, has been shown to decrease infection of normal cells in the presence of IFN-β but has no effect on replication in IFN-unresponsive tumour cells (154). The effects of this strategy can range from subtle for complex poxviruses such as VV and myxoma virus (MYXV), which encode a plethora of
immune evasion proteins (155, 156), to drastic for simple viruses such as VSV, which becomes exquisitely sensitive to IFN upon mutation of the M-protein (122).

1.2.3 Mechanism(s) of action

At the onset of OV research, the anti-tumour efficacy was attributed primarily to the lytic properties of the viruses. OVs have rapidly lytic lifecycles that lead to the accumulation of viral progeny within infected host cells. The exact mechanism of cell death and progeny release varies between viruses. Several OVs, such as MV and NDV, express fusogenic proteins that lead to the formation of large multi-nucleated syncytium. This allows for efficient cell-to-cell spread and eventual cell death due to apoptosis (157, 158). Ad encodes the adenovirus death protein (ADP), which is required for cell lysis, late in its lifecycle (159). Other OVs, such as γ134.5-deleted HSV recombinants, induce both apoptotic and non-apoptotic tumour cell death, depending on the host cell type (160). Alternatively, VV encodes numerous anti-apoptotic proteins, which prevent infected cells from dying until the end of the virus lifecycle. It is generally thought that cells lyse secondary to cytoplasmic accumulation of thousands of viral progeny, however VV has also been shown to induce early apoptotic cell death in melanoma cells (161) and macrophages (162). A more recent study suggests that VV kills through a programmed necrotic pathway in some ovarian cancer cell lines (163). It is likely that large complex viruses such as VV and HSV are capable of inducing a variety of different cell death pathways and that the dominate mechanism will vary between cell type and virus backbone. Whether the primary mechanism of virus-induced cell death is apoptosis, autophagy or lysis, the reality is that in vivo anti-tumour mechanisms of action are far more complex. OVs do replicate in and kill in vivo tumour cells in the aforementioned manners, however additional mechanisms, including anti-tumour and anti-virus immune responses (discussed in 1.2.4.5.1) and vascular disruption (discussed in 1.2.4.5.2) have increasingly been identified as critical to the overall efficacy of oncolytic VT.

1.2.4 Vaccinia virus

Vaccinia virus (VV) is a linear dsDNA virus belonging to the Orthopoxvirus genus in the Poxviridae family. The approximately 190 kb genome, encoding ~ 250 genes, is packaged inside an enveloped brick-shaped virion. Scientific interest in VV can be traced back to Edward Jenner, a renowned physician who in 1776 performed one of the first successful vaccinations on a young
boy using material isolated from cowpox lesions on the hands of milkmaids (164). For over a century the material used in this original vaccine was passaged repeatedly until, in the 1930s, it was discovered that the virus no longer resembled the original cowpox virus from which it was thought to be derived. The new virus species, whose precise origins are unclear, was called vaccinia virus and subsequently adopted as the preferred choice for smallpox vaccination. Today, there exist several VV strains in development as OVs, each with different levels of attenuation and virulence in humans and other mammals (Table 1.1).

1.2.4.1 VV biology and lifecycle

The VV lifecycle can be broken down into the following steps: entry, early mRNA transcription, uncoating, DNA replication, intermediate/late mRNA transcription, translation, assembly, DNA packaging, maturation, Golgi wrapping and virion egress (Figure 1.4). The lifecycle takes place entirely within the cytoplasm, which is made possible by the encoding of viral DNA and RNA polymerases. VV exists in 2 infectious forms: the intracellular mature virion (IMV) and extracellular enveloped virion (EEV). The IMV consists of the virus core (DNA genome, RNA polymerase, capping, methylation and polyadenylation enzymes) surrounded by a lipoprotein shell and is a relatively stable particle adept at transmission between hosts. The EEV is comprised of the IMV surrounded by an additional cell-derived lipoprotein envelope. Prior to entry, EEVs shed the outermost envelop in a non-fusogenic event that involves 2 viral glycoproteins (A34 and B5) and cell surface glycosaminoglycans (GAGs) (165). The released IMV then binds to the cell surface GAGs and enters the cell through fusion of the cell and virus membrane either at the cell surface or within the endosome (166, 167). Viral RNA-polymerase and transcription factors delivered in the virion core direct early mRNA transcription, which occurs prior to DNA replication (within approximately 20 min). Early mRNA transcripts make up approximately half of the viral genome and encode for proteins involved in DNA replication, nucleotide biosynthesis, intermediate viral gene expression and host interactions. Following completion of viral uncoating, viral DNA replication commences. By this point (approximately 2 h after entry) there is widespread shutdown of host-cell DNA, RNA and protein synthesis (168). Intermediate transcripts encode transcription factors for late genes and late transcripts encode structural proteins and enzymes that are packaged into the virion. Assembled IMVs either remain inside the cell until release following cell lysis (approximately 24 h post infection) or are transported by microtubules to the trans-Golgi
network where they are wrapped in a double layer membrane. These intracellular enveloped viruses (IEVs) represent only a small fraction of the particles produced within a cell. IEVs are transport via microtubules to the cell surface and egress from the cell via membrane fusion. A portion of egressing IEVs remain attached to the cell membrane to become cell-associated enveloped virions (CEV) while the remaining will fully detach to become EEVs. Laboratory manufactured VV is predominately comprised of the IMV form, as the isolation process destroys the membrane of the more delicate EEV.

**Table 1.1. Vaccinia virus strains currently under investigation as oncolytic agents.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent Virus</th>
<th>Origins</th>
<th>Att (Y/N)</th>
<th>Virulence</th>
<th>Immuno-genicity</th>
<th>Prototypic OV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lister</td>
<td>n/a</td>
<td>Calf lymph vaccine developed in UK during WHO SEC</td>
<td>N</td>
<td>++</td>
<td>++</td>
<td>GLV-1h68</td>
</tr>
<tr>
<td>Dryvax (Wyeth)</td>
<td>New York City Board of Health Vaccinia</td>
<td>Calf lymph vaccine used in USA for SEC until 2008 when replaced by ACAM2000</td>
<td>N</td>
<td>+</td>
<td>++</td>
<td>JX-594</td>
</tr>
<tr>
<td>Western Reserve (WR)</td>
<td>Wyeth</td>
<td>Laboratory strain derived from serial passage in mouse brain</td>
<td>N</td>
<td>+++ (mouse)</td>
<td>+++</td>
<td>vvDD</td>
</tr>
<tr>
<td>Copenhagen</td>
<td>n/a</td>
<td>Used to create NYVAC</td>
<td>N</td>
<td>++</td>
<td>+++</td>
<td>VV-Cop</td>
</tr>
<tr>
<td>Modified Vaccinia Ankara</td>
<td>VV Ankara</td>
<td>Created from 100s of passages in chicken embryo fibroblasts</td>
<td>Y</td>
<td>+</td>
<td>+++</td>
<td>MVA</td>
</tr>
<tr>
<td>NYVAC</td>
<td>Copenhagen</td>
<td>Derived from Copenhagen strain with 18 ORFs deleted</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>NYVAC</td>
</tr>
</tbody>
</table>

Abbreviations: Att, attenuated; ORFs, open reading frames; WHO, World Health Organization; SEC, Smallpox eradication campaign. Adapted from (169-171).
Figure 1.4 Vaccinia virus lifecycle. Viron entry occurs within approximately 20 minutes of initial infection (based on cell culture). Early mRNA transcription occurs prior to uncoating and DNA replication (approximately 1-2 h post-infection). Intermediate and late mRNA transcription occurs in peri-nuclear regions called viral factories. Virus assembly leads to the formation of immature virions, which undergo maturation to form intracellular mature virions. Virions are wrapped in a double membrane and begin egressing from the cell approximately 6 h post-infection. Cell lysis occurs within approximately 24 h post-infection. Abbreviations: CEV, cell-associated enveloped virion; EEV, extracellular enveloped virion; h, hour; IEV, intracellular enveloped virion; IMV, intracellular mature virion; IV, intracellular virion; mins, minutes; TGN, trans-Golgi network. Adapted from (168).

1.2.4.2 VV advantages and disadvantages

VV has many features that make it an ideal oncolytic agent. Firstly, VV has a broad host range lending to its utility in numerous preclinical animal models and application in humans. Secondly, techniques for easy manipulation of its DNA genome have been described in detail (reviewed in (172)) and strong naturally-occurring or synthetic viral promoters that drive high levels of transgene (therapeutic or marker) expression are available. Thirdly, VV has a strictly
cytoplasmic lifecycle yielding a limited risk of chromosomal integration. Fourthly, VV shows a strong natural tumour tropism in vivo, likely related to the large particle size that can only extravasate in tissues with leaky vasculature, as is characteristic of tumours (173). Fifthly, VV spreads efficiently from cell-to-cell (174) facilitating dissemination within a tumour. Lastly, VV’s safety profile in humans (summarized in Table 1.2) has been well established due to its long history of use as the smallpox vaccine (175).

### Table 1.2 Adverse events associated with smallpox vaccination.

<table>
<thead>
<tr>
<th>Common acute reactions</th>
<th>Common systemic reactions</th>
<th>Complications (approx. incidence per million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion at injection site; local edema; swelling of regional lymph nodes; satellite lesions</td>
<td>Fever; malaise, myalgia, local lymphadenopathy, erythema ringing at vaccination site; rashes</td>
<td>Inadvertent inoculation (529); generalized vaccinia (242); eczema vaccinatum (10-39); vaccinia keratitis; progressive vaccinia (1-2); post-vaccinial encephalitis (3-12); fetal vaccinia (&lt;50 ever reported); death (1-2)</td>
</tr>
</tbody>
</table>

Adapted from data reported by (175).

One potential disadvantage of the use of VV as an oncolytic agent is related to pre-existing immunity. Given that the majority of North American adults over age 50 will have had previous exposure to VV through the smallpox vaccination, it has been postulated that lingering immunity to the virus may inhibit treatment efficacy; this is not, however, supported by the evidence. There is no clear consensus on the duration of immunity following smallpox vaccination. For some time it was believed to last < 5 to 10 y (175, 176); US military personnel are currently required to update their smallpox vaccination every decade. More recent data suggests that immunity lasts significantly longer, with studies reporting up to 75-88 years post-vaccination, with a half-life of 8-15 y (177, 178). Despite this, there has been no correlation identified between baseline antibody titers and efficacy, toxicity, virus replication or gene expression in patients treated with oncolytic VV (179). Furthermore, antibodies against the virus developed rapidly in all subjects, regardless of initial vaccination status, and this did not preclude sustained virus replication after multiple cycles of administration (180).
1.2.4.3 VV tumour-specificity

VV has been shown to replicate well in a broad range of human and mouse cancer cell lines (181) and primary human tumours (182). Tumour-specificity is governed by the cellular and tissue tropism of the virus. Given that virus entry is not facilitated by a specific cell-surface receptor (as is typical of many other viruses) but rather by ubiquitously expressed GAGs, cellular tropism occurs downstream of entry. Transcription of early genes is carried out exclusively by viral proteins, however DNA replication and intermediate/late transcription requires participation of host factors. The presence of these host factors and/or ability of VV to stimulate their production are two of the major determinants of cellular tropism. Several of the pathways implicated as playing important roles in supporting VV replication are also frequently deregulated in numerous cancer types. Wild-type (wt) VV activates both the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase 1/2 (ERK1/2)(183) and phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)(184) pathways after infection; inhibition of either pathway significantly reduces virus replication (183). The MAPK/ERK pathway is constitutively activated in approximately one-third of cancers and is thought to play an important role in the pathogenesis, progression and oncogenicity of CRC (115).

1.2.4.4 Double-deleted vaccinia virus as an oncolytic agent

Double-deleted VV (vvDD) is a recombinant Western Reserve strain OV engineered for improved tumour specificity through deletion of 2 non-essential viral genes – thymidine kinase (TK) and vaccinia growth factor (VGF) (123). Human TK is a critical enzyme in the salvage pathway of DNA precursor biosynthesis that is responsible for the catalytic conversion of the nucleoside thymidine into thymidine monophosphate (dTMP) in cells preparing to undergo cell division. Viral TK serves the same function and allows VV to sustain the nucleotide pool necessary to support virus DNA replication in the context of virus-induced shut-off of host protein synthesis. In normal cells, TK activity is tightly regulated in a cell-cycle dependent fashion with peak activity during S-phase and little to no activity during other phases (185). In malignant cells, which require a steady supply of DNA precursors, TK activity is characteristically upregulated (186, 187). During wt VV infection of normal cells, viral TK is necessary to support virus replication, whereas, in malignant cells viral TK activity is redundant.
Deletion of TK from VV significantly attenuates its replication in normal cells leading to decreased toxicity in mice (123, 188). VGF is a secreted peptide with significant homology to epidermal growth factor (EGF) (189, 190). VGF specifically binds and activates EGF receptors stimulating DNA replication and cellular proliferation of uninfected cells (191, 192). In the absence of VGF, *in vitro* infectivity and replication of VV was significantly impaired in resting cells but not dividing cells and *in vivo* cytopathic effects were reduced (193).

Deletion of both TK and VGF results in further attenuation of virus replication in normal cells without loss of tumour-specific cytotoxicity (123). In nude tumour-bearing mice, systemic vvDD maintained wt levels of replication in the tumours, while infection of normal tissues was markedly reduced. Whereas IP administration of the wt virus was uniformly lethal in tumour-free athymic nude and C57BL/6 mice, vvDD was well-tolerated in both mouse strains at injected doses as high as $10^9$ plaque forming units (pfu). Improved safety of vvDD was further confirmed in non-human primate studies wherein vvDD showed significantly reduced viral loads in normal tissues and decreased liver toxicity and other pathology relative to the wt virus (194). Viruses based on the vvDD backbone have been successfully delivered by multiple routes (IT, IP, IV, IC) in a variety of tumour and non-tumour bearing animal models (194-203).

### 1.2.4.5 VV – Host interactions

Favourable virus-host interactions are paramount to the success of therapy. Systemically delivered viruses must contend with both the systemic immune system as well as the many components of the tumour microenvironment. The tumour microenvironment plays a critical role in the efficacy of oncolytic VV therapy, one that is only beginning to come to light. Components of the microenvironment can act as mediators of OV therapy as well as obstacles that need to be overcome. For viruses with proven preclinical anti-tumour activity, the interaction with the tumour microenvironment may have one of the greatest influences on therapy efficacy.

#### 1.2.4.5.1 Immune system

The importance of the immune response when administering a biological agent cannot be underestimated. From the time of injection until eventual clearance a virus is in constant contact with various components of the body’s natural anti-viral defenses. The interaction between VV and the immune system is particularly complex given that the virus encodes an array of immune
evasion proteins that help it escape immune detection (155). Firstly, VV efficiently evades complement-mediated destruction by encoding a soluble complement control protein as well as by incorporating host complement control proteins into the outer envelope of the EEV particle. Secondly, VV encodes a comprehensive group of proteins that act on multiple levels to inhibit the production and action of IFNs, including those that: decrease the production and recognition of pathogen-associated molecular patterns (PAMPs), block signaling by pathogen recognition receptors (PRRs), are secreted from infected cells to bind and block IFN(R)s, inhibit the intracellular downstream signaling through IFNRs and block host cell protein synthesis. Thirdly, VV regulates the action of pro-inflammatory cytokines (e.g. IL-1, TNF, IL-18) and chemokines, either by blocking the signaling that leads to their production (e.g. NF-κB) or encoding soluble proteins that bind to them in the extracellular space. Lastly, VV encodes numerous anti-apoptotic proteins, which inhibit Fas-L and TNF-α, the mediators of CTL-induced apoptosis (204). Deletion of immunomodulatory viral proteins typically leads to a virus that induces greater immune infiltration and is less virulent. In some instances, though, the increased immune response can itself be responsible for increased pathogenicity. Clearance of an active wild-type VV infection requires induction of the adaptive immune response, including both humoral CD4+ T-cell-dependent antibody responses and CD8+ cytotoxic T cell responses (205). Attenuated VVs are less pathogenic and can be cleared in the absence of functional T- and B-cells, as is apparent from the use of oncolytic VVs in nude and NOD/SCID mouse tumour models (123, 206, 207).

The immune response also plays a critical role in facilitating OV therapy. The induction of innate and/or specific anti-tumour adaptive responses is one of the mechanisms by which VV exerts its therapeutic effect, particularly in the case of third-generation VVs that have been engineered to encode immunomodulatory proteins (discussed in Section 1.2.5).

1.2.4.5.2 Vasculature

The vasculature is vital to the growth and spread of tumours. In the absence of a blood supply tumour nodules can grow to approximately 1-2 mm in diameter; beyond this, further tumour growth becomes angiogenesis-dependent (208, 209). The tumour blood supply has a dual role in mediating OV therapy. For systemically-delivered OVs, adequate tumour perfusion at the time of therapy is required to achieve high levels of virus distribution within the tumour.
Increasing vascular permeability through exposure to hyperthermic conditions was found to significantly increase the delivery of an oncolytic VV to tumours and improve the anti-tumour response relative to virus-treated controls (210). Similarly, viruses that depend on a robust anti-tumour immune response to mediate their effect require functional blood vessels to deliver the infiltrating immune cells (211, 212). Conversely, virus tropism for the tumour vasculature represents another important mechanism by which VV exerts its anti-tumour effects. VV appears to have innate antiangiogenic properties; it has been shown to colocalize with CD31+ tumour endothelial cells as well as decrease vascular density and tumour perfusion (154, 198, 213). In mice, decreased tumour perfusion has led to increased apoptosis in uninfected areas of subcutaneous CRC tumours (198). Arming VVs to encode anti-angiogenic cytokines (eg. GM-CSF) or molecules that target angiogenic pathways (e.g. anti-VEGFR antibodies or soluble VEGF) has further improved the efficacy of these viruses over their unarmed counterparts (197, 214-217) (discussed in 1.2.5). The anti-vascular mechanism of VV is not fully understood, but both clinical and preclinical data show that normal vessels are unaffected by OV treatment (217). In vitro, endothelial cells must be activated with a growth factor (eg. VEGF) in order to support high levels of virus replication and transgene expression, which suggests that endothelial cell killing is specific to tumour vessels (217).

1.2.4.5.3 Cancer stem cells

The concept of cancer stem cells (CSC) was first introduced over half a century ago (218). CSCs are a unique subpopulation of undifferentiated cells within a tumour that possess ‘stem-like’ properties, namely they are capable of self-renewal and responsible for producing differentiated progeny that make up the bulk of the tumour yet have limited proliferative ability. While there is overwhelming evidence for the existence of CSCs both in hematological and solid tumours, their role in cancer development, maintenance and spread, remains somewhat controversial, as does an understanding of how they can be accurately and reliably identified. Nevertheless, it is widely believed that for durable cures to occur the CSC population must be eliminated, thereby preventing recurrence at a later time. Unfortunately, traditional therapies seem to be particularly ill-suited for targeting these cells, which may exist in a low proliferative state (e.g. leukemic CSCs) and may also share some of the properties of normal stem cells, such as expression of multi-drug resistant efflux proteins (219).
Over the past 5 years there has been growing evidence that OVs can infect and kill CSCs with similar or greater affinity compared to their more differentiated tumour cell counterparts (220). The majority of studies to date have looked at CSC targeting abilities of HSV (221-229), however there are a handful of studies that examine the abilities of poxviruses to kill CSCs (230-232). In one study looking at the potential of VV therapy (GLV-1h68), CSCs were identified in a human breast cancer cell line by characterization of ALDH1 activity, tumourgenic potential in nude mice, mammosphere formation, asymmetric division, resistance to chemo- and radio-therapy, stem cell markers and invasive capacity (231). The authors demonstrated that VV replicates more efficiently in the CSC-enriched population in vitro and in vivo, resulting in a greater tumour growth reduction in vivo relative to the non-stem-like population. VV also led to more efficient replication in and eradication of tumours driven to undergo epithelial-mesenchymal transition (EMT) by TGF-β1 treatment compared to untreated controls. In a glioma model study, 5 of 5 brain tumour-initiating cell lines tested were permissive for VV (JX-594) infection and oncolysis, although to a lesser extent than the highly susceptible U87 glioma cell line (232). Using a previously established lymphoma residual disease model wherein residual tumour cells have stem-like properties, Contag et al. demonstrated that VV (vvDD) significantly decreased the rate of relapse when delivered by cytokine-induced killer (CIK) carriers (233). The rabbitpox virus MYXV has also shown potential as a CSC-targeting agent for cancer patients undergoing autologous blood and marrow transplant (230, 234). These studies used MYXV to purge leukemia and myeloma stem and progenitor cells from patient samples ex vivo prior to engraftment into immunocompromised mice. The authors suggest that MYXV specificity was determined by its ability to bind cancerous cells but not to normal hematopoietic cells (234, 235).

1.2.5 Third-generation OVs

Second-generation OVs displaying the best tumour-specificity and oncolytic potency as well as the least toxicity have been further developed to encode therapeutic genes. These third-generation viruses serve as both tumour-specific gene therapy vectors as well as cytolytic agents. One of the greatest challenges in OV therapy to date has been achieving sufficiently widespread tumour infection. It is thought that the modest efficacy observed in early OV clinical trials was likely due to single intratumoural injections producing small areas of initial infection combined with poor virus spread (236, 237). Several different arming strategies that maximize the effect of OVs on the tumour and its microenvironment have been pursued to address this limitation. These
include arming OVs to (1) improve virus spread (238), (2) promote an anti-tumour immune response (239), (3) disrupt tumour vasculature (240), and (4) improve combination therapy strategies (discussed in Section 1.4) by encoding prodrug activating enzymes (238) or drug targetable cell-membrane proteins (241) (discussed in Section 1.4.2.1). The above strategies have been applied to a range of OVs, including VV.

Mathematical modeling and preclinical data have shown widespread infection throughout the core and rim regions is required to ensure complete destruction of a tumour (236) and that infection of just 1% of cells is sufficient to eradicate a tumour if those cells are homogenously distributed (242). Solid tumours are typically associated with poor fluid clearance and excessive secretion of extracellular matrix (ECM), which serve as significant obstacles to achieving optimal distribution of cancer therapies (243). Many third-generation OVs have been engineered accordingly to encode proteins that act on the ECM to enhance virus spread. VV expressing matrix metalloproteinase (MMP)-9 (GLV-1h255) was responsible for significant degradation of collagen IV in infected subcutaneous human prostate tumours and improved tumour response compared to the parental virus (244). This strategy has been applied to other viruses, including HSV and Ad, engineered to encode hyaluronidase (245), various MMPs (246, 247) as well as the collagen protease-inducing hormone relaxin (137).

As previously discussed, investigators have long understood that the immune response plays a critical role in the overall efficacy of OV therapy. Different components of the immune system act at different stages of virus infection (delivery, replication, spread, etc.) and have the potential to either suppress or promote tumour cell killing. The role of the immune system in OV therapy is a precarious one; the anti-viral immune response must be delayed or suppressed enough to permit sufficient virus replication, yet robust enough to prohibit uncontrolled off-target replication. There is also mounting evidence that exploiting the natural anti-tumour immune response stimulated by OVs (198, 248-253) can significantly improve the therapeutic outcome (239). OVs have been constructed to encode a range of cytokines with the objective of promoting the desired CTL dominated anti-tumour immune response. For example, granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to stimulate CD4^+ and CD8^+ T cell-dependent lasting anti-tumour immunity when combined with an irradiated tumour cell vaccine (254). JX-594, a tk-deleted Wyeth strain VV engineered to encode GM-CSF (255), has shown promising results in intratumoural (179, 180, 256, 257) and intravenous phase I studies.
Other GM-CSF-armed viruses, including HSV, MV, NDV and Ad, have also induced therapy enhancing anti-tumour immune response, particularly T cells, in preclinical models and patients (259-271). VV has been engineered to encode a variety of other immune-modulating cytokines (e.g. IFN-β (154, 211), IL-6 (272)), chemokines (e.g. CCL5 (203, 273)), chemokine antagonists (274), costimulatory molecules (275), and a T cell engaging bispecific antibody (212).

Tumour angiogenesis is a critical and sometimes rate-limiting step in tumour growth. Currently approved anti-angiogenic drugs on the market are all antibody-based agents (ex. bevacizumab), whose performance can be hindered by poor tumour penetration (243). Arming OVs with genes encoding anti-vascular molecules has the potential to overcome issues of delivery by facilitating high levels of expression directly at the tumour site. A comprehensive review by Angarita et al. discusses the numerous strategies used to create armed anti-vascular OVs using VV, HSV and Ad backbones (240). VVs designed to inhibit the angiogenic VEGF/VEGF-R pathway by encoding anti-VEGF antibody (214, 215, 276) or a soluble VEGF-R (197) have led to decreased tumour vessel density and improved tumour response relative to their parental viruses in subcutaneous tumour models. Alternatively, VV encoding the synergistically anti-angiogenic proteins angiostatin and endostatin successfully inhibited tumour angiogenesis and growth after both IT and IV administration (277). Engineered HSVs and Ads have also been designed to target angiogenic pathways and cytokines including VEGF/VEGF-R (278-280), EGF/EGF-R (281), fibroblast growth factor (FGF)/FGF-R (282) and IL-8 (283) as well as encode anti-angiogenic factors and cytokines, including vasculostatin (284, 285), endostatin (286-291), canstatin (292), vascular endothelial growth inhibitor (VEGI)(293), plasminogen (294), arresten (142), thrombospondin-1 (295), angiostatin (296), IL-12 (297, 298), IL-18 (299, 300), IL-24 (301) and PF4 (302).

Disruption of the tumour vasculature can eventually lead to areas of poor perfusion, low pH and high interstitial pressure. This can be problematic for conventional therapies as drug delivery to these areas is restricted and hypoxic tumour cells are generally more resistant to both chemotherapy and radiation therapy (303). Conversely, VV and other OVs have been shown to thrive in this type of abnormal tumour microenvironment (166, 167, 200, 222, 304-307).
1.2.6 Clinical safety and efficacy of OV

OVs belonging to a spectrum of virus families have been investigated in the clinic. Table 1.3 summarizes the published results of most clinical trials involving OV. The majority of trials have been phase 1, with safety as the primary endpoint; efficacy has often been evaluated as a secondary endpoint. In most phase I trials, dose-limiting toxicities (DLTs) have not been observed and the maximum tolerable dose (MTD) has not been identified. Often dose escalation is curtailed due to manufacturing limitations rather than toxicity. The most common adverse event across all trials has been grade 1/2 flu-like symptoms including fever, chills, fatigue, myalgia, nausea and vomiting.

Early clinical investigations of OV faced significant hurdles. ONYX-015 was one of the first OV to be given to patients, but despite demonstrated safety it showed minimal anti-tumour activity (308, 309). Unfortunately, this poor performance, compounded with the death of a young man enrolled in an Ad gene therapy trial, led to a halt of all further clinical investigation into adenovirus vectors for some time in North America. The rights to ONYX-015 were sold to a Chinese company that later reported on a phase III trial of 160 patients with squamous cell carcinoma of the head and neck or esophageous (310); the results of which (overall response rate of >50%) led to its licensing in China for use in combination with platinum-based chemotherapy.

The clinical performance of third-generation viruses has been greatly improved over their first- and second-generation counterparts. For example, GM-CSF armed viruses have performed well in phase I/II trials demonstrating both efficacy and safety (179, 180, 216, 217, 257, 258, 311-314). Ad5-Δ24-GMCSF (a.k.a. CG00700) resulted in complete responses (CRs) in 17 of 35 patients (311). The GM-CSF expressing herpes virus, OncoVex(GM-CSF) induced complete and partial responses in 4 and 10 of 17 patients, respectively (312). Similarly, JX-594 led to complete and partial responses in 1 and 3 of 30 patients, respectively, and stable disease in 10 patients (257). Building on the results of these trials, there are currently several phase III trials underway including the OPTIM trial looking at OncoVex(GM-CSF) in patients with unresectable melanoma (315) and an integrated phase II/III trial of CG00700 in patients with invasive bladder cancer (NCT01438112).
<table>
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<tr>
<th>Study Authors</th>
<th>Study Design</th>
<th>Disease</th>
<th>Response</th>
<th>Toxicity</th>
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<tr>
<td><strong>Adenovirus</strong></td>
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<tr>
<td>Ad5/3-Δ24</td>
<td>Phase I (10) Dose escalation</td>
<td>Recurrent ovarian ca (IP)</td>
<td>SD (6/8)(^6); PD (2/8)(^6)</td>
<td>15 vector-related AEs (5/10 patients) 1: fluid-like symptoms(^3)</td>
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<td>Kim et al. (2013)(316)</td>
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<tr>
<td>CG0070 (Ad5-Δ24-GMCSF)</td>
<td>Phase I (35) Dose escalation</td>
<td>Nonmuscle invasive bladder ca. (intravesical)</td>
<td>CR (17/35); median response duration (10.4 mo)</td>
<td>Grade 1/2: dysuria, hematuria, urinary frequency, urgency, bladder spasm, nocturia, flu-like sympotms(^9), arthralgia, bladder discomfort, abdominal pain  Grade ≥3 (3 patients): pollakiuria, lymphopenia (DLT), dysuria, urgency, nocturia</td>
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<td>Burke et al. (2012)(311)</td>
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<tr>
<td>Cerullo et al. (2011) (317)</td>
<td>Retrospective (43)</td>
<td>Treatment-refractory solid tumours (IT ± CP)</td>
<td>64% show possible clinical benefits (SD or better) OS, PFS improved in CP (IV ± oral) + virus vs. virus alone cohort</td>
<td>Grade 1/2: flu-like symptoms(^3)  Grade ≥3: hyponatremia, abdominal pain</td>
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<td>Koski et al. (2010) (318)</td>
<td>Phase I (21)</td>
<td>Treatment-refractory solid tumours (4/5 of dose IT or IP + 1/5 dose IV ± CP)</td>
<td>MR (2/12)(^7); SD (6/12)(^7); PD (4/12)(^#)</td>
<td>Grade 1/2: flu-like symptoms(^3), injection site pain, abdominal pain, anorexia, heartburn, anemia, leukocytopenia, thrombocytopenia, limb edema, increased ALT &amp; AST, hyperbilirubinemia, hypokalemia, hyperkalemia, hyponatremia, glucose imbalance, cough.  Grade ≥3: cholecystitis, anemia, neutropenia, increased ALT &amp; AST, hyponatremia, hyperbilirubinemia</td>
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<tr>
<td>Ad5-Δ24-RGD &amp; Ad5-Δ24-RGD-GMCSF</td>
<td>Non-randomized series (16)</td>
<td>Chemo-refractory solid tumours (4/5 of dose IT or IP + 1/5 dose IV)</td>
<td>Ad5-Δ24-RGD: PD (9/9)(^#) Ad5-Δ24-RGD-GMCSF: SD (3/6)(^#); PD (3/6)(^#)</td>
<td>Grade 1/2 (15/15): flu-like symptoms(^3), increased AST and ALP, hyperbilirubinemia, hyponatremia, increased INR, decreased haemoglobin, leukocytopenia, thrombocytopenia, anorexia, cough, injection site and abdominal pain, hypotension  Grade ≥3 (7/15): increased ALP, hyponatremia, increased INR, lymphocytopenia, fever, pulmonary embolism</td>
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<td>Pesonen et al. (2012) (268)</td>
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<tr>
<td>Ad5-Δ24-RGD</td>
<td>Phase I (21)</td>
<td>Gynecologic disease (IP)</td>
<td>SD (15/21)(^#); PD (6/21)(^#)</td>
<td>Grade 1/2-flu-like symptoms(^3), abdominal pain, etc.  Grade ≥3 (not vector-related): abdominal pain, respiratory, shortness of breath, chest pain, nausea/vomiting,</td>
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<tr>
<td>Drug</td>
<td>Study Details</td>
<td>Phase</td>
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<td>Outcome</td>
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<td>Telomelysin</td>
<td>Nemunaitis et al. (2010) (321)</td>
<td>Phase I (16)</td>
<td>Treatment-refractory solid tumours (IT)</td>
<td>SD (11/14)(^a); PD (3/14)(^a)</td>
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<td>KH901</td>
<td>Chang et al. (2009) (322)</td>
<td>Phase I (23)</td>
<td>Recurrent head and neck ca. (IT)</td>
<td>SD (12/19); PD (7/19)</td>
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<tr>
<td>H103</td>
<td>Li et al. (2009) (323)</td>
<td>Phase I (27)</td>
<td>Treatment-refractory solid tumours (IT)</td>
<td>PR (3/27); MR (1/27); SD (18/27); PD (5/27)</td>
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<tr>
<td>Ad5-(\gamma)CD/(\mu)TK(_{SR30})Fep-ADP</td>
<td>Freytag et al. (2007) (324)</td>
<td>Phase I (9)</td>
<td>Prostate ca (intraprostatic) with IMRT and S-FC/GCV</td>
<td>Neg. biopsy at 6 mo. (5/8)</td>
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<tr>
<td>Ad5-CD-TKrep</td>
<td>Freytag et al. (2007) (325) Freytag et al. (2002) (326)</td>
<td>Phase I (16) Dose escalation and Five-year follow up</td>
<td>Recurrent prostate Ca (intraprostatic) with S-FC/GCV</td>
<td>Neg. biopsy at 1 y. (2/16) 5-y survival (13/16, deaths unrelated to cancer) PFS at 5-y (12/16)</td>
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<tr>
<td>CG7870</td>
<td>Small et al. (2006) (327)</td>
<td>Phase I (23)</td>
<td>Refractory metastatic prostate Ca (IV)</td>
<td>25-49% decrease in PSA (5/23)</td>
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<tr>
<td>Onyx-015</td>
<td>Galanis et al. (2005) (328)</td>
<td>Phase I/II (6)</td>
<td>Advanced sarcoma (IT) combined with MAP chemotherapy</td>
<td>PR (1/6); SD (4/6); PD (1/6)</td>
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<td></td>
<td>Xia et al. (2004) (310)</td>
<td>Phase III (160)</td>
<td>Squamous cell Ca of head and neck or esophagus (IT) with ORR 48/66 virus + chemo vs. 23/57</td>
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<tr>
<td>Study (Year)</td>
<td>Phase</td>
<td>Type of Cancer</td>
<td>Treatment</td>
<td>Response</td>
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<td>Chiocca et al. (2004) (309)</td>
<td>Phase I (24)</td>
<td>Recurrent malignant glioma (resection cavity)</td>
<td>CIS or adriamycin plus 5-FU</td>
<td>SD (1/24); PD (23/24)</td>
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<tr>
<td>Reid et al. (2001, 2002, 2005) (329-331)</td>
<td>Phase I (11) Dose escalation Phase II (27)</td>
<td>GI carcinoma metastatic to liver (hepatic artery) with 5-FU/leucovorin</td>
<td>PR (3/27); MR (4/27); SD (9/27); PD (11/27)</td>
<td>Grade 1/2: flu-like symptoms, increased ALP, ALT, SGOT and AST, hypophenia, increased LDH, bilirubinemia, abdominal pain, asthenia, hypochromic anemia, granulocytosis, diarrhea, tachycardia, anemia</td>
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<td>Vasey et al. (2002) (308)</td>
<td>Phase I (16) Dose escalation</td>
<td>Recurrent ovarian Ca (IP)</td>
<td>PD (15/16); DLT (1/16)</td>
<td>Grade 2: flu-like symptoms, diarrhea, abdominal pain</td>
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<tr>
<td>CV706 DeWeese et al. (2001) (332)</td>
<td>Phase I (20) Dose escalation</td>
<td>Recurrent prostate Ca (intraprostatic)</td>
<td>PSA ≥30% (13/20)</td>
<td>Grade 1/2: flu-like symptoms, injection site pain, hematuria, genitourinary symptoms, skin rash, increased AST/ALT</td>
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<td>Herpes Simplex Virus</td>
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<td>NV1020 Sze et al. (2012) Geeverghese et al. (2010) (333, 334)</td>
<td>Phase I/II (22) Dose escalation</td>
<td>Treatment refractory colorectal Ca with liver metastasis</td>
<td>SD (14/22); PR (1/22)</td>
<td>Grade 1/2: fever/chills, nausea, myalgia, fatigue, etc.</td>
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<td>HF10 Nakao et al. (2011) (335)</td>
<td>Phase I (6) Dose escalation</td>
<td>Advanced non-resectable pancreatic Ca (IT)</td>
<td>PR (1/6); SD (3/6); PD (2/6)</td>
<td>Grade 1/2: none</td>
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<td>OncoVex-GM-CSF (T-Vec) Harrington et al. (2010) (312)</td>
<td>Phase I/II (17) Dose escalation</td>
<td>Untreated stage III/IV squamous cell ca. of head and neck (IT) with cisplatin and radiotherapy</td>
<td>CR (4/17); PR (10/16)</td>
<td>Grade 1/2: pyrexia, fatigue</td>
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<td>Hu et al. (2006) (336)</td>
<td>Phase I (30) Dose escalation</td>
<td>Breast, head and neck, GI Ca with s.c. or cutaneous deposits (IT)</td>
<td>SD (3/30)</td>
<td>Grade 1/2: Pyrexia, rigor, hypotension, tachycardia, anorexia, flu-like symptoms, constipation, diarrhea, insomnia, pneumonia, abdominal pain</td>
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<tr>
<td>Senzer et al. (2009) Kaufman et al. (2010) (259, 313)</td>
<td>Phase II (50)</td>
<td>Unresectable metastatic melanoma</td>
<td>CR (8/50); PR (5/50); SD (10/50)</td>
<td>Grade 1/2: flu-like symptoms, pyrexia, diarrhea, anemia, anorexia, gastroesophageal reflux, rash, abdominal pain, increased blood urea, constipation, cough, vertigo, erythema,</td>
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<tr>
<td>Study</td>
<td>Design Description</td>
<td>Adverse Events</td>
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<td>NV1020</td>
<td>Fong et al. (2009) (337, 338) Kemeny et al. (2006)</td>
<td>Grade ≥3 (not treatment related): fatigue, pain, flu-like symptoms, diarrhea, anemia, arthralgia, back pain, abdominal pain, asthenia, vertigo, edema, atrial fibrillation, increase blood amylase, dyspnea, chest pains, hypertension, hyponatremia, hypoxia, bowel obstruction, leukopenia, increased lipase, palpitations, pneumonia, pleural infection, syncope, thrombocytopenia</td>
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<td>HSV1716</td>
<td>Mace et al. (2008) (339)</td>
<td>Grade 1/2: flu-like symptoms, pyrexia, rigors, leukocytosis</td>
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<tr>
<td>HF10</td>
<td>Nakao et al. (2007) (340)</td>
<td>Grade ≥3: increased GGT, gastroenteritis, leukocytosis</td>
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<td>Vaccinia Virus</td>
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<tr>
<td>JX-594</td>
<td>Heo et al. (2013) (257)</td>
<td>Grade 1/2: flu-like symptoms, rigor, anorexia, skin pustules, increased transaminase</td>
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<td>Grade ≥3: nausea, vomiting, lymphopenia</td>
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<td>Breitbach et al. (2011 &amp; 2013) (217, 258)</td>
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<td>Hwang et al. (2011) (180)</td>
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<td>Park et al. (2008) (179)</td>
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<td>GL-ONC1</td>
<td>Lauer et al. (2013)(341)</td>
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<tr>
<td>Reovirus</td>
<td>Zeh et al. (2014)(342)</td>
<td>Grade 1/2: pyrexia, musculoskeletal pain, fatigue, nausea and vomiting, pustule</td>
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<td>Grade ≥3: transaminitis (DLT)</td>
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<td>Reference</td>
<td>Phase</td>
<td>Treatment Details</td>
<td>Dose Escalation</td>
<td>Toxicity</td>
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<td>Galanis et al. (2012) (343)</td>
<td>Phase II (21)</td>
<td>Metastatic melanoma (IV)</td>
<td>SD (6/21)</td>
<td>Grade 1/2:</td>
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<td>Grade ≥3:</td>
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<tr>
<td>Adair et al. (2012) (344)</td>
<td>Phase 0 (10)</td>
<td>CRC with liver metastases (IV) prior to scheduled surgical resection</td>
<td>n/a</td>
<td>Grade 1/2:</td>
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<td>Grade ≥3:</td>
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<tr>
<td>Karapanagiotou et al. (2012) (345)</td>
<td>Phase I/II (31)</td>
<td>Advanced malignancies (IV) with carboplatin and paclitaxel</td>
<td>CR (1/31); PR (6/31); SD (9/31); PD (8/31)</td>
<td>Grade 1/2:</td>
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<td></td>
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<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Comins et al. (2010) (346)</td>
<td>Phase I (24)</td>
<td>Advanced malignancies (IV) with docetaxel</td>
<td>PR (4/16); MR (3/16); SD (7/16)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Harrington et al. (2010) (347)</td>
<td>Phase I (23)</td>
<td>Advanced solid malignancies (IT) with palliative radiotherapy</td>
<td>Low dose RT: PR (2/7); SD (5/14); High dose RT: PR (5/7); SD (2/7)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Vidal et al. (2008) White et al. (2008) (348, 349)</td>
<td>Phase I (33)</td>
<td>Advanced solid malignancies (IV)</td>
<td>SD (8/33)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Reovirus serotype 3 Forsyth et al. (2008) (350)</td>
<td>Phase I (12)</td>
<td>Recurrent glioblastoma multiforme (IT)</td>
<td>SD (1/11); PD (10/11)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Seneca Valley Virus SVV-001 Rudin et al. (351)</td>
<td>Phase I (30)</td>
<td>Advanced solid tumours with neuroendocrine features (IV)</td>
<td>MR (5/30); SD (1/30)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose escalation</td>
<td></td>
<td>Grade ≥3:</td>
</tr>
<tr>
<td>Measles Virus MV-CEA</td>
<td>Phase I (21)</td>
<td>Recurrent ovarian Ca (IP)</td>
<td>SD (14/21)</td>
<td>Grade 1/2:</td>
</tr>
<tr>
<td>Study</td>
<td>Phase</td>
<td>Dose Escalation</td>
<td>Disease</td>
<td>Response</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>----------------</td>
<td>---------</td>
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</tr>
<tr>
<td>Galanis et al. (2010) (352)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV-EZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heinzerling et al. (2005) (353)</td>
<td>Phase I (5)</td>
<td>Cutaneous T-cell lymphoma (IT)</td>
<td>CR (1/5); PR (3/5); MR (1/5)</td>
<td>Grade 1/2: erythema at injection site, itching</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Grade ≥3: none</td>
</tr>
<tr>
<td>Newcastle Disease Virus</td>
<td>Phase I (18)</td>
<td>Treatment refractory solid tumours (IV)</td>
<td>CR (1/18); PR (3/18); MR (2/18); SD (5/18)</td>
<td>None</td>
</tr>
<tr>
<td>PV701</td>
<td></td>
<td></td>
<td></td>
<td>Grade 1/2: flu-like symptoms(^4), diarrhea, anorexia, dyspepsia, constipation, abdominal distention, increased ALT &amp; APT, hypotension, increased bilirubin, thrombocytopenia, neutropenia, leukopenia, anemia, increased PTT, hypotension, tachycardia, hypertension, cough, rhinitis, dyspnea, pharyngitis, pain, hypokalemia</td>
</tr>
<tr>
<td>Hotte et al. (2007) (354)</td>
<td></td>
<td></td>
<td></td>
<td>Grade ≥3: none</td>
</tr>
<tr>
<td></td>
<td>Phase I (16)</td>
<td>Treatment refractory solid tumours (IV)</td>
<td>SD (5/14); PD (9/14)</td>
<td>Grade 1/2: fever, chills, fatigue, headache, GI symptoms, hypotension, anemia, pain, thrombocytopenia, dyspnea, increased transaminases</td>
</tr>
<tr>
<td>PV701</td>
<td></td>
<td></td>
<td></td>
<td>Grade ≥3: none</td>
</tr>
<tr>
<td>Laurie et al. (2006) (355)</td>
<td></td>
<td></td>
<td></td>
<td>Grade 1/2: fever</td>
</tr>
<tr>
<td>NDV-HUJ</td>
<td>Phase I/II (11)</td>
<td>Recurrent glioblastoma multiforme (IV)</td>
<td>CR (1/11) TTP: 2-53 wk OS: 3-66 wk</td>
<td>Grade ≥3: leukopenia, neutropenia</td>
</tr>
<tr>
<td>Freeman et al. (2006) (356)</td>
<td></td>
<td></td>
<td></td>
<td>Grade ≥3: none</td>
</tr>
<tr>
<td>PV701</td>
<td>Phase I (79)</td>
<td>Treatment refractory advanced solid tumours (IV)</td>
<td>CR (1/62); PR (1/62); SD (14/62)</td>
<td>Grade 1/2: flu-like symptoms(^4), anorexia, diarrhea, hypotension (DLT), leukopenia, anemia, thrombocytopenia, neutropenia, increased PTT, hypotension, dyspnea, cough, increased ALT/AST, increased bilirubin, pain, hypokalemia</td>
</tr>
<tr>
<td>Pecora et al. (2002) (357)</td>
<td></td>
<td></td>
<td></td>
<td>Grade ≥3: fever, diarrhea, dyspnea, increased ALT/AST, increased bilirubin, pain, hypoxia (DLT), dehydration (DLT), tremors (DLT)</td>
</tr>
</tbody>
</table>

Abbreviations: no., number; IP, intraperitoneal; SD, stable disease; PD, progressive disease; CA-125, carcinoma antigen-125; AE, adverse events; Ca, cancer; DLT, dose limiting toxicity; CR, complete response; IT, intratumoural; OS, overall survival; PFS, progression free survival; IV, intravenous; CP, cyclophosphamide; MR, minor response; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; INR, international normalized ratio; Neg., negative; mo, months; y, year; CPK, creatine phosphokinase PSA, prostate-specific antigen; MAP, methotrexate, cisplatin, and doxorubicin; CIS, cisplatin; 5-FU, 5-fluorouracil; ORR, overall response rate; GI, gastrointestinal; SGOT, serum glutamic oxaloacetic transaminase; SPGT, serum glutamic pyruvic transaminase; s.c., subcutaneous; CRC, colorectal cancer; GGT, gamma-glutamyl transferase; DOD, dead of disease; LDH, lactate dehydrogenase; RT, radiotherapy; CK-MB, creatine kinase-MB; TTP, time to progression. \(^4\)RECIST; \(^5\)Symptoms including: fever, chills, fatigue, nausea/vomiting, headache, myalgia; \(^\ast\)CHOI, \(^\ast\)EORTC, \(^\ast\)modified WHO criteria
1.2.6.1 Oncolytic vaccinia virus in clinical trials

VV has only recently moved into clinical investigation as an oncolytic vector. Its previous widespread clinical use as a vaccine generated a wealth of toxicity information (discussed in 1.2.4.2) that supports its use. To date, 3 oncolytic VVs have been studied in patients: JX-594 (Jennerex), a tk-deleted Wyeth strain VV expressing GM-CSF (180, 217, 257, 258), vvDD (tk- and vgf-delted Western Reserve stain VV) (342) and GL-ONC (aka GLV-1h68; Genelux), a Lister strain VV expressing GFP (341).

Patients with treatment-refractory metastatic melanoma (180) and advanced solid tumours (179, 217, 257, 258) have been treated with intratumoural or systemic JX-594 at doses up to 3 x 10^9 pfu (179). Two patients with liver tumours receiving IT injections at the highest dose (3 x 10^9 pfu) experienced dose-limiting hyperbilirubinaemia due to tumour swelling, therefore 1 x 10^9 pfu was deemed the MTD (179). Tumour responses and virus replication were observed at all doses in the injected tumours as well as in some uninjected tumours (179). Systemic delivery of doses between 1 x10^5 pfu/kg and approximately 1.5 x 10^7 pfu/kg was carried out safely without reaching the MTD (258). Virus dose correlated with viral genomes in the blood (0-4 h post infusion), as well as with detection of virus in the tumours, antibodies against the β-gal marker protein as well as tumour response.

Further investigation of the mechanism of action were conducted on histological samples from these patients based on preclinical findings that VV infects tumour vasculature and substantially compromises tumour perfusion (198, 358). Infection of CD31+ endothelial cells was confirmed in 5 of 8 tumour biopsies from patients with a variety of tumour histologies (217). MRI analysis of patients with liver tumours (HCC or CRC) recruited to the phase II trial (257) showed a durable decrease in tumour perfusion (up to 8 wk post-treatment) in both injected and non-injected tumours (217). The overall disease control rate for this trial was 46% by modified Response Evaluation Criteria in Solid Tumours (mRECIST)(359, 360). Development of anti-tumoural immunity was demonstrated in 11 of 16 patients who showed complement-dependent cytotoxicity against at least 1 of 4 HCC cell lines. Additionally, T-cells directed against β-gal were present in high dose patients up to 1.5 y post-treatment. To date, none of the known serious
adverse reactions associated with smallpox vaccination (discussed in 1.2.4.2) have been observed in patients receiving JX-594.

Preliminary results of the ongoing phase I/II GL-ONC1 trial in patients with unresectable and chemotherapy resistant PC were reported at an American Society for Clinical Oncology meeting in 2013 (341). Of the 33 patients treated, there was one DLT (grade 3 transaminitis) at the $1 \times 10^9$ pfu dose. Stable disease (SD) was reported in 6 patients at $>24$ wk.

1.3 Radiation Therapy

1.3.1 Overview

Radiation therapy (RT) of malignancies can be classified into 3 general categories based on the location the radiation source: external beam radiation therapy (EBRT), brachytherapy, and systemic radionuclide therapy. EBRT, the most commonly applied type of radiotherapy, involves a radiation source outside of the body that is directed towards a specific treatment area. One of the major limitations of EBRT, and radiation therapy in general, is accurate and reliable tumour targeting. It is the goal of radiation oncologists to achieve the maximum possible dose to the tumour while minimizing the dose to the surrounding normal tissues. Almost all of the advances in radiation therapy have focused on addressing this limitation using different strategies for different tumours and anatomical regions.

Progress in the field of medical imaging, including development of computed CT, magnetic resonance imaging (MRI) and positron emission tomography (PET) have significantly improved the ability to accurately locate tumours within soft tissues. Integration of these images into computer software designed to generate precise volumetric data and create the optimal beam pattern allows for what is known as 3-dimensional conformal radiation therapy. Additionally, using a technique called intensity-modulated radiation therapy, individual radiation beams are divided into smaller ‘beamlets’ of varying intensities in order to avoid harmful doses to critical neighbouring structures (e.g. nerves and vessels). Given the challenge that is presented by the movement of patients and involuntary visceral motion (e.g. breathing, digestion) during treatment, as well as tumour movement over the course of treatment (361), integration of imaging modalities into radiation delivery systems has allowed for image-guided radiation therapy. Combination of all these technological advances to EBRT has significantly improved
tumour-targeting accuracy. EBRT is useful primarily in the treatment of superficial tumours, however it can also be applied intraoperatively to reach deeper tissues. Four-dimensional EBRT, which involves the mapping of visceral movements over the course of treatment, is particularly useful for the treatment of tumours in the lung and liver.

Brachytherapy utilizes a sealed radiation source that is permanently or temporarily implanted in (interstitial brachytherapy) or near (contact brachytherapy) the tumour. In tumour types that are amenable to brachytherapy (e.g. breast, cervix, prostate, skin, head and neck and others), this treatment has many advantages over EBRT. Advances in radionuclide production, core and shell development, imaging technologies, surgical techniques, seed delivery systems (afterloading) and dosimetry have maintained brachytherapy as a relevant and effective treatment since its first uses over a century ago. The localized delivery system minimizes damage to the surrounding normal tissues and ensures that the dose will be maintained even with organ and tumour movement. Furthermore, unlike EBRT, which is typically fractionated into daily doses administered over the course of several weeks, brachytherapy requires significantly fewer hospital visits.

Systemic radionuclide therapy (i.e. molecularly-targeted RT) represents another strategy to improve the therapeutic index of RT by improving delivery to tumour tissues. Targeted RT achieves its high level of specificity by linking radionuclides to either monoclonal antibodies (mAb) or peptides that bind tumour-specific cell surface markers. Targeted RT has the advantage of being able to target tumours in anatomical locations not amenable to either EBRT or brachytherapy (e.g. micro-metastases) as the radiolabeled peptide or mAb can be delivered systemically. Pioneering work in radiolabeled antibody therapy began in the 1950s and was first used in humans over 30 y ago (362) for imaging carcinoembryonic antigen (CEA)-positive tumours. Since then, radiolabeled mAb have become an important treatment option primarily for hematologic malignancies (e.g. non-Hodgkin’s lymphoma) (363), although it has also shown promise in solid tumours in the context of CRC minimal residual disease (MRD) (364-366). Peptide receptor radiotherapy (PRRT) is in principle similar to radiolabeled mAb therapy, however, using small (3-11 amino acid) peptides instead of mAb as the radionuclide delivery vehicle has several important advantages. These include: significant reductions in production costs and time; non-immunogenicity; deeper tumour penetration; low bone marrow penetration; and quick blood clearance. PRRT is a powerful treatment option for a subset of inoperable and
metastatic solid tumours wherein the radiation payload is delivered directly to the tumour cell surface where it is internalized by tumour-specific receptors, leading to specific accumulation of the radiation dose in the tumour bed.

1.3.2 Peptide-receptor radiotherapy

Peptide-based radiopharmaceuticals are most commonly targeted towards members of the large and ubiquitous family of 7-transmembrane proteins, also known as G-protein coupled receptors (GPCR). The GPCR family includes many receptors that are specifically overexpressed on a range of cancer types (367) and therefore represent potential tumour targets. GPCRs, including somatostatin receptor (SSTR) and bombesin receptor 2 (BB2; also referred to as gastrin releasing peptide receptor, GRP-R), have been the subject of investigation in the development of radiolabeled peptides.

Radiolabeled peptides were originally developed for imaging of SSTR-positive tumours. Unfortunately, early peptides performed poorly primarily due to a lack of in vivo stability. However, advanced structural and functional analysis of SSTR led to the development and clinical use of somatostatin (SS) peptide analogues (368, 369) with improved stability and tumour accumulation (Table 1.4). The first radiolabeled peptide approved for clinical use was the radiolabeled SS analogue $[^{111}\text{In-DTPA}]$octreotide (Octreoscan®), which continues to be used for imaging of SSTR-positive tumours. Building on the success of radiolabeled peptides for diagnostic imaging, investigators looked to their potential as therapeutic agents. Today, radiolabeled SS analogues remain the only peptides approved for imaging and PRRT in North America, with analogues of bombesin (gastrin-releasing peptide, GRP) labeled with 99m-technetium (370) or 68-gallium (371) also being investigated in early phase clinical trails.

1.3.2.1 Peptide properties for PRRT

There are many important considerations that must be addressed during the development of peptides for PRRT. Firstly, the peptide target must be differentially expressed in tumour versus normal tissue. This criteria can be met either through selection of an endogenous peptide and known receptor target with previously demonstrated tumour-associated overexpression or through screening of peptide libraries for specific binding to tumour cell lines over controls. Secondly, alterations to the peptide, such as conversion of L-isomer amino acids to D-isomers or
Table 1.4. Binding affinities of endogenous and synthetic somatostatin peptides

<table>
<thead>
<tr>
<th></th>
<th>SSTR1</th>
<th>SSTR2</th>
<th>SSTR3</th>
<th>SSTR4</th>
<th>SSTR5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous SS-like peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-14</td>
<td>0.1-2.26</td>
<td>0.2-1.3</td>
<td>0.3-1.6</td>
<td>0.3-1.8</td>
<td>0.2-0.9</td>
</tr>
<tr>
<td>SS-28</td>
<td>0.1-2.2</td>
<td>0.2-4.1</td>
<td>0.3-6.1</td>
<td>0.3-7.9</td>
<td>0.05-0.4</td>
</tr>
<tr>
<td><strong>Short synthetic peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octreotide</td>
<td>290-1140</td>
<td>0.4-2.1</td>
<td>4.4-34.5</td>
<td>&gt;1000</td>
<td>5.6-32</td>
</tr>
<tr>
<td>Lanreotide</td>
<td>500-2330</td>
<td>0.5-1.8</td>
<td>43-107</td>
<td>66-2100</td>
<td>0.6-14</td>
</tr>
<tr>
<td>Vapreotide</td>
<td>&gt;1000</td>
<td>5.4</td>
<td>31</td>
<td>45</td>
<td>0.7</td>
</tr>
<tr>
<td>Seglitide</td>
<td>&gt;1000</td>
<td>0.1-1.5</td>
<td>27-36</td>
<td>127-&gt;1000</td>
<td>2-23</td>
</tr>
</tbody>
</table>

Adapted from (372).

C- and N-terminus modifications, may be required to improve its in vivo stability. Thirdly, consideration must be given to the physiological activity of the peptide. Given that many of the target GPCRs have been shown to play a role in carcinogenesis and metastasis (367), it is vital to ascertain whether the peptide is capable of affecting a biological response. Whether the peptide is an agonist or antagonist, the downstream effects on tumour progression must be evaluated. Fourthly, the selected radionuclide should have properties suitable to its desired application and its incorporation should not interfere with the receptor-binding site of the peptide. Lastly, modifications may be required to improve the pharmacokinetics and toxicity profile of radiolabeled peptides. Kidney damage is the major toxicity associated with all radiolabeled peptides (373). Elimination of lysine residues from the peptide sequence has been shown to decrease renal uptake and retention (374), as has coadministration of lysine with the radiolabeled peptide (375).

1.3.2.2 Radionuclides for peptide labeling

Radionuclide selection should reflect the specific application of the radiolabeled peptide. Radionuclides have specific properties that make some suitable for imaging or therapy exclusively and others useful in both applications (at different doses). The properties of some of the more commonly-used radionuclides are presented in Table 1.5. Gamma-photon and positron emitting radionuclides are used for single-photon emission computed tomography (SPECT) and PET imaging, respectively. Alternatively, emission of α-particles, β-particles or Auger electrons
is required for a radionuclide to be considered as a therapeutic agent. These particles have a much higher linear energy transfer (LET) than $\gamma$-photons and better tissue radiation absorbed dose deposition.

**Table 1.5 Properties and applications of selected radionuclides used in peptide labeling**

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>$T_{1/2}$</th>
<th>Radiation emitted</th>
<th>Energy in keV (abundance)</th>
<th>Max tissue penetration of particle emissions</th>
<th>Clinical use</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}$F</td>
<td>1.82 h</td>
<td>$\beta^+$</td>
<td>634 (97%)</td>
<td>2.3 mm</td>
<td>PET</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>511 (194%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>12.7 h</td>
<td>$\beta^+$</td>
<td>653 (18%)</td>
<td>2 mm</td>
<td>PET, radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\beta^-$</td>
<td>579 (39%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>511 (35.12%), 1345 (0.475%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>67.6 min</td>
<td>$\beta^+$</td>
<td>1900 (87.7%), 822 (1.2%)</td>
<td>8.9 mm</td>
<td>PET, SPECT, radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>511 (178%), 1077 (3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>2.7 d</td>
<td>$\beta^-$</td>
<td>2280 (100%)</td>
<td>12 mm</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>$^{94m}$Tc</td>
<td>52 min</td>
<td>$\beta^+$</td>
<td>2470 (69%)</td>
<td></td>
<td>PET</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>511 (138%), 871 (94%), 875 (1%), 993 (2.2%), 1521 (4.5%), 1869 (5.5%), 2740 (3.5%), 3128 (1.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>6 h</td>
<td>$\gamma$</td>
<td>140 (88.5%)</td>
<td></td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>2.8 d</td>
<td>Auger</td>
<td>&lt;30</td>
<td>10 um</td>
<td>SPECT, radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>171 (90.2%), 245 (94.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13.2 h</td>
<td>Auger</td>
<td>&lt;30</td>
<td>&lt; 10 um</td>
<td>SPECT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>159 (83.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>4.2 d</td>
<td>$\beta^+$</td>
<td>1543 (11.3%), 2146 (11.3%)</td>
<td></td>
<td>PET</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>511 (46%), 603 (61%), 723 (10%), 1691 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radionuclide</td>
<td>Half-Life (d)</td>
<td>Emission Type</td>
<td>Energy Modalities</td>
<td>Penetration</td>
<td>Application</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
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<td>-------------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>60.2</td>
<td>Auger</td>
<td>$&lt;30$ keV</td>
<td>10 nm</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>35.5 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8.05</td>
<td>$\beta^+$</td>
<td>248 (2.1%), 334 (7.2%), 606 (89%)</td>
<td>2 mm</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>80.2 (2.6%), 163 (1.1%), 284 (6.1%), 364 (81%), 637 (7.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>6.7</td>
<td>$\beta^-$</td>
<td>177 (11.6%), 385 (9.1%), 498 (79.3%)</td>
<td>2 mm</td>
<td>SPECT, radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>113 (6.2%), 208 (10.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{188}$Re</td>
<td>17</td>
<td>$\beta^-$</td>
<td>1487 (1.65%), 1965 (25.6%), 2120 (71.1%)</td>
<td>8 mm</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>155 (15.2%), 478 (1%), 633 (1.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>7.2</td>
<td>$\alpha$</td>
<td>5870 (41.8%)</td>
<td>50 - 100 µm</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Auger</td>
<td>$&lt;20$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: h, hour; d, day; min, minutes; PET, positron emission tomography; SPECT, single photon emission computed tomography. Adapted with permission from (241).

One of the key differences between the different radioactive particles is tissue penetration defined by the path length. Alpha-particles are relatively large positively charged particles consisting of 2 protons and 2 neutrons. They have low kinetic energy leading to short tissue penetration (approximately 50-100µm) but have high LET (80-100 keV/µm) and are extremely ionizing. Emitted alpha particles will have one of a defined number of kinetic energies that is equal to the difference between nuclear energy states of the parent and daughter nucleus. Beta-particles ($\beta^-$) are negatively charged electrons released from a decaying nucleus. These lightweight particles, with the same mass as an electron, have deeper tissue penetration (2-12 mm) but much lower LET relative to $\alpha$-particles. Beta-emitters represent the majority of radionuclides currently used in the clinic for targeted radiation therapy. Auger electrons are low energy ($< 30$ keV) particles produced through a specific type of decay called electron capture. Electron capture results in the release of many Auger electrons (approximately 5-30) from a
single decaying atom. As a result of their extremely low energies, Auger electrons have very short path lengths (< 10 µm) and consequently relatively high average LET, which approaches that of more energetic α-particles. Use of radionuclides with longer path lengths allows for the possibility that neighbouring tumour cells not specifically bound by the radiolabeled peptide may still receive ionizing radiation (this is known as the cross-fire effect). Alternatively, short path length radionuclides minimize the potential for damage to surrounding normal tissue when the tumour target is small. Generally, α-particles are indicated primarily for treatment of small tumour nodules and micrometastases while β-particles may be more effective for larger tumours. The effect of Auger electrons is generally localized to the point of decay, which means they mostly affect the specific cell in which the the radionuclide has been internalized; a cross-dose effect on immediately neighbouring cells is also possible. Radionuclides such as $^{64}$Cu, $^{68}$Ga, $^{111}$In and $^{177}$Lu emit multiple types of radiation and can be used for both imaging and therapeutic applications.

### 1.3.3 Radiobiology of radionuclide therapy

Radionuclide therapy differs from traditional EBRT and brachytherapy in a very fundamental way; whereas EBRT and brachytherapy deliver radiation in the form of photons, radionuclide therapy delivers particulate radiation. Ionization is the process by which charged particles (α or β) transfer energy to an electron from another atom causing it to escape. As discussed above, different particles have different ionizing properties and therefore can have varying biological effects. As with other forms of radiation therapy, formation of DNA lesions is believed to be the primary mediator of radionuclide-induced cell death. The type and distribution of the DNA lesions created are dependent on the type of particulate radiation emitted. DSB and multiply damaged sites (MDS) are they most damaging to cells, as they are less amenable to DNA repair than the other types of lesions (e.g. single-strand breaks [SSB] and DNA-protein crosslinks). Highly ionizing particles, such as Auger electrons and α-particles, induce more DSBs than the less ionizing β-particles, and the distribution of lesions is quite different. Whereas α-particle ionizations occur with high density at multiple sites along a linear path reaching a maximum near the end of the track (Bragg’s peak), β-particle ionizations occur primarily at the end of the path length and Auger electron ionizations are distributed irregularly around the decaying atom (reviewed in (376)). In all cases ionization must occur within the nucleus to cause direct DNA damage, which usually only occurs for high LET radiation such as α-particles or
Auger electrons. Ionizing radiation most commonly causes indirect damage through the formation of free radicals which themselves can lead to molecular lesions. Given that the path length of α and β̂ particles can exceed the diameter of a single cell, ionization events may occur in cells not specifically targeted by the radiolabeled mAb or peptide. This phenomenon is called the radiation cross-fire effect and is critical to ensuring a majority of tumour cells, including those not bound by the radiolabeled mAb or peptide, receive a potentially lethal dose of radiation (Figure 1.5).

The downstream biological effects of ionizing radiation are several-fold. One of the early cellular responses to radiation-induced DNA damage is engagement of DNA-repair mechanisms. The fate of a cell is a function of the extent of DNA damage and the ability to repair that damage. Time also plays a critical role, as DNA repair occurs at a specific rate and the rate of dose delivery (dose rate) can affect the likelihood of inducing lethal DNA damage. Sub-lethal lesions, such as SSBs, can be repaired with relatively high fidelity resulting in increased probability of cell survival. Lethal lesions occur with greater frequency following exposure to high LET radiation and with faster delivery rates (less time for DNA repair).

During the process of DNA repair, cells will become arrested at various checkpoints of the cell-cycle. Arrest occurs in both surviving and non-surviving cells and its duration is dose- and genotype-dependent (377). Cells in different stages of the cycle show different delay responses, leading to an accumulation of cells in G2/M and G1/S. Recovery from arrest is dependent on the ability to repair any DNA damage the cell has sustained. Cells with irreparable damage will activate apoptotic pathways and undergo programmed cell death.

Different tumours are differentially susceptible to radiation-induced cell death. This is partially related to additional factors that can contribute to protecting cells from radiation-induced cell death. The oxygenation status of a tumour can greatly affect its sensitivity, with hypoxic cells being more radio-resistant than normoxic cells. This is due to the prominent role of oxygen free radicals in mediating the effects of radiation-induced damage. Mutations in genes involved in DNA-damage sensing and repair, apoptosis and cell survival, as well as cell-cycle check points, can all also significantly affect a cells response to radiation.
Figure 1.5. Maximizing the radiation crossfire effect using rational radionuclide selection. The maximum distance radiation travels within tissues is defined by the radionuclide path length. Depending on the type of radiation emitted from a given radionuclide, the majority of energy will be deposited at the end of the path length or spread out along the entire distance of the path length. (A) Radiation emitted from the radionuclide will travel a given distance (x, y) and deposit its energy in cells along its path length. While radiolabeled peptide binding is receptor specific, radiation damage due to the cross-fire effect has the potential to effect both tumour and normal cells. Therefore, a radionuclide with a shorter path length (x) would be more suitable for treatment of smaller tumours (B), whereas one with a longer path length (y) would be more effective for larger tumours (C). Rational selection of radionuclides for radiopharmaceutical labeling based on the approximate size of the tumour and the radionuclide path length could minimize unwanted radiation damage to surrounding normal cells. Adapted from (241).
1.3.3.1 Radiation bystander effect

In addition to direct and indirect ionization-induced molecular lesions, exposure to radiation can also induce a biological bystander effect. Communication between damaged cells and cells not directly impacted by an ionization event occurs either through gap-junctions (378) or secreted soluble factors (379, 380). The concept that the bystander effect plays a role in targeted radiation-induced cell killing is relatively new and most investigations have looked at it in terms of EBRT, where there is a high dose rate (HDR) with a relatively homogenous distribution throughout the radiation field. Targeted radionuclide therapy delivers low dose rate (LDR), non-uniform radiation over a prolonged period and recent data shows that the contribution of the bystander effect to overall cell killing likely varies between LDR and HDR radiation (381, 382). Whereas downstream biological effects can largely be attributed to dose-dependent DNA damage following HDR radiation, unexpected effects of LDR non-uniform radiation that do not fit the linear non-threshold model, combined with evidence of radiation-induced cell stress responses, has led to the widely held opinion that the bystander effect plays a critical role mainly at low doses. In fact, data suggest that the bystander effect may become saturated at doses as low as 1 Gy (383).

Bystander effects have been implicated in numerous and sometimes opposing cellular response, including: cell death (384), cell survival (385), cell proliferation (386), genomic instability (387), DNA damage (388), cell-cycle alterations (384), and carcinogenesis (389). In the context of cancer therapy, the biological bystander effect can represent either a therapeutic advantage or liability depending on the downstream outcome (e.g. cell death vs survival) and target (e.g. tumour vs. normal cells). Whereas gap-junction-mediated bystander effects are localized - inducing effects in cells in close proximity to the radiation-damaged cell, bystander effects mediated by soluble factors can occur on a systemic scale. This can be due to the systemic circulation of these factors and/or their downstream effects on systemically circulating cells such as innate and adaptive immune cells. The mediators of the bystander effect are not fully understood, but it is known that they largely belong to a collection of stress-response pathways, and include: damage-associated molecular patterns (DAMPs; e.g. HMGB) (390), cytokines (391-393), p53 (394) and reactive oxygen and nitrogen species (395).
In vitro experiments have demonstrated that media transferred from cells exposed to radionuclide therapy significantly affects the clonogenic survival of nonirradiated cells (379). Some studies suggest that the bystander effect from LDR radiation shows a dose- and LET-dependent relationship (396, 397), although others have suggested such a statement cannot be broadly applied due to the contribution of many factors including genetics, epigenetics and tissue (malignant vs. normal) or tumour type on downstream outcomes of the bystander effect (381). Nevertheless, data has confirmed the importance of the biological bystander effect in LDR radionuclide therapy in in vivo preclinical tumour models (398). Media transfer experiments have also consistently shown that conditioned media from cells treated with α- and/or β-emitters induces cell killing in non-irradiated cells (384, 388, 393, 399).

Tumour xenografts containing small fractions of either pre-irradiated cells or receptor-expressing cells (e.g. NAT) that can be targeted by radiolabeled peptides have been used to demonstrate in vivo bystander effects. In both cases, significant tumour growth reduction beyond that expected based on the proportion of irradiation cells was observed (396, 398). Animal data have also highlighted the important role of macrophages and other immune cells in mediating the radiation bystander effect – albeit in the context of EBRT. Given that induction of cytokines is one of the cellular responses to radiation exposure, it is not surprising that EBRT leads to immunomodulation. Macrophages are themselves relatively radioresistant, showing minimal cell death or transcriptional activation of cell death pathways shortly after in vitro irradiation (400). Comparison of mouse bone marrow-derived macrophage activation following in vitro and in vivo irradiation shows that while direct in vitro irradiation did not significantly affect markers of activation (e.g. nitric oxide synthase (NOS)-2 or arginase), full body irradiation significantly altered macrophage activation in a genotype-dependent manner (400). There is ample evidence that mouse strains with different genetic backgrounds produce different categories of cytokines (e.g. Th1 vs. Th2 or M1 vs. M2) in response to radiation, leading to different downstream effects (401). At present, a better understanding of the mediators of the biological bystander effect as well as the genetic and epigenetic factors that influence its outcome is still required. In the future, this knowledge could be combined with pretreatment dosimetry to develop personalized treatment plans with improved clinical outcomes.
1.3.4  Somatostatin imaging and PRRT

1.3.4.1  Introduction

Somatostatin (SS) receptor scintigraphy (SRS) is an imaging technique developed in 1989 (402), following the successful use of SS analogues in the treatment of acromegaly (403). SRS allowed clinicians to visualize SSTR-positive tumours using gamma camera imaging after systemic administration of radiolabeled SS analogues. SRS continues to be one of the best imaging techniques for the diagnosis and staging of SSTR-positive tumours (404). Development of novel peptides labeled with α- or β-emitting radionuclides led to the first preclinical and clinical studies of SSTR PRRT (405-408). Today, SSTR PRRT is used for the treatment of a variety of SSTR-positive cancers and new radiolabeled analogues with improved affinity, stability, pharmacokinetics and toxicity profiles continue to be investigated (409, 410).

1.3.4.2  Somatostatin and somatostatin receptors

Somatostatin (SS) is a small cyclic peptide naturally existing in 2 active forms (SS-14 and SS-28) that result from the proteolytic cleavage of the 116 amino acid propresomatostatin precursor (411, 412). SS binds to SSTRs of which there are 5 known subtypes (SSTR1-5) (413-416). Ligand binding triggers these GPCRs to associate with intracellular G proteins and results in the activation of a variety of intracellular signal transduction pathways. SS is an inhibitory molecule that is involved in a variety of diverse biological functions (417) including: 1) inhibiting the secretion of numerous hormones, pancreatic enzymes, bile and colonic fluids; 2) inhibiting GI motility and nutrient absorption; 3) modulating CNS neurotransmission; and 4) autocrine and paracrine regulation of cell proliferation. Each SSTR subtype has been demonstrated to mediate unique downstream signaling cascades (reviewed (372)) and the entire SS/SSTR system has been implicated in a variety of disease states including cancer (reviewed in (418-420)).

SSTR expression is present in organs throughout the body with each receptor subtype showing a different pattern of expression and different physiological functions. One or more receptor subtypes are expressed in the brain, pituitary, pancreas, stomach, salivary gland, liver, kidney, lymphocytes, lungs, testes, ovaries, thyroid, immune cells, intestines and myocardium. SS itself is produced both in the central nervous system (hypothalamus, deep cortex, limbic
tissue, striatum and periaqueductal central grey) as well as in organs throughout the body (pancreas, gut, thyroid, adrenals, kidneys, prostate and placenta) (372). Typically, SS-expressing cells are morphologically similar to either neurons or secretory cells (δ cells). SS production also occurs in various immune and tumour cells, albeit upstream activation – likely mediated by proinflammatory cytokines (421) – is typically required and the quantities produced are much lower than in other SS-producing cells (418, 421-423).

1.3.4.3 Somatostatin and somatostatin receptors in cancer

Elevated SSTR expression is present in numerous primary tumours and cancer cell lines. SSTR overexpression occurs predominately in neuroendocrine and nervous system tumours, however various epithelial tumours, soft tissue sarcomas, melanomas, lymphomas and thymomas have also demonstrated high levels of SSTR. Of the different subtypes, SSTR2 is the most commonly detected in tumours (424).

Due to their ability to inhibit hormone secretion, SS and SS-analogues were originally investigated for use in treatment of acromegaly, a hormone hyper-secreting tumour (403). Observations of accompanying tumour shrinkage led to more in-depth investigations of the downstream targets of SSTR signaling (425). SS has since been found to induce anti-proliferative and apoptotic effects in tumour cells by direct or indirect mechanisms (reviewed in (420, 426)). Signaling through SSTRs down-regulates other pathways, including the MAPK cascade, leading to direct inhibition of tumour cell proliferation (424). Alternatively, inhibition of hormone and growth factor secretion from normal cells indirectly limits tumour growth (427). Not surprisingly, loss of SSTR expression may be linked with a growth advantage in some tumours (428), likely due to the loss of the inhibitory effects mediated by SS signaling.

1.3.4.4 Radiolabeled somatostatin analogues

In the late 1980s, the first-generation SS analogue, octreotide, was modified through radio-iodination to yield the first radiolabeled somatostatin analogue (402, 429). Octreotide shows similar affinity for SSTR2 as endogenous SS (430), but demonstrates greater in vivo stability and improved inhibition of hormone production (431). The other first generation SS analogues developed were lanreotide and vapreotide, which show different binding affinities and toxicity profiles (432). Second-generation radiolabeled SS analogues were modified with metal
chelators such as DTPA and DOTA in order to facilitate their labeling with heavy metal radionuclides such as $^{111}$In, $^{177}$Lu and $^{90}$Y. The addition of the metal chelators and radionuclides as well as modification of the C-terminal amino acid in [Tyr$^3$]octreotide to give the more negatively charged [Tyr$^3$]octreotate, significantly affected the affinity profiles of the peptides (430). For example [DOTA$^0$,Tyr$^3$]octreotate (DOTATATE) has the highest affinity for SSTR2 with [DTPA$^0$,Tyr$^3$]octreotate, [DTPA$^0$,Tyr$^3$]octreotide and [DOTA$^0$,Tyr$^3$]octreotide (DOTATOC) having sequentially lower binding affinities (430). Figure 1.6 provides a schematic representation of the structure of various SS analogues. In clinical studies both $^{111}$In- and $^{177}$Lu-DOTATATE internalization were increased relative to $^{111}$In- and $^{177}$Lu-DOTATOC when uptake of paired radioligands was evaluated in the same patient (433, 434). Overall, the difference in tumour uptake between the peptides is marginal and varies between patients and tumours (435). It is important to note that DOTATATE is also associated with increased kidney and spleen uptake (434-438). Similar findings have also been reported for preclinical mouse and rat tumour models (436, 439).

1.3.4.5 SSTR imaging and dosimetry

Imaging using radiolabeled SS-analogue tracers plays an important role in diagnosis and staging of SSTR-positive tumours. Additionally, serial imaging is used to perform dosimetric calculations, which are critical to the development of a treatment plan. Dosimetry studies are performed in patients receiving tracer quantities of radiolabeled peptides in order to calculate the estimated dose to critical normal tissues as well as the tumour when administered at a higher therapeutic dose of radioactivity. The ratio of absorbed dose in critical tissues and tumour can be used to determine whether the toxicity risks are acceptable and/or whether a patient is likely to benefit from PRRT (440, 441).

When SSTR-based imaging was first introduced, the primary imaging modality was full-body planar gamma scintigraphy. This is sufficient for identifying SSTR-positive tumours and determining kinetics of retention and elimination; but the 2-dimensional images lack the volumetric data required for accurate dosimetric calculations. Today, SPECT and SPECT/CT tomographic imaging are used to provide greater resolution of intra-organ uptake. A specialized
Figure 1.6. Structures of selected somatostatin analogues and chelators. (A) Octreotide has been modified through substitution of phenyalanin at position three with tyrosine to give [Tyr\(^3\)]octreotide and by oxidation of the C-terminal amino acid to give [Tyr\(^3\)]octreotate. (B) Chemical structure of the metal chelator DOTA. (C) Structure of \(^{177}\)Lu-DOTATOC.

Software program (OLINDA) calculates dose estimates to individual organs based on the Medical Internal Radiation Dose schema. Due to significant inter-patient variability in the uptake of radiolabeled peptides, pretreatment dosimetry studies must be performed on an individual basis. Nevertheless, synthesis of data from clinical dosimetry studies using \(^{177}\)Lu-DOTATATE and \(^{90}\)Y-DOTATOC has revealed several generally-applicable principles of PRRT with SS...
analogues, as reported by Cremonesi et al. (442): (1) whole body exposure is low due to rapid blood clearance and renal elimination; (2) highest absorbed doses occur in the kidneys, spleen and liver; and (3) renal impairment is the DLT. Assessment of the potential toxicity risks requires consideration of several factors in addition to the estimated absorbed dose, including: tissue radiosensitivity, dose rate, fractionation and patient risk factors.

Dosimetry based on tissue biodistribution also has an important role in preclinical studies. It provides investigators with information about how well a given radiolabeled peptide is performing \textit{in vivo}. Various tumour-to-normal tissue ratio calculations can be used to predict efficacy and toxicity, thereby identifying novel treatments with potential and those that require further modification before moving forward. Preclinical dosimetry and biodistribution studies typically involves measuring activity in tissues harvested at several time points post-radioligand delivery. With the development of new micro-imaging technologies (443-445), designed for preclinical animal models, it is now also possible to obtain biodistribution information through imaging studies. The advantage of imaging-based biodistribution studies is that they generate data from an individual mouse over time, but unfortunately these studies can be very costly. Consistent with clinical findings (405, 441, 446, 447), preclinical studies (448) have also identified the kidneys as the primary critical organ and bone marrow as the secondary site of potential toxicity following SS-analogue PRRT.

The complexity of dosimetric calculations can vary based on the specific radionuclide used as well as the question(s) the study aims to address. Due to the fact that the radiation source is internal, the most accurate dose calculations have to take into consideration cross-organ radiation. Cross-organ radiation calculations in preclinical models are relevant for understanding how a particular radioligand behaves in that model, but these calculations are less relevant in projecting how it will behave in patients. The volumes and distances separating organs are substantially smaller in preclinical animal models relative to humans; as a result, a greater proportion of the absorbed dose in any given organ will be due to cross-organ radiation from a proximal organ in preclinical models. Furthermore, for radionuclides with extremely short path lengths (e.g. Auger electron-emitters), cross-organ radiation may not be a concern, even in preclinical models.
In order for preclinical dosimetry data to most closely resemble what is expected in patients, it is important that the model also reflect the clinical disease. The pharmacokinetics and pharmacodynamics of a radiolabeled peptide can be substantially altered based on the location of a tumour (e.g. subcutaneous vs. intracranial) and extent of disease burden, as the ability of the agent to access the tumour is likely to be different. For example, tumours with high levels of receptor expression can significantly alter the blood pharmacokinetics by sequestering the radiolabeled peptide. Similarly, blood pharmacokinetics often differ substantially between non-tumour-bearing and tumour-bearing mice.

1.3.4.6 SSTR PRRT

Several phase I and II clinical trials have looked at PRRT in patients with SSTR-positive gastroenteropancreatic neuroendocrine tumours. A variety of different radiolabeled peptides have been investigated including: $^{111}$In-DTPAOC (407, 449-452), $^{90}$Y-DOTATOC (441, 446, 453-460), $^{90}$Y-DOTALAN (406), $^{90}$Y-DOTATATE (461-463) and $^{177}$Lu-DOTATATE (464, 465). The results of recent trials using $^{177}$Lu- and/or $^{90}$Y- labeled DOTA-TOC and/or -TATE are summarized in Table 1.6. Overall the results of these trials have been favourable. In 2 of largest studies to date, 46% and 34% of patients showed either a complete, partial or minor response following treatment with $^{177}$Lu-DOTATATE (465) or $^{90}$Y-DOTATOC (441), respectively. Furthermore, patients treated with $^{90}$Y-DOTATOC or $^{177}$Lu-DOTATATE showed a significant survival advantage compared to historical controls (441, 457, 465). A multi-centre phase III trial comparing $^{177}$Lu-DOTATATE and Octreotide in patients with advanced midgut carcinoids is currently recruiting patients (NCT01578239).

In the 504 patients treated with $^{177}$Lu-DOTATATE, the major common acute adverse effects were nausea (25% of administrations), vomiting (10% of administrations) and abdominal discomfort (10% of administrations) (465). Sub-acute grade 3/4 hematological toxicity occurred in 9.5% of patients and reversible grade 1 hair loss occurred in 62% of patients. They also reported serious delayed toxicities in 9 patients, although most were considered unrelated to treatment. Toxicities included: renal insufficiency (2 patients), serious liver toxicity (3 patients) and myelodysplastic syndrome (1 patient). Three patients experienced treatment-related myelodysplastic syndrome between 2 to 3 y after $^{177}$Lu-DOTATATE.
<table>
<thead>
<tr>
<th>Study Authors (year of publication)</th>
<th>Phase (no. patients)</th>
<th>Disease</th>
<th>Response</th>
<th>Major Toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>90Y-DOTATOC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waldherr et al. (2001) (454)</td>
<td>2 (41)</td>
<td>GEP NETs and bronchial tumours</td>
<td>WHO: 1 CR; 9 PR; 5 MR; 20 SD; 6 PD</td>
<td>Grade 3: pancytopenia</td>
</tr>
<tr>
<td>Waldherr et al. (2002) (455)</td>
<td>2 (39)</td>
<td>GEP NETs and bronchial tumours</td>
<td>WHO: 2 CR; 7 PR; 37 SD; 3 PD</td>
<td>Grade 3: lymphocytopenia, anemia, renal insufficiency</td>
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<td>Bodei et al. (2003) (456)</td>
<td>1 (40)</td>
<td>SSTR2+</td>
<td>n/a</td>
<td>Grade 3/4: lymphocytopenia</td>
</tr>
<tr>
<td>Valkemia et al. (2006) (457)</td>
<td>1 (58)</td>
<td>GEP NETs</td>
<td>5 PR; 7 MR; 29 SD; 17 PD OS 29.3 mo</td>
<td>2 DLTs: hepatic toxicity, thrombocytopenia Chronic: MDS, end-stage renal disease</td>
</tr>
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<td>Imhof et al. (2011) (441)</td>
<td>2 (1,109)</td>
<td>Metastatic NETs</td>
<td>Morphologic response, 34.1%; biochemical response, 15.5%; clinical response, 29.7% RECIST (applied to 159 ptns): 7 CR; 72 PR; 80 SD Median OS 94.6 mo</td>
<td>Severe transient: hematologic, acute kidney failure, tumour lyses syndrome Myeloproliferative: MDS, AML Severe permanent: renal toxicity</td>
</tr>
<tr>
<td>Savelli et al. (2013) (458)</td>
<td>2A (38)</td>
<td>GEP NETs</td>
<td>RECIST: 17 PR; 10 SD; 11 PD</td>
<td>Severe permanent: renal toxicity</td>
</tr>
<tr>
<td>Marincek et al. (2013) (459)</td>
<td>1 (359)</td>
<td>Metastatic NETs</td>
<td>Low dose (n = 60): 11 SD/PR Int dose (n = 77): 21 SD/PR High Dose (n = 222): 83 SD/PR</td>
<td>Grade 3/4: leukopenia, anemia, thrombocytopenia Myeloproliferative: AML Severe permanent: renal toxicity</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Cohort</td>
<td>NETs</td>
<td>Results</td>
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<tr>
<td>Sowa-Staszczak et al. (2011) (461)</td>
<td>Cohort (50)</td>
<td>NETs</td>
<td>$^{90}Y$ (n= 25): 26.2 mo $^{90}Y/^{177}Lu$ (n =25): not reached</td>
<td>Severe transient: leukopenia, thrombocytopenia, anemia</td>
</tr>
<tr>
<td>Vinjamuri et al. (2013) (462)</td>
<td>Retrospective (57)</td>
<td>Metastatic NETS</td>
<td>RECIST: PR 14, SD 21, PD 4</td>
<td>Severe transient: leukopenia, thrombocytopenia, anemia</td>
</tr>
<tr>
<td>Romer et al. (2014) (463)</td>
<td>Comparative Cohort (910)</td>
<td>NETs</td>
<td>Median OS: $^{90}Y$ (n= 910): 35.9 mo $^{177}Lu$ (n =141): 45.5 mo</td>
<td>Severe transient: haematotoxicity ($^{90}Y &gt; ^{177}Lu$) Severe permanent: renal toxicity ($^{90}Y = ^{177}Lu$)</td>
</tr>
<tr>
<td>Kwekkeboom et al. (2008) (464)</td>
<td>2 (504)</td>
<td>GEP NETs, carcinoids, NETs (unknown origin)</td>
<td>SWOG (310 evaluable patients): 5 CR; 86 PR; 51 MR; 107 SD; 61 PD</td>
<td>Grade 3/4: haematotoxicity Serious delayed: renal insufficiency, hepatic toxicity, MDS</td>
</tr>
<tr>
<td>Bodei et al. (2011) (465)</td>
<td>1/2 (51)</td>
<td>Metastatic SSTR2+</td>
<td>RECIST: 1 CR; 14 PR; 13 SD; 9 PD</td>
<td>Severe transient: lymphocytopenia, thrombocytopenia Long term: decreased creatinine clearance</td>
</tr>
</tbody>
</table>

Abbreviations: GEP, gastroenteropancreatic; NET, neuroendocrine tumour; WHO, World Health Organization (response criteria); RECIST, Response Evaluation Criteria in Solid Tumours; SSTR, somatostatin receptor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; MR, minor response; OS, overall survival; mo, months; MDS, myelodysplastic syndrome; AML, acute myelocytic leukemia; Int, intermediate; SWOG, Southwestern Oncology Group (response criteria)
1.4 Combination Treatment Strategies

Cancer is a complex and heterogeneous disease for which successful treatment are rarely simple. Today, there are very few therapies that are administered alone. Combination strategies were born out of lessons learnt from the antibiotic treatment of tuberculosis; use of drug combinations, each with a different mechanism of action, sig improves therapy efficacy. This is a lesson in medicine that has played out particularly in diseases where there is an element of evolution, change or development of resistance as is the case with cancer. OVs have been investigated in combination with different conventional and experimental cancer therapies including chemotherapy, therapy, biologic therapies (reviewed in (466); see Appendix 1.1) and surgery (199).

Combining OVs with conventional therapies is appealing, as it is more cost effective faces fewer regulatory hurdles than do experimental therapies. Rational pairing of viral other therapies, based on considerations of their individual mechanisms of action and the specific cancer type, should maximize the potential for discovering synergistic com Broadly defined, synergy is an effect greater than the expected additive effect of 2 or more is the ultimate objective when investigating combination therapies as this will typically dose reductions, decreased toxicity and delayed development of resistance. The combin index (CI) developed by Chou and Talalay is one of the most widely-used methods for quantifying synergy (467). Based on the median effect equation, this method provides a mechanism- and unit-independent means of determining synergy using a relatively small number of data points. This method has been used frequently to describe the relationship between and chemo- or radiation therapy, in many instances identifying synergistic interactions. often than not, the exact mechanism of interaction is not fully understood, but because t and Talalay combination index is mechanism-independent this does not preclude dete of synergy. Interactions can be broadly categorized in the following ways without know the exact molecular mediators/pathways involved: (1) drug/radiation-induced cellular leading to enhanced virus replication/spread; (2) drug/radiation-mediated enhancement replication/spread through direct action on the virus; (3) virus-mediated sensitization of cells to drug/radiation treatment; and (4) virus-mediated sensitization of uninfected cell: drug/radiation treatment.
1.4.1 OVs and chemotherapy

Viruses engineered to encode prodrug-activating enzymes allow for targeted combination therapy by directing specific accumulation of the cytotoxic drug in areas of tumour infection. This approach, termed gene-directed enzyme prodrug therapy (GDEPT), is attractive as it promotes a bystander effect wherein non-infected tumour cells are killed while minimizing drug-induced toxicity in uninfected normal cells. One of the more common prodrug-activating enzyme used is cytosine-deaminase (CD), which converts non-toxic 5-fluorocytosine (5-FC) to cytotoxic 5-FU (196, 468-485). Tk-deleted and double-deleted VVs encoding CD have demonstrated improved therapeutic effect when combined with 5-FC in murine models of primary (475) and metastatic (486) CRC and ovarian carcinomatosis (196). The addition of 5-FU allowed for virus dose reductions in vivo while still achieving comparable tumour growth inhibition (475). While the results with the tk-deleted mutant were complicated by some virus-associated toxicity (475), vvDD-CD was well-tolerated in both immunosuppressed and immunocompetent models (196).

Viruses encoding CES, the enzyme responsible for conversion of CPT-11 into SN-38, have also been engineered to increase the concentration of the cytotoxic metabolite at the tumour site (478, 487-489). An oncolytic HSV vector encoding both secreted human iCES and CYP2B1 – the enzyme responsible for activation of CPA – has been tested for efficacy and safety in models of glioma (478, 488). Individually, these prodrugs significantly improved both in vitro and in vivo glioma cell killing relative to virus alone and this effect was further enhanced by delivery of both prodrugs (488). In CRC cells, infection with a CRAd expressing secreted human CES2 sensitized cells to CPT-11 treatment despite drug-induced decreases in virus replication (487).

There are many other examples of OVs being successfully combined with common chemotherapy drugs belonging to a range of drug classes including alkylating agents [cisplatin, OX, temozolamide, cyclophosphamide (CPA) and MMC], nucleotide analogues (5-FU and GCV), cellular cytoskeleton modifiers (paclitaxel and docetaxel), topoisomerase inhibitors (CPT-11, doxorubicin, mitoxantrone, etoposide) and cytostatic agents (rapamycin).

One of the most well-studied examples of OV combination therapy synergy is that of γ34.5-deleted HSVs with various chemotherapy drugs, including cisplatin (490), MMC (491-494), temozolimide (491) and external beam radiation (discussed in 1.4.2). When combined with these treatments, γ1,34.5-deleted HSVs have consistently demonstrated improved virus replication
and therapeutic efficacy. The mechanism of this interaction was first described with reference to EBRT and is discussed in the following section.

VV therapy has also been enhanced through combination with paclitaxel (495), cisplatin (496), gemcitabine (496), rapamycin (195, 232) and cyclophosphamide (195, 497). In most of these cases the precise mechanism of interaction is not fully understood. VV has complex mechanisms of action and determining the mechanism of interaction can be made all the more challenging when the drug also has multiple targets. For example, in addition to its alkylating property cyclophosphamide has many different immunodulatory properties, including decreasing neutralizing antibodies (498), immune cell infiltration (195, 499) and Treg function (500), all of which can lead to increased virus replication and spread (195). In the case of paclitaxel, VV was found to sensitize cells to drug-mediated apoptosis (495). vvDD and paclitaxel synergistically improved cell killing in a panel of murine and human tumour cell lines in vitro and the combination significantly improved survival relative to either monotherapy in nude mice bearing SC human CRC tumours. Synergy occurred despite an overall decrease in virus gene expression and replication following paclitaxel treatment. Further investigations revealed that conditioned media from infected cells taken at 6 hpi and 24 hpi (but not 12 hpi) was capable of sensitizing cells to paclitaxel. Sensitization was attributed, at least in part, to virus-induced release of IFN early after infection (6 hpi) and high-mobility group box 1 (HMGB1) release following virus-induced cell lysis (24 hpi). This is an example of how a virus-induced bystander effect can synergize with a secondary therapy, be it chemo- or radiation-therapy, while having no/minimal direct therapeutic effect (Figure 1.7).

To date, there have been only a handful of studies investigating OVAs in combination with the CRC drugs CPT-11 (488, 501-506) and OX (504, 507, 508). The relationship between OVAs and CPT-11 appears to vary widely between virus and cell type. CPT-11 increased Ad cytotoxicity in a replication-dependent manner in glioma cell lines (506) and a replication-independent manner in pancreatic cell lines (503). In an ovarian carcinomatosis model, improved survival following CPT-11 and oncolytic sindbis virus therapy occurred only in the presence of functional NK cells (505). In one study, synergistic improvements in CRC cell killing were observed following HSV and CPT-11 treatment in vitro (504). In contrast, another study found
Figure 1.7. The biological bystander effect mediated by radiation/chemotherapy damage or viral infection. Danger signals and their downstream responses induced by the either virus, radiation or chemotherapy can sensitize unaffected cells to therapy. Reprinted with permission from (241).

that HSV replication and oncolysis was inhibited by treatment with CPT-11 in CRC cells (509). Using a gene therapy approach, CPT-11 was combined with HSV encoding secreted hCES with the goal of increasing the concentration of SN-38 metabolite at the site of the tumour (488). Currently, to our knowledge there are no published studies looking at the interaction between VV and CPT-11 (or any other Top1 inhibitor).

The older platinum-based drug cisplatin has been studied fairly extensively in combination with OVs (466, 510), yet investigations into the combination with OX remain few. OX resulted in an additive improvement in CRC cell killing when combined with reovirus (508) and synergistic improvement when combined with HSV (504). In both studies the mechanism of
interaction was not defined. When combined with VV expressing TNF-related apoptosis inducing ligand (TRAIL), OX led to increased induction of apoptosis and improved survival of tumour bearing mice in a transgene-dependent fashion (507).

1.4.2 OVs and radiation therapy

The combination of virotherapy and radiation therapy for the treatment of cancer has been investigated in numerous preclinical models using a variety of different viruses and radiation sources (466, 511). Interactions between EBRT and OVs have been investigated with Ad (512), HSV (513-519), VSV (520), MV (521, 522) and VV (523-526) and are generally virus-, dose-, model- and timing-dependent (516, 517, 523, 526). The mechanisms underlying these interactions vary and not all are fully understood. The most well-characterized synergistic interaction is that between radiation and γ134.5-deleted HSVs (513-519). The γ134.5 gene, encoding for the ICP 34.5 protein, is deleted in many attenuated oncolytic HSVs due to its involvement in neurotoxicity (527). Deletion of the γ134.5 gene improves the safety profile of oncolytic HSVs, but this comes at the cost of slightly decreasing replication efficiency in tumour cells (528). Interestingly, ICP 34.5 shows structural homology to growth arrest and DNA damage protein 34 (GADD34) (529), a protein involved in DNA repair that is upregulated in response to radiation-induced DNA damage. Numerous studies have shown that combining γ134.5-deleted HSVs with EBRT synergistically improves tumour cell killing in a GADD34-dependent manner (513, 514, 516-518), increases virus replication (513, 514, 516, 517, 519) and improves in vivo tumour response (513-519) (Figure 1.8).

Synergistic interactions between EBRT and VV have been attributed to enhancement of virus replication with an unattenuated LIVP strain (523) but not with the more attenuated versions of the same strain (524-526). In the latter cases, combination therapy significantly increased tumour cell apoptosis in head and neck (524), malignant melanoma (525) and pancreatic tumour models (526). This interaction was cell line-dependent in all models and associated with the presence of specific BRAF mutations in the melanoma cell lines (525). These studies highlight the potential for synergistic interactions between radiation therapy and OVs. Even so, as discussed previously, due to the off-target effects in surrounding normal tissues, EBRT is not suitable for cancers with widespread tumour dissemination. Therefore, a more targeted approach to combining OVs with radiation is required.
1.4.2.1 Viruses and PRRT

Currently, effective application of PRRT in a more diverse range of cancers is limited by the reliance on the receptor expression status of the tumour; many cancers are in fact associated with

![Diagram](image)

**Figure 1.8. Model of the mechanism of synergy between γ34.5-deleted herpes viruses and conventional cancer therapies.** γ34.5 shows significant structural homology to a portion of human GADD34, a protein involved in the cells response to DNA damage. γ34.5 is responsible for dephosphorylation of the eukaryotic translation initiation factor eIF-2α, which is required for translation of both host and viral proteins. Radiation or chemotherapy-induced upregulation of GADD34 functionally replaces the γ34.5 protein in infected tumour cells leading to increased viral protein synthesis and production of infectious virus particles. Reprinted with permission from (466).
overexpression of specific receptors. That said, there is significant heterogeneity in which receptors are overexpressed between cancer types and even between patients with the same cancer type. Additionally, heterogeneity of receptor expression within an individual patient and/or loss of receptor expression can also limit the efficacy of PRRT. Combining PRRT with a gene therapy approach can effectively eliminate the requirement of endogenous overexpression of a tumour-specific receptor. Viral vectors encoding a ‘receptor of choice’ can specifically direct receptor expression in tumour cells thereby converting a previously receptor-negative tumour. To date, viral delivery of 5 different receptors has been investigated as a strategy to make receptor-negative tumours amenable to radiolabeled peptide imaging or therapy. These receptors are: SSTR2, sodium iodide symporter (NIS), GRP-R, norepinephrine transporter (NET) and dopamine receptor (DRD2) (Table 1.7). NIS is unique among these receptors as its ligand is the radionuclide itself (radioactive iodine); therefore, strictly speaking it cannot be classified as PRRT.

The above-described receptors have been delivered to tumours using both non-replicating and replicating viruses. The former gene transfer strategy has been accomplished using replication incompetent Ad and retroviruses. While the use of non-replicating viruses for delivery of sstr2 (530-533), nis (534-536), net (537, 538), grp-r (539-541), and d2r (542, 543) genes has resulted in specific accumulation of radionuclides in both tumour cell lines and in vivo tumour models, this strategy is significantly limited by the poor transduction efficiency, tumour penetration, and persistence these vectors have displayed. This limitation was demonstrated in a study that compared in vivo$^{99\text{Tc}}\text{O}_4^-$ uptake following local injection of an hNIS-expressing replication-competent or -incompetent Ad. The replication-competent virus resulted in higher overall gene expression, greater spread from the site of injection and longer persistence (536).

Tumour-specific replicating viruses are less susceptible to the limitations of replication-incompetent vectors and can themselves induce anti-tumour effects. Replicating viruses have
### Table 1.7 Overview of virally-delivered receptors/transporters used with PRRT

| Abbreviations: GRPR, G-protein coupled receptor; NET, neuroendocrine tumour; RCC, renal cell carcinoma; CRC, colorectal cancer; SS, somatostatin; DTPA, diethylene triamine pentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOTATOC, DOTA-[Tyr³]octreotide; DOTALAN, DOTATATE, DOTANOC, DOTANOC-ATE, DOTA-BOC-ATE, SOM230, P2045, SMT 487; 68Ga, 90Y, 90mTc, 90Re, 111In, 177Lu, 186Re, 18F, 64Cu, 68Ga, 99mTc, 111In, 125I, 11C, 123I, 124I, 131I, 211At. Adapted from (241). |

<table>
<thead>
<tr>
<th><strong>Distribution in Normal Tissues</strong></th>
<th><strong>Distribution in Cancers</strong></th>
<th><strong>cDNA Size</strong></th>
<th><strong>Peptide Ligands</strong></th>
<th><strong>Radionuclides</strong></th>
<th><strong>Viral Vectors</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SSTR2</strong></td>
<td>Brain, pituitary, pancreas, islets, stomach, kidney, spleen, adrenal glands</td>
<td>NETs, epithelial tumours (breast, lung, pancreaticobiliary tract, liver, colorectal, follicular thyroid and prostate), meningioma, glioma, soft tissue sarcoma, malignant melanoma, lymphoma, thymoma, medulloblastoma, RCC, hepatoma, inactive pituitary adenoma, growth hormone-producing pituitary adenoma, gastric carcinoma</td>
<td>3000 bp</td>
<td>SS-14, SS-28, Octreotide, Lanreotide, DTPA-octreotide (Pentetreotide), DOTATOC, DOTALAN, DOTATATE, DOTANOC, DOTANOC-ATE, DOTA-BOC-ATE, SOM230, P2045, SMT 487</td>
<td>68Ga, 90Y, 90mTc, 90Re, 111In, 177Lu, 186Re</td>
</tr>
<tr>
<td><strong>GR-P-R</strong></td>
<td>Brain, brain stem, spinal cord, testis, stomach, smooth muscles of gastrointestinal and urogenital tracts</td>
<td>Glioblastoma small lung cell carcinoma, non-small lung cell carcinoma, gastrointestinal carcinoids, CRC, prostate cancer, cervical cancer, RCC</td>
<td>1227 bp</td>
<td>Bombesin, Tyr³-Bombesin, mIP-Bombesin, Gastrin releasing peptide</td>
<td>18F, 64Cu, 68Ga, 99mTc, 111In, 125I</td>
</tr>
<tr>
<td><strong>D2R</strong></td>
<td>Brain, lymphocytes, lungs</td>
<td>Esophageal squamous cell carcinoma, lung carcinoma</td>
<td>1372 bp</td>
<td>Raclopride, Nemonapride, Spiperone, N-methylspiperone, FESP</td>
<td>3H, 11C, 18F</td>
</tr>
<tr>
<td><strong>NIS</strong></td>
<td>Thyroid, salivary gland, stomach, lactating mammary glands, nasal mucosa, placenta, thymus, hair follicles, prostate, ovaries</td>
<td>Differentiated thyroid cancer, breast cancer</td>
<td>3595 bp</td>
<td>N/A</td>
<td>99mTc, 123I, 124I, 125I, 131I, 211At</td>
</tr>
<tr>
<td><strong>NET</strong></td>
<td>Brain, sympathetic nervous system, placenta, adrenal gland</td>
<td>Pheochromocytoma, gastrointestinal NETs</td>
<td>1915 bp</td>
<td>MIBG, mHED</td>
<td>11C, 123I, 124I, 131I</td>
</tr>
</tbody>
</table>

**Abbreviations:** Ad, adenovirus; VV, vaccinia virus; VSV, vesicular stomatitis virus; MV, measles virus.
additional benefits over their non-replicating counterparts. In an effort to achieve maximal transduction efficiency, non-replicating viruses are almost exclusively administered intratumourally. On the other hand, replicating viruses can be administered systemically leading to gene transfer into both the primary tumour and distant metastases. Given that metastases are typically less likely to be amenable to surgical resection, therapies that reach these tumours could significantly improve patient outcomes. Replication-incompetent viruses continue to be studied as receptor delivery vehicles, including in a recent phase I trial of an sstr2-expressing Ad (544), interest, however, appears to be moving increasingly towards the use of OVs. Studies have focused primarily on the delivery of $nis$ (536, 545-571) likely due to the commercial availability of clinically-approved radioligands that can be readily used for imaging and/or radiotherapy.

Expression of NIS from a range of oncolytic viruses, including Ad (536, 547-556), MV (557-567), VSV (568), VV (569, 570) and HSV (571) has permitted investigation of noninvasive imaging (536, 547-551, 553-560, 563-571) and combined radiovirotherapy (548-552, 558, 559, 561, 564, 568-571) using $^{99m}$Tc or radioiodine. NIS expressing viruses have been studied in a range of preclinical tumour models, including traditionally radiosensitive models of multiple myeloma (563, 568), glioma (567), medulloblastoma (561) and breast cancer (550, 569, 570), as well as in models of disease that are not traditionally treated with radiation therapy such as peritoneally disseminated ovarian cancer (555, 565). Intraperitoneal delivery of MV-NIS (565) or AdAM7 (Ad expressing NIS) (555) to mice bearing intraperitoneal ovarian tumours resulted in specific-tumour accumulation of $^{99m}$Tc demonstrated by gamma camera imaging and SPECT/CT. Importantly, the strong signal observed in the stomach, an expected site of non-specific $^{99m}$Tc uptake (572), did not prevent visualization of the nearby intraperitoneal tumours; even when using a planar imaging modality (565).

Several important factors have been borne out in the OV-NIS studies that can be applied to any targeted radiovirotherapy strategy. Firstly, there is likely a minimum threshold of virus required in the tumour to permit detectable tumour-specific accumulation of a tracer radioligand and mediate therapeutic efficacy when combined with a therapeutic radioligand (551, 552, 556). Secondly, dosimetric calculations are important in order to confirm that the adsorbed dose to the tumour is sufficient to induce a substantive radiological effect and to identify the normal organs that represent the DLT (553, 568). Lastly, the timing of therapeutic radioligand delivery relative
to virus is critical and should correspond to the timing of maximum radioligand uptake, which may lag maximum receptor/symporter mRNA expression (556, 561, 567).

To date the only publications investigating OV delivery of sstr2 as part of a PRRT strategy have come from work done by our group in collaboration with Dr. Raymond Reilly (Appendix 1.3) (573, 574). vvDD expressing SSTR2 (vvDD-SSTR2) under control of a late promoter (PsynL) resulted in specific accumulation of $^{111}$In- pentetreotide in mouse CRC cells \textit{in vitro} and in a subcutaneous syngeneic CRC tumour model (574). The same virus engineered to express red fluorescent protein (RFP) (vvDD-SSTR2-RFP) was also investigated \textit{in vitro} in combination with $^{111}$In and $^{177}$Lu labeled DOTA TOC (573). Addition of either $^{111}$In- and $^{177}$Lu-DOTATOC significantly improved \textit{in vitro} CRC cell killing relative to virus alone.

\section*{1.5 Rationale}

\subsection*{1.5.1 Knowledge gap}

VV is moving into clinical trial for the treatment of patients with PC of a variety of origins, including CRC. While initial clinical investigation will likely be single agent, future trials may explore combinations with current standard of care therapies. Clinical trials combining VV with standard chemotherapy drugs are already underway for other cancers and, pending the results of ongoing single-agent trials in PC patients, it is likely that combination therapy trials in these patients will be initiated within the next 5 years. At present, very little is known about the interaction between VV and two of the most effective CRC chemotherapy drugs, OX and CPT-11. In order to design trials with a greater chance of success, it is necessary to better understand the interactions between these treatments. Preclinical investigation can provide insight into the optimal timing, dose, and route of administration as well as any potential toxicities related to the combination.

Translation of SSTR2-expressing VV with PRRT into clinical trial may be further from fruition given that neither is approved for use in patients with CRC; there is, though, significant evidence to support this line of investigation. In a recently published phase I clinical trial, Ad expression of SSTR, detected by gamma camera imaging following $^{111}$In-pentetreotide administration, was used as a marker of virus replication in patients with recurrent gynecologic cancers (544). This study provides evidence that intraperitoneally delivered virus can be used to
direct tumour-specific uptake of radiolabeled SS analogues and therefore warrants further investigation.

1.5.2 Objective

The objective of this project is to investigate novel oncolytic virus-based combination therapies for the treatment of colorectal PC. Understanding how VV interacts with standard CRC chemotherapy drugs and radiolabeled peptides will enable rationale- and evidence-based design of future clinical trials.

1.5.3 Hypothesis

We hypothesize that:

1) vvDD will specifically replicate in and kill CRC cells resulting in improved survival in immunosuppressed and immunocompetent murine models of PC.

2) vvDD anti-tumour effects will be enhanced when combined with CPT-11 or OX in a dose dependent manner

3) vvDD expressing SSTR2 will enable the imaging and treatment of IP CRC tumours with low endogenous SSTR using radiolabeled somatostatin analogues.

1.5.4 Specific aims

1) Investigate the efficacy of oncolytic vaccinia virus in \textit{in vitro} and \textit{in vivo} models of colorectal peritoneal carcinomatosis by evaluating virus replication/spread, biodistribution, tumour cell death and survival.

2) Identify synergistic virus-drug interactions through \textit{in vitro} screening and evaluate these combinations in \textit{in vivo} models of colorectal peritoneal carcinomatosis.

3) Demonstrate specific uptake of radiolabeled SS analogues in intraperitoneal CRC tumours treated with an oncolytic SSTR-expressing vaccinia virus.

4) Evaluate the efficacy of oncolytic virotherapy combined with PRRT in murine models of colorectal peritoneal carcinomatosis.
Chapter 2

Tumour vascularization is critical for vaccinia virus treatment of peritoneal carcinomatosis

Chapter 2 was published as:


Contributions:

KOP and JAM contributed to the overall study design. Individual experiments were devised by KOP with the assistance of JAM. The majority of experiments were performed by KOP with the assistance of NT, RH, NC, SAA and FAA. In vivo fluorescent imaging was performed by RD. FAA performed all window chamber experiments. RA and YC performed in vitro experiments involving infection of HUVEC cells in the laboratory of JB. Data analysis and manuscript preparation were performed by KOP with critical review by JAM.
2.1 Abstract

Peritoneal carcinomatosis (PC) represents a significant clinical challenge for which there are few treatment options. Oncolytic viruses are ideal candidates for PC treatment because of their high tumour specificity, excellent safety profile and suitability for peritoneal delivery. Here, we described the use of vvDD-SR-RFP, a recombinant vaccinia virus, in xenograft and syngeneic models of colorectal PC. Colorectal cancer cell lines were highly susceptible to vvDD-SR-RFP replication and cytotoxicity. Intraperitoneal delivery of vvDD-SR-RFP on Day 12 to mice with colorectal carcinomatosis significantly improved survival whereas survival was not improved following virus treatment on Day 8, when tumours were smaller. Immunohistochemistry revealed early tumours had a poorly distributed network of blood vessels and lower proliferation index compared to later tumours. Virus infection was also restricted to tumour rims following Day 8 treatment, whereas it was disseminated in tumours treated on Day 12. Additionally, direct infection of tumour endothelium was observed and virus infection correlated with a loss of endothelial staining and induction of cell death. Our results demonstrate that tumour vasculature has a critical role in virus delivery and tumour response. This will have significant implications in the clinical setting, both in understanding timing of therapies and in designing combination treatment strategies.
2.2 Introduction

Colorectal cancer (CRC) is the third most common cancer in North America and the third leading cause of cancer-related deaths (575, 576). Peritoneal carcinomatosis (PC), a late-stage disseminated disease, is present in 7% of CRC patients at the time of diagnosis and 40–80% of patients at the time of death (17). Surgical resection combined with chemotherapy is the best treatment option available, achieving 5-year survival rates of up to 54%. Unfortunately complete resection is often impossible. Despite advances in the spectrum of available drugs, 5-year survival rates are still <10% in patients with inoperable tumours (577). Novel therapies with lower associated toxicities and a greater ability to reduce tumour burden are required.

Oncolytic viruses (OVs) are a promising new therapy for refractory cancers because of their ability to specifically kill tumour cells with minimal toxicity to normal tissues. OVs can target tumours too close to vital organs to allow for safe surgical excision—thus representing ideal candidates for PC therapy. Vaccinia virus (VV) is a potent OV with excellent efficacy in numerous preclinical solid tumour models (123, 195-197, 578). Encouragingly, Phase I and II trials of various VVs have shown measurable tumour responses and minor toxicity (179, 182, 216).

vvDD, a recombinant VV with deletions of the viral thymidine kinase (tk) and vaccinia growth factor (vgf) genes, has a significantly improved safety profile in murine models relative to the wild-type (wt) or single-deleted mutants while still maintaining its oncolytic potency (123). Virus safety has also been demonstrated in rhesus macaques (194) and a Phase I trial of a virus with the vvDD backbone is currently underway in patients with superficial solid tumours (NCT00574977).

vvDD has previously been reported to inhibit growth of subcutaneous colorectal tumours (123) as well as improve survival in both immunosuppressed and immunocompetent models of ovarian carcinomatosis (196). In immunocompetent CRC PC models, combination therapy with VV expressing tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and oxaliplatin improved survival over either agent alone (507) and delivery of low dose vvDD in preimmunized mice was improved when combined with immunosuppression and carrier cells (579).
In our study, we report the use of vvDD in two orthotopic models of colorectal PC and are the first to report improved survival in a syngeneic model following intraperitoneal (IP) virus delivery. In addition, we identify factors that are critical to therapeutic success, namely the presence of adequate tumour vasculature and highly proliferating cells at the time of treatment. Finally, we examine the relationship between VV infection, tumour cell death and the loss of tumour vasculature.

2.3 Materials and Methods

**Cell lines.** Human colorectal adenocarcinoma cell lines HT29, Lovo, DLD1 and H508, normal human intestinal epithelial cell line FHs-74-Int and monkey kidney fibroblast cell line CV-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). MC38 murine colon adenocarcinoma and 24JK murine sarcoma cell lines were obtained from the National Institutes of Health (NIH) (Bethesda, MD). CT26 murine colorectal adenocarcinoma cells were a kind gift from Dr. Brian Lichty (McMaster University, Hamilton, ON, Canada). Cells were cultured at 37°C with 5% CO2 in media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON, Canada) and 1% antibiotics–antimycotic (Invitrogen, GIBCO, Grand Island, NY). All cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO) except Lovo cells, maintained in F12-K media (Invitrogen, GIBCO) with 2 mM l-glutamine (Invitrogen, GIBCO), H508 cells, maintained in RMPI-1640 medium (Sigma Aldrich) and FHs-74-Int maintained in Hybri-Care Media (ATCC) supplemented with recombinant human epidermal growth factor (Sigma Aldrich).

**Vaccinia virus.** The previously described vvDD expressing the reporter gene human somatostatin receptor subtype 2 (SR) (574) was modified by insertion of a red fluorescent protein (RFP) gene encoding DsRed (Invitrogen) into the tk locus under control of the synthetic early/late vaccinia promoter, Psel, using standard techniques (580). After homologous recombination with the parental virus VSC20 (193) (a kind gift from Dr. B. Moss, NIH), vvDD-SR-RFP underwent selection in mycophenolic acid (Calbiochem, EMD Biosciences, San Diego, CA), xanthine, and hypoxanthine (Invitrogen) (Supporting Information Appendix 2.1). Virus was propagated in 24-JK cells, purified by ultracentrifugation over a sucrose cushion and titered on CV-1 cells (580).
**In vitro infections.** Tumour cells were seeded at $5 \times 10^5$/well in 6-well plates and incubated overnight. FHs-74-Int cells were seeded in 24-well plates and incubated until confluent, at which point the number of cells per well was determined. Cells were preinfected with vvDD or mock-infected with media alone (2.5% FBS) and incubated at 37°C for 2 hr, shaking every 10 min. Cells were then supplemented with media (10% FBS) and incubated. Neither imaging nor therapy targeting SR was utilized in the current experiments.

**Virus replication.** Multistep growth curves were generated from cells infected at a multiplicity of infection (MOI) of 0.1. Cells were collected in triplicate immediately after the preinfection and every 24-hr to 72-hr postinfection (hpi). Samples were exposed to three freeze–thaw cycles and sonicated. Virus was quantified by plaque assay on CV-1 cells.

**Monolayer cytotoxicity.** Cell viability was quantified by trypan blue assay as per the manufacturer's protocol (Invitrogen, GIBCO). Cells were harvested 72 hpi and counted on a hemocytometer following trypan blue staining. Viability was expressed as the percent of viable cells in the infected wells relative to viable cells in the mock wells. Mock- and virus-infected cells were also stained with crystal violet.

**Spheroid culturing, infection and cytotoxicity.** Spheroids were cultured as previously described (581). Briefly, cells were harvested using Accutase (Sigma Aldrich) and seeded at 1,000 cells/well in round-bottom 96-well plates coated with 1% poly-HEMA (Sigma Aldrich). Plates were centrifuged at 1,500 rpm for 10 min and incubated for 48 hr. vvDD (MOI = 1.0) or media alone was added and infection was confirmed by fluorescence microscopy. Virus-induced cytotoxicity was determined by clonogenic assay. Briefly, 20–50 spheroids were collected, pooled and dissociated into a single cell suspension in 0.25% trypsin (Invitrogen, GIBCO). Cells were seeded in triplicate (25–100 cells/well) in 6-well plates and incubated for 10 d. Plates were stained with crystal violet and colonies (>50 cells) were counted. Surviving fraction was expressed as the number of colonies per well divided by the number of cells originally plated.

**Infection of patient samples.** Tumour and normal (when available) tissue was collected at the time of laparotomy under an REB approved protocol. Tumours were maintained in DMEM (10% FBS). Tumours were sliced into small segments (approximately 1 mm × 5 mm), weighed and infected with $7.2 \times 10^5$ pfu of vvDD-SR-RFP/average mg of tissue in 0.3 mL of DMEM (2.5% FBS) for 2 hr. Wells were then supplemented with DMEM (10% FBS) and incubated.
**In vitro HUVEC infection.** Human umbilical vein endothelial cells (HUVEC) were subcultured in Endothelial or Microvascular Endothelial Cell Growth Medium-2 (Lonza Group, Basel, Switzerland). Endothelial cells used in experiments were not passaged more than three times since resuscitation. Cells were plated in MCDB131 medium (Invitrogen, GIBCO) in 12-well plates and pulsed with 50 ng/mL VEGF165 (R&D Systems, Minneapolis, MN) in the presence of vvDD (MOI = 0.01). Fluorescent images were taken 24 and 48 hpi. Cells and supernatant were collected 48 hpi and virus was titered by plaque assay.

**Microscopy.** Infected cells and patient samples were imaged using a Zeiss AxioObserver microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Series 120Q Fluorescence Illumination unit (EXFO, Quebec City, QC, Canada). Images were acquired with a Coolsnap HQ camera (Roper Scientific, Tucson, AZ).

**Mice.** Female NOD/SCID (Animal Resource Center in house colonies, University Health Network (UHN), Toronto, Canada) and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used for the xenograft and syngeneic PC models, respectively. Male C57BL/6 mice (Taconic Farms, NY) were used in the window chamber model. Mice were housed under standard conditions and given food and water ad lib. Experiment protocols were approved by the Animal Care Centre, UHN, Toronto, Canada. Survival endpoint criteria were defined as severe abdominal distension, cachexia, or development of subcutaneous injection site tumour greater than 1.5 mm² in diameter.

**Xenograft model.** NOD/SCID mice were injected IP with $10^6$ HT29 cells. Twelve days post-tumour inoculation, mice were injected IP with vvDD ($10^9$ pfu) in 1 mL Hanks-Buffered Saline Solution (HBSS; Invitrogen, GIBCO) or HBSS alone. Mice were followed for survival or sacrificed 6 ds postinfection (dpi) for biodistribution or immunohistochemistry (IHC).

**Syngeneic model.** C57BL/6 mice were injected IP with $10^5$ MC38 cells. Tumours from untreated mice were harvested and fixed on Day 8 ($n = 3$) and 12 ($n = 3$) for IHC (multiple tumours per mouse were harvested where possible). Remaining mice received IP injections of vvDD ($10^9$ pfu) in 1 mL HBSS or HBSS alone, 8 or 12 d post-tumour inoculation. Mice were followed for survival or sacrificed for biodistribution and IHC.
**Virus biodistribution.** Tumour and normal tissues were harvested 6 dpi and stored at −80°C in HBSS. Tissues were homogenized, exposed to three freeze–thaw cycles and sonicated before titering on CV-1 cells. Protein levels were determined by BCA assay (Product #23252, Thermo Scientific, Rockford, IL).

**In vivo optical imaging.** In vivo imaging was performed using a prototype handheld optical imaging system (PRODIGI™, developed by Drs. RS DaCosta, BC Wilson and K Zhang, Ontario Cancer Institute). Fluorescence images were acquired using 405 nm excitation light provided by two (single-wavelength) high-power LED arrays (estimated total optical output 80 mW/cm2) in combination with proprietary fluorescence filters (Chroma Technology Corp., VT) to detect specific fluorescence emission wavelength bands (500–550 nm for green autofluorescence, and >600 nm for red fluorescence). High-resolution digital images were acquired by an integrated digital camera equipped with a Super HAD CCD (Model T900 Cybershot, Sony Corp., Japan).

**Immunohistochemistry.** Tissues were fixed in 10% formalin for 48–72 hr and then transferred to 70% ethanol. Samples were paraffin embedded, sectioned and stained using the following primary antibodies: polyclonal goat anti-mouse CD31 (PECAM-1 M-20, SC-1506, 1/2,000 dilution; Santa Cruz, Dallas, TX), monoclonal rabbit anti-mouse Ki67 (RM-9106, clone SP6, 1/1,000 dilution; Neomarkers, Thermo Fisher Scientific, Fremont, CA), polyclonal rabbit anti-VV (ab35219, dilution 1/1,000; Abcam, Cambridge, MA), monoclonal rat anti-mouse F4/80 (clone A3.1, dilution 1/50, Serotec), and monoclonal rat anti-mouse Ly-6B.2 (clone 7/4, dilution 1/500, Serotec). Standard H&E and TUNEL staining were also performed (as previously described[20]). Slides were scanned using ScanScope XT (Aperio Technologies, Vista, CA) and staining was quantified using ImageScope's Positive Pixel algorithm (Aperio Technologies).

Relative vessel distribution was determined on CD31-stained pretreatment slides using a vessel distribution index (VDI, Appendix 2.2) developed for this experiment. For each tumour analyzed, two regions of interest were drawn: an inner area (Ai) and an outer area (Ao). Ai comprised approximately 50% (range 49.01–50.99) of the total area (AT) of a given tumour. The number of positive staining pixels in the inner (Pi) and outer (Po) areas was analyzed. VDI was defined as follows: VDI = [Po/Ao]/[Pi/Ai].
A “VDI = 1” indicates the distribution of positive staining was homogenous throughout $A_T$. VDI $< 1$ indicates a greater proportion of positive staining in the $A_i$ region and VDI $> 1$ indicates a greater proportion of positive staining in the $A_o$ region.

VV staining distribution was quantified in tumour sections harvested 72 hpi and sectioned at three levels, 100–250 µm apart (depending on the size of the tissue) prior to staining. Regions $A_i$ and $A_o$ were drawn on VV stained tumours to determine the percentage of VV staining in $A_i$ (see Supporting Information Fig. 2). The percent positive staining in $A_i$ on all three levels was averaged and used to determine the mean percent positive staining for all tumours of each individual mouse. The average percent positive staining between mice treated on Day 8 or 12 was then calculated and compared.

**Dorsal window chamber model.** The window chamber technique was performed as previously described (582). Briefly, a flap of skin was dissected from the dorsal skin-fold of anesthetized C57BL/6 mice leaving an intact vascularized fascia. The skin window was held vertically away from the dorsum by two titanium frames (APJ Trading Co, Ventura, CA) and sealed with a glass cover slip. Forty-eight hours later, $1.5 \times 10^3$ MC38 cells expressing GFP were injected subcutaneously. Five days after tumour injection, mice received $10^9$ pfu of vvDD-SR-RFP IP. Seventy-two hpi mice were imaged with a Zeiss LSM510 Meta Multiphoton Confocal Fluorescence Microscope (Zeiss Microsystems; Jena, Germany). Blood vessels were visualized by injecting allophycocyanin (APC) conjugated rat anti-mouse CD31 antibody (Phar mingen, San Diego, CA) into the tail vein 30–45 min before imaging.

**Statistical analysis.** Data were analyzed using the Students two-tailed t-test where applicable. Survival curves were analyzed using the log-rank test. D'Agostino & Pearson omnibus normality test, followed by Pearson's correlation analysis were performed on pretreatment IHC data using Prism 5 software (GraphPad Software, La Jolla, CA). Data are presented as the mean ± SD or SEM and statistical significance was defined as $p < 0.05$. 
2.4 Results

2.4.1 vvDD-SR-RFP infects and kills CRC cells

To demonstrate the oncolytic potency and tumour-specificity of vvDD-SR-RFP, a panel of CRC cell lines was infected and compared with FHs-74-Int cells, serving as a normal control. Both the CRC cells and normal controls were near confluent at the time of infection. Viral RFP was detected in all tumour cell lines at all MOIs (0.01–5) 72 hpi (Figure 2.1A). HT29, Lovo, H508 and MC38 cells supported the highest levels of RFP expression, which increased in a dose-dependent manner. This corresponded with the appearance of cytopathic effect (CPE) in these cells at an MOI of 0.01 (Figure 2.1B). DLD1 and CT26 cells showed lower levels of RFP expression with only moderate dose-dependent increases (Figure 2.1A) and minimal CPE (Figure 2.1B). At all MOIs < 5, RFP expression and virus-induced CPE was greater in the tumour cell lines relative to the FHs-74-Int normal control.

Multiple-step low MOI growth curves were performed in all cell lines (Figure 2.1C). HT29, Lovo, H508 and MC38 cells supported high levels of virus replication showing a 2- to 4-log increase by 72 hpi and this was significantly increased at all time points compared to FHs-74-Int cells (p < 0.05) (Figure 2.1C). Replication in DLD1 and CT26 cells was similar to that in FHs-74-Int cells and lower relative to the other tumour cell lines. At 72 hpi there were minimal signs of cytotoxicity in FHs-74-Int control cells at MOIs ≤ 1 by crystal violet (Figure 2.1D). In contrast, HT29, Lovo, H508 and MC38 cells showed virus-induced cytotoxicity at MOIs ≥ 0.01 while cytotoxicity was observed at MOI ≥ 0.1 in CT26 and DLD1 cells (Figure 2.1D). Cell viability by trypan blue was significantly decreased in MC38, HT29, Lovo and H508 at all MOI ≤ 1 relative to FHs-74-Int (p < 0.05) (Figure 2.1E).

2.4.2 vvDD-SR-RFP infects and kills CRC spheroids

To evaluate the ability of vvDD-SR-RFP to spread through a solid tumour mass, MC38 and HT29 cells were cultured as multicellular tumour spheroids. Dispersed foci of RFP were detected at 48 hpi by fluorescence microscopy (MOI = 1) in both MC38 and HT29 spheroids (Figure 2.2A). At 120 hpi, virus had spread over the entire spheroid area and inhibited growth of MC38 spheroids (Figure 2.2A). By 192 hpi infected HT29 spheroids showed a loss of both RFP
**Figure 2.1. VV infects and kills colon cancer cells.** (a) Fluorescence microscopy 72 hpi with increasing MOIs. Magnification 10×. (b) Bright field microscopy showing induction of cytopathic effect in cells infected at MOI 0.01 compared to mock-infected cells 72 hpi. Magnification 10×. (c) Multistep growth curve of cells infected at MOI = 0.1. The 0 h time point represents the initial virus dose. Data shown are the mean of triplicate values ± SD. Virus titers in the tumour cell lines were compared to those in the control cells at each time point. *p < 0.05 at all time points (24–72 hr). (d) Crystal violet staining of tumour and control cells infected with increasing MOIs. Plates were stained 72 hpi. (e) Virus induced cytotoxicity in tumour and control cells assessed by trypan blue assay 72 hpi. Values are expressed as a percentage of viable cells in the infected well relative to viable cells in the mock-treated wells. Data represent the mean of triplicate values ± SD.
**Figure 2.2.** VV kills multicellular tumour spheroids and specifically infects human peritoneal carcinomatosis surgical specimens. (a) Bright field (left and middle panels) and fluorescence (right panel) microscopy of MC38 and HT29 spheroids infected at MOI 1. Magnification 10×. (b) Clonogenic assay of cells from virus (MOI = 1) and mock-infected MC38 and HT29 spheroids harvested at 120 hpi. The surviving fraction is the number of colonies (>50 cells) formed relative to the number of cells plated after 10 d of incubation. Data represent the mean of triplicate values ± SD. *p < 0.05. (c) Bright field and fluorescence microscopy of vvDD infected (+) and mock-infected (−) tumour and normal tissue (omentum) from three patients (P1–P3) with adenocarcinoma of the bowel. Tissue sections were infected at $7.2 \times 10^5$ pfu/mg tissue (approximately MOI = 1) and imaged at 24 and 72 hpi. Magnification 5x.
expression and structural integrity relative to mock-infected controls. Virus-induced cytotoxicity resulted in viability of <15% in MC38 and 4% in HT29 cells at 120 hpi (Figure 2.2B).

2.4.3 vvDD-SR-RFP specifically infects human PC surgical specimens

Tumour tissue from three patients (P1–P3) with PC from adenocarcinoma of the intestine and normal tissue (omentum) from P3 were collected at the time of laparotomy. In patients with PC, the omentum is one of the primary sites of tumour growth therefore it represents the neighboring normal tissue. vvDD-SR-RFP treated tumour samples showed high levels of RFP expression that increased over time (24–72 hpi) indicating successful infection and spread (Figure 2.2C). RFP expression in normal tissue samples was limited to individual cells without spreading consistent with the mechanism of tumour specificity attributed to vvDD; it can infect but does not replicate well in normal cells.

2.4.4 vvDD-SR-RFP improves survival in a xenograft model of CRC PC

In vivo studies were designed to reflect the current clinical knowledge regarding effective treatment of PC. On the basis of strong evidence that IP chemotherapy is superior to intravenous delivery (17), we chose to treat with IP virus. NOD/SCID mice bearing IP HT29 tumours received an IP injection of vvDD-SR-RFP (10⁹ pfu) or vehicle alone on Day 12 post-tumour cell inoculation. This virus dose was selected based on previous studies in similar IP models using IP delivery (123). Virus-treated mice survived significantly longer than vehicle-treated controls (median survival 37.5 vs. 24.5 d, p = 0.02) (Figure 2.3A). All of the untreated mice developed bloody ascites prior to sacrifice whereas virus-treated mice showed no signs of ascites (Figure 2.3B), rather they were sacrificed due to development of subcutaneous injection site tumours. Virus biodistribution was evaluated 6 dpi. With the exception of the ovaries (known targets of VV replication (123, 188)), the mean tumour titer was at least 3-logs higher than all other normal tissues (Figure 2.3C). Despite being an immunosuppressed model minimal virus was detected in the most vulnerable proliferative tissue compartments (gastrointestinal tract and bone marrow) and no signs of gastrointestinal distress were observed. To investigate the mechanism of tumour cell death we performed IHC on tumours harvested 6 dpi. Quantification of TUNEL staining
Figure 2.3. Efficacy and specificity of vvDD in a xenograft model of colorectal PC. NOD/SCID mice were injected IP with HT29 cells and treated with vvDD (10^9 pfu) or vehicle alone (HBSS) on Day 12. (a) Kaplan–Meier survival curves show a significant increase in survival of virus (n = 6) compared to vehicle (n = 7) treated mice (37.5 vs. 24.5 d, *p = 0.02). (b) Number of mice in (a) with bloody ascites at the time of euthanasia (upper panel). Representative images 6 dpi of mice treated with HBSS or virus (lower panel). (c) Virus biodistribution in tumour and normal tissues of virus-treated mice (n = 5). Tissue titers for each mouse are shown (dots) as well as the mean of all mice (line). Values are expressed as the number of pfu per mg of total protein. (d) Immunohistochemistry on tumours harvested 6 dpi. Representative images of H&E and TUNEL stained tumours from each treatment group (top) and quantification of TUNEL staining (bottom) in tumours from virus- (n = 5) and vehicle-treated (n = 3) mice (*p = 0.0185).
revealed significantly higher levels of cell death in virus- relative to vehicle-treated mice 
\( p = 0.0185 \) (Figure 2.3D).

2.4.5 vvDD-SR-RFP improves survival in a syngeneic model of CRC PC

C57BL/6 mice bearing IP MC38 tumours treated on Day 12 with an IP injection of
vvDD-SR-RFP (\( 10^9 \) pfu) survived significantly longer than control mice (36.5 vs. 26.5 d; 
\( p = 0.0007 \)) (Figure 2.4A). In vivo fluorescence imaging 6 dpi showed viral RFP expression was
limited to tumour tissues of treated mice (Figure 2.4B) and was capable of detecting tumours as
small as 1 mm, otherwise difficult to identify under white light (Figure 2.4B, arrowheads).
Imaging results were confirmed by assaying for virus in tissues 6 dpi (Figure 2.4C). Mean
tumour titers were 2–3 logs increased over normal tissues. Virus titers were very low (0–1.25 × 
\( 10^3 \) pfu/mg) in all normal tissues, likely due to immune clearance, and mice showed no overt
signs of virus-associated toxicity. Day 12 treatment significantly increased survival, however, all
mice were eventually sacrificed due to severe abdominal distension secondary to tumour growth.
We hypothesized that earlier treatment, when tumour burden is lower, would improve virus
efficacy. Surprisingly, early treatment (Day 8) did not improve survival over controls (Figure
2.4D).

2.4.6 Vessel distribution and tumour proliferation are critical for virus 
delivery and spread

To determine the mechanism(s) governing the difference in therapeutic efficacy, IHC was
performed on Day 8 and Day 12 tumours prior to, as well as immediately following virus
treatment. Pretreatment tumours were stained for markers of cellular proliferation (Ki67),
vascular endothelial cells (CD31) and immune cells. Cellular proliferation was significantly
increased at Day 12 relative to Day 8 (\( p = 0.0077 \)) (Figure 2.5A). As well, the distribution of
CD31+ vessels (see Supporting Information Appendix 2.2 for method of analysis) differed
significantly between the two time points. CD31 staining was evenly distributed in Day 12
tumours (VDI = 0.920 ± 0.469, \( p = 0.303 \), one sample \( t \)-test, theoretical mean = 1), whereas
tumours harvested on Day 8 had a greater proportion of CD31 in the outer region
(\( VDI = 1.239 ± 1.029, p = 0.034 \), one sample \( t \)-test, theoretical mean = 1) (Figure 2.5B).
Figure 2.4. Efficacy and specificity of vvDD in a syngeneic model of colorectal PC.

C57BL/6 mice bearing IP MC38 tumours were treated with vvDD ($10^9$ pfu) or vehicle (HBSS) alone. (a) Kaplan–Meier survival curves of mice treated on Day 12 show a significant increase in the median survival of virus ($n = 6$) relative to vehicle ($n = 6$) treated mice (36.5 vs. 26.5 d, $p = 0.0007$). (b) White light (WL, left) and fluorescence (FL, right and below) imaging of virus-treated mice 6 dpi. RFP expression is detected in nodules as small as 1 mm (arrow heads) and in larger tumours with a pattern indicative of multiple sites of infection (arrow). Scale bar = 5 mm. (c) Virus biodistribution in tumour and normal tissues harvested 6 dpi ($n = 5$). Shown are tissues titers for individual mice (dots) as well as the mean value for each organ (line). Values are expressed as the number of pfu per mg of total protein. (d) Kaplan–Meier survival curves of mice treated with vvDD ($10^9$ pfu, $n = 6$) or vehicle (HBSS, $n = 6$) on Day 8. Survival of virus-treated mice was not significantly improved relative to control mice.
Figure 2.5
Figure 2.5. Virus delivery is improved in tumours with well-distributed vessels. (a) Representative images and quantification of Ki67 staining in untreated MC38 tumours harvested on Day 8 (D8, n = 6) or Day 12 (D12, n = 3). Presented is the mean percentage of Ki67+ pixels relative to the total number of pixels per tumour. *p = 0.016 (b) Representative images and quantification of CD31+ endothelial cell distribution in untreated tumours harvested on Day 8 [D8, n = 3 (86 tumours)] or Day 12 [D12, n = 3 (31 tumours)]. VDI = 1 indicates equal distribution of CD31+ staining across the entire tumour area. VDI < 1 indicates more staining in the inner area, while VDI > 1 indicates more staining in the outer area of the tumour. Shown is a floating box plot where the box represents the range of the data and the horizontal line indicates the mean. A one-sample t-test compared the mean VDI for each group to a theoretical mean of 1 (assuming even distribution). *p = 0.034. (c) Representative images and quantification of macrophage (F480) staining in untreated D8 (n = 3) and D12 (n = 3) tumours. Presented is the mean ± SEM. *p = 0.0042. (d) Representative images of VV stained tumours treated on Day 8 or 12 and harvested at the indicated time point. (e) Quantification of VV staining in the inner area of tumours harvested 72 hpi [D8, n = 2 (19 tumours); D12, n = 3 (28 tumours)]. Representative images and a detailed description of the method are found in Appendix 2.2. Briefly, tumour blocks were cut at three depths 100–250 µm apart (depending on the size of the tissue) and the percent positive VV staining in the inner 50% of the tumour area was determined. Presented is the mean percentage for each tumour at all depths ± SEM. Scale bar, 500 µm or as indicated.
The presence of immune infiltrates prior to treatment was determined by staining for CD3 (T cells), B220 (B cells) and F480 (macrophages). No significant difference in lymphocyte infiltration was observed (data not shown), however, there was a significant increase in macrophage staining in Day 12 relative to Day 8 tumours (Figure 2.5C).

VV delivery to Day 8 and Day 12 tumours was investigated shortly after injection (Figure 2.5D). In Day 8-treated mice, virus staining was observed predominately in the tumour rim and not in the center at all time-points (Figure 2.5D, left panel). In mice treated on Day 12, virus staining was found predominately in the periphery at 12–24 hr, however, by 48–72 hr, virus was detected both on the rim as well as in the center (Figure 2.5D, right panel). Virus distribution in the inner 50% of tumour area was quantified 72 hpi in Day 8- and Day 12-treated tumours (Figure 2.5D). To ensure that our findings were representative of the entire tumour volume, three sections at increasing depths (100–250 µm apart) were stained and used in the analysis. VV was significantly increased in the inner area of Day 12-treated tumours relative to Day 8-treated tumours (13.64% ± 1.64% vs. 2.51% ± 0.41%, p = 0.0054) (Figure 2.5E). Detection of virus in the interior of tumours without a direct link to staining on the periphery suggests that virus gained access to the tumour interior through the vasculature and not by direct spread from the infected cells in the periphery. This was supported by the detection of virus DNA in the blood, by real-time PCR, as early as 1-hr post-IP injection (Supporting Information Appendix 2.3). Furthermore, the pattern of virus staining in Day 12-treated tumours was consistent with the pattern of pretreatment CD31 staining.

2.4.7 vvDD-SR-RFP correlates with increased TUNEL and decreased tumour vasculature

Due to the significant heterogeneity in the levels of viral infection observed in tumours, even amongst those from the same mouse (Figure 2.6A), correlation analyses between TUNEL, CD31 and virus staining was performed on tumours harvested 6 dpi (Figure 2.6B). We observed a significant positive correlation between the amount of virus and TUNEL staining (p = 0.0233, r = 0.703). Consistent with previous data showing VV-induced apoptosis correlated with decreased tumour perfusion in a subcutaneous model (198), we found a significant negative correlation between virus infection and tumour CD31 staining (Figure 2.6C, p = 0.0081, r = −0.778). IHC was performed to determine whether VV directly infected CD31+ tumour
Figure 2.6
Figure 2.6. vvDD infection correlates with TUNEL and loss of tumour endothelial cells. C57BL/6 mice bearing IP MC38 tumours were treated with vvDD (10⁹ pfu, n = 3, tumours = 10) or vehicle (HBSS, n = 3, tumours = 10) on Day 12. Tumours were harvested 6 dpi and immunohistochemistry was performed. (a) Representative images of tumours from virus-treated mice stained for VV (pink) or TUNEL (brown). Variability in VV and TUNEL staining was observed when comparing tumours from the same mouse (i vs. ii) or from different mice (i and ii vs. iii). (b, c) To account for variability in virus infection amongst tumours from virus-treated mice correlation analysis was performed. VV staining had a strong positive correlation with TUNEL staining (b) (r = 0.703, p = 0.0233) and a significant negative correlation with CD31 staining (c) (r = -0.778, p = 0.0081). (d) Immunohistochemistry for CD31 (left) and VV (right) on sequential slices of a virus-treated tumour harvested 6 dpi (scale bar, 100 µm) shows colocalization of virus and endothelial cells. (e) Quantification of staining for infiltrating neutrophils (Ly-6B.2) in tumours from mice treated on Day 8 (HBSS, n = 3, tumours = 55; vvDD, n = 2, tumours = 19) or Day 12 (HBSS, n = 3, tumours = 39; vvDD, n = 2, tumours = 24). (f) Virus titers (48 hpi) and RFP expression in HuVEC cells. HuVECs cultured in the presence of Vegf165 prior to infection (MOI = 0.001) showed significantly increased RFP expression and viral titers compared to controls (p = 0.003). (g) Representative images of mice bearing MC38 tumours within a window chamber and treated with vvDD IP (10⁹ pfu/mouse). (i) Bright field picture of window chamber apparatus on a tumour-bearing mouse. (ii–v) Confocal fluorescence microscopy of GFP-expressing tumours (T) and CD31 positive endothelial cells (arrow heads) 72-hr post-treatment (ii–iv) with vvDD. Virus infection is detected throughout the tumour (ii) and in vessel-like structures (iii, arrows). Simultaneous imaging for the APC conjugated anti-CD31 antibody (iv, left) and virus RFP (iv, middle) shows colocalization (merge, right).
endothelial cells, which may account for the loss of CD31 staining. Tumours harvested 6 dpi showed colocalization of CD31 and VV staining in addition to VV infection of surrounding tumour tissues (Figure 2.6D). VV and CD31 colocalization was not observed at earlier time points (12–72 hpi, data not shown) suggesting endothelial infection is not necessary for virus extravasation. Differences in the inflammatory response between Day 8- and Day 12-treated mice were also investigated. Neutrophil infiltration was significantly increased relative to controls in tumours of mice treated with vvDD-SR-RFP on Day 12 (1.58 ± 0.39% vs. 0.182 ± 0.03%; p = 0.002) but not in those treated on Day 8 (0.471 ± 0.17% vs. 0.907 ± 0.19%; p = 0.09) (Figure 2.6E).

The potential for endothelial cell infection was examined in two additional models. In vitro HuVECS were incubated in the absence or presence of VEGF prior to infection to mimic normal and activated tumour vessels, respectively. Stimulation with VEGF significantly increased virus titers and viral RFP expression relative to non-activated controls (Figure 2.6F). In vivo endothelial cell infection was observed by confocal fluorescence microscopy in vvDD-SR-RFP treated mice bearing subcutaneous MC38 tumours within a window-chamber (Figure 2.6G). Tumour vessels were visualized within the context of the GFP-expressing MC38 window chamber tumour macroscopically (Figure 2.6G, image i) and using an APC-conjugated anti-CD31 antibody. Viral RFP was detected throughout the tumour (Figure 2.6G, image ii) as well as in distinct vessel-like structures (Figure 2.6G, image iii, arrows). After injection of the CD31 specific antibody we saw clear colocalization of the viral RFP signal within CD31+ endothelial lined blood vessels (Figure 2.6G, image iv).

2.5 Discussion

The evolution in our understanding of OVs has enabled the engineering of second and third generation viruses with enhanced specificity, improved cytotoxicity, antitumour immunostimulatory properties, and excellent safety profiles. OVs are well suited for the treatment of such advanced disease like PC. They consistently demonstrate excellent tumour specificity, can be delivered by multiple routes, act on pathways unique from those targeted by standard therapies, and can potentially target residual tumours left behind after surgical debulking. In our study, we described the effect of vvDD-SR-RFP in two orthotopic models of CRC PC, demonstrating both specificity and therapeutic efficacy. Furthermore, we report the
importance of an adequate tumour blood supply to enable virus delivery and affect an antitumour response.

Treatment in both the xenograft and syngeneic models demonstrated the ability of vvDD to target and kill peritoneal CRC tumours and improve survival. In the xenograft model, virus-treated mice showed minimal intra-abdominal tumour burden and were sacrificed due to subcutaneous injection site tumours. Furthermore, complete inhibition of ascites, a clinical feature of the disease associated with poor prognosis (583) and diminished quality of life (584), suggests a potential role for palliative VV therapy. However, given that the efficacy of an OV is dependent not only on its direct oncolytic potency but also on the induction of an innate (198) and/or adaptive antitumour immune response (161, 248, 251, 585), it is the syngeneic model that may be most clinically relevant. An intact immune system will invariably affect virus delivery and clearance, thereby influencing its efficacy and toxicity profile. For these reasons we focused primarily on the syngeneic model for understanding the effects of VV treatment.

Biodistribution studies demonstrated virus specificity for tumour tissues, and confirmed previous observations that mouse ovaries are targets for VV replication (123, 188). The leaky nature of ovarian follicles (586) is thought to cause this tropism. However, studies in non-human primates found no virus in the ovaries at 6 d or 6 wk postinfection (194) suggesting this may be a species-specific phenomenon. Normal tissue titers, particularly in the ovaries, were substantially lower in the syngeneic model compared to the xenograft model. This difference is attributed to the absence of critical immune system components (functional B- and T-cells) in the xenograft model that play an important role in virus clearance (205).

It has been recognized that tumour vasculature is important in VV-based treatments (198), but the exact nature of the relationship is not fully understood. The finding that early viral treatment was inferior to later administration was unexpected. However, examination of the differences between early and late tumours suggests certain tumour features that contribute to treatment efficacy. Day 12 tumours showed higher levels of cellular proliferation, a better distributed vessel network and increased macrophage infiltration prior to treatment. We hypothesize that the reason for early treatment failure is two-fold: (i) inadequate virus delivery mediated by a poorly distributed tumour blood supply and (ii) minimal virus replication and spread due to low levels of tumour cell proliferation. The proposed mechanisms of early
treatment failure are not mutually exclusive given the crucial interdependent relationship between tumour cell proliferation and vasculature (208, 209). Expansion of tumours from single cells into small nodules can occur independent of angiogenesis, however growth beyond approximately 1–2 mm becomes angiogenesis-dependent. The low proliferation rate of Day 8 tumours can therefore be attributed to their size (mean diameter = 1.58 mm ± 0.89) and their poor vessel distribution, whereas increased vessel distribution in Day 12 tumours allowed for greater cellular proliferation and tumour growth (mean diameter = 3.06 mm ± 2.21). Virus delivery was improved in the Day 12 tumours and the actively dividing cells provided abundant targets for productive virus replication.

Interestingly, unlike the unresponsive Day 8 tumours our tumour spheroid study showed complete infection and significant cell death. However, there are important differences between this and in vivo models that affect infection efficiency. Tumour spheroids share many of the barriers to virus spread found in in vivo tumours, including: a complex 3D architecture, areas of differential growth kinetics (rapid replication, senescence and necrosis) (587), altered metabolism (588) and different sensitivities to standard therapies relative to monolayer cultured cells (589, 590). However, spheroids lack all non-tumour components of the tumour microenvironment (e.g., vasculature, immune cells and fibroblasts). The smaller size of the spheroids (~0.5 mm compared to 1.58 mm for Day 8 tumours), the lack of tumour stromal tissue, as well as the use of in vitro infection protocols optimized for virus infection likely contributed to their improved infection efficiency.

Our study focused primarily on differences in tumour cell proliferation and vasculature; yet, there are likely other factors that contribute to the disparity in therapeutic efficacy between early and late treatment. Given the evidence that a virus-induced antitumour immune response is critical to therapy efficacy (161, 198, 248, 251, 585), it is possible that impaired immune cell infiltration also contributed to therapy failure. Indeed, we observed lower levels of macrophage infiltration prior to treatment and decreased neutrophil infiltration 6 dpi in Day 8 relative to Day 12-treated tumours. The role of the immune response in this model requires further investigation.

The potential of VV to elicit an adaptive antitumour response could prove a great asset in the treatment of late-stage disease such as PC, which are often accompanied by metastatic
tumour spread. Previous studies have demonstrated that mice with complete tumour response following VV treatment subsequently reject tumour rechallenge (154).

Vasculature is not only critical for virus delivery early after injection but also plays an important role in mediating tumour killing at later time points after treatment. Previous studies have shown that VV can directly infect tumour endothelial cells leading to a collapse of tumour blood supply (154) and that VV-induced neutrophil accumulation in tumour vessels blocks tumour perfusion resulting in apoptosis due to ischemia (198). Interactions between VV and tumour vasculature have been reported in humans as well, where systemic treatment with JX-594 (Wyeth strain VV) in a Phase 1 trial of patients with hepatocellular carcinoma led to a significant reduction in tumour blood perfusion (216). Here, we show that VV infects tumour endothelial cells and that increased infection of tumours corresponds with a loss of vascular endothelial cells and increased cell death in an IP model.

Despite improved survival in the Day 12-treated syngeneic model, all mice were eventually sacrificed due to tumour growth. Treatment failure is attributed to incomplete infection of all tumour nodules rather than acquired resistance. IHC analysis 6 dpi showed substantial heterogeneity in the extent of infection among tumours from the same mouse (Figure 2.6A). MC38 cells are highly susceptible to VV infection showing high levels of cell death in both in vitro monolayer and spheroid models. We have no evidence to suggest that there is anything fundamentally different about the cells themselves in the tumours that are not well infected (i.e., acquired resistance). It is also important to note that while Day 12-treated tumours were on average better vascularized and more proliferative than Day 8 tumours, individual mice could have in excess of 20 tumours at the time of treatment with heterogeneity in tumour stroma (fibroblasts, vasculature, immune cells etc.) and consequently virus delivery. It is expected that these poorly infected tumours represent those that continue to grow after virotherapy and ultimately lead to treatment failure.

The heterogeneity of tumour development in individual mice also makes it challenging to investigate how the complex interaction between growing tumours and developing vasculature affects OV therapy. For this reason we established the window-chamber model. Here, we use real-time in vivo imaging to show the interaction between tumour and its associated vasculature and VV infection in tumour endothelial cells and the surrounding tumour. Further investigations
are underway in this model to better understand VV infection of endothelial cells, the role of endothelial cell infection on response and the potential role of immune infiltrates on oncolytic virotherapy.

Clinically, IP chemotherapy for PC has significantly improved therapeutic outcomes compared to intravenous therapy (17, 95). To reflect the existing clinical data regarding the optimal route of delivery, we chose to administer the virus IP. In a subcutaneous MC38 tumour model, maximum vvDD gene expression levels were the same regardless of the route of administration (IV vs. IP vs. IT) (475). In another study, VV gene expression in MC38 liver metastases was higher following IP versus IV delivery (591). These studies provide strong evidence that virus delivered IP is taken up and delivered to tumours by the blood supply, which was further supported here by detection of VV in the blood 1 hr after injection. Given that IP virus delivery permits bidirectional treatment (initially from the peritoneum and then via the vasculature after absorption) and evidence that VV gene expression in peritoneal tumours is higher after IP versus IV delivery (475), we felt there would be improved therapeutic benefit of IP delivery in this IP model.

The notion that an inadequate tumour blood supply results in treatment failure could have serious implications for the use of VV as an adjuvant therapy in the clinic. Many investigators have proposed adjuvant OV therapy to target residual tumours following surgical cytoreduction. Disease left behind after surgery typically consists of tumours that are microscopic or inoperable due to location. Given an adequate tumour blood supply disease left behind may be amenable to virus treatment. However, small, avascular tumours may escape treatment. The data presented herein suggest that the presence of tumour vasculature is critical at early time points for virus delivery and spread, and at later time points to effect a tumour response. Understanding the underlying mechanisms behind VV's interaction with the tumour vasculature will be crucial to moving this therapy forward in the clinic.
Chapter 3

Vaccinia virus synergizes with irinotecan and improves treatment of peritoneal carcinomatosis

Chapter 3 has been submitted for publication as:


Contributions:
Experiments were designed and executed by KOP. SAF, FAA, CS, SZ and NT assisted in the performance of experiments. Data analysis and manuscript preparation were performed by KOP. JAM contributed to the overall study design and manuscript preparation.
3.1 Abstract
Metastatic colorectal cancer (CRC) is complex clinical challenge for which there are limited treatment options. Chemotherapy with or without surgery provides moderate improvements in overall survival and quality of life; nevertheless the 5-year survival remains below 30%. Oncolytic vaccinia virus (VV) therapy has shown strong anti-tumour activity in orthotopic murine models of CRC, however transient delays in disease progression are insufficient to lead to long-term survival. Here we examined the efficacy of combining VV with oxaliplatin or SN-38 (active metabolite of irinotecan) in three CRC cell lines in vitro and VV with irinotecan in an orthotopic xenograft model of metastatic CRC. Synergistic improvements in in vitro tumour cell killing were not due to drug-mediated enhancement of viral production. Combination therapy was well tolerated in DLD1 tumour-bearing mice and the median survival was significantly increased relative to monotherapy despite a drug-dependent decrease in the mean virus titer. Increased apoptosis following in vitro and in vivo combination therapy was observed. In vitro cell cycle analysis showed increases in S-phase cells following infection occurred in both infected and uninfected cell populations. This suggests that uninfected virus-treated cells should be more sensitive to SN-38-induced apoptosis than untreated cells; which was supported by a 4-fold greater increase in apoptosis in the uninfected cells than infected cells following combination therapy. Combination treatment strategies continue to be among the best options for patients with advanced cancers. VV is currently under clinical investigation in patients with CRC and the data presented here suggesting that its combination with irinotecan may provide benefit to a proportion of CRC patients. Further, investigation of this combination is necessary to determine the tumour characteristics responsible for mediating synergy.
3.2 Introduction

Cancer is a complex and heterogeneous disease for which successful treatment regimens are rarely simple. Today, there are few chemotherapeutics administered alone. Combination chemotherapy and multi-modality cancer treatment strategies (surgery and/or radiation therapy) have significantly improved survival for many cancer types; unfortunately, for advanced diseases such as metastatic colorectal cancer (CRC), these traditional approaches are still inadequate (8) for the majority of patients. Novel combination therapy strategies that include targeted agents with multiple mechanisms of action (MOAs), increased specificity and decreased toxicity are required to improve outcomes in diseases where effective traditional therapies are lacking.

Oncolytic viruses (OVs) are tumour-specific agents that take advantage of the many deregulated pathways characteristic of malignant cells. OVs have multi-mechanistic modes of action, including direct oncolysis, induction of anti-tumour immune responses (161, 248), and anti-angiogenesis (198, 358). OVs have been studied in a wide variety of preclinical models and show promising results in early phase clinical trials (592). As with the vast majority of cancer therapies, OVs are unlikely to be delivered alone but rather as part of a rationally designed, combination therapy regimen (466). OVs have been combined with surgical resection (199, 569, 593, 594), radiation(523, 524), and numerous chemotherapy drugs(470, 495, 501, 503, 504, 507, 595, 596) in preclinical models. Combination OV and chemotherapy studies have demonstrated that particular combinations can be highly synergistic in specific tumour models. In many cases it is still unclear what exact MOA underlies these synergistic interactions.

In this study we investigate the efficacy of an attenuated oncolytic vaccinia virus (VV) combined with chemotherapy in models of colorectal carcinomatosis; a clinically challenging, locally disseminated disease with limited treatment options. Currently, the 5-year survival rate in patients with inoperable tumours is less than 10% (8). CRC chemotherapy drugs including oxaliplatin (OX) a platinum-based DNA-crosslinker, irinotecan (also known as CPT-11) which is activated by hydrolysis to SN-38 a topoisomerase I inhibitor, and 5-fluorouracil (5-FU)/leucovorin (LV) a pyrimidine analog and its enhancer, are used to reduce tumour burden and palliate symptoms. Addition of OX and CPT-11 to the standard 5-FU regimen has significantly improved survival in patients with peritoneally disseminated CRC (597). Furthermore, intraoperative heated intraperitoneal chemotherapy with a combination of 5-FU,
LV, OX and/or mitomycin C (DNA-crosslinker) has resulted in reported 5-year survival rates between 19 – 31% in select patient populations (598).

It has previously been shown that OX synergistically improves killing in CRC cells when combined with VV expressing tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (507). Combination therapy significantly increased induction of apoptosis and improved survival relative to monotherapy in models of disseminated CRC in a transgene-dependent manner. Other platinum-based agents have also been shown to synergize with VV primarily through enhanced anti-tumour immune responses (496, 599). While CPT-11 has been demonstrated to interact synergistically with oncolytic herpes simplex virus(504), adenovirus (503) and reovirus (501) its effect when combined with oncolytic VV is unknown.

We have previously shown that ‘double-deleted’ VV (vvDD)(123) significantly improved survival in peritoneally disseminated models of metastatic CRC, however no long term survival was observed (600). In this study we identify virus/drug combinations that exhibit synergistic interactions and uncover a novel mechanism of virus-induced sensitization to CPT-11 therapy. Additionally we report improved survival with combination therapy in an orthotopic model of metastatic CRC.

3.3 Materials and Methods

Cells lines and drugs. Human colorectal adenocarcinoma (HT29 and DLD1) and monkey kidney fibroblast (CV-1) cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). MC38 murine colorectal adenocarcinoma and 24-JK murine sarcoma cell lines were obtained from National Institutes of Health (Bethesda, Maryland, USA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON, Canada) and 1% antibiotic-antimycotic (Invitrogen, GIBCO, Grand Island, NY, USA) at 37°C with 5% CO₂. Stocks of oxaliplatin (0.5mg/ml in water; Sigma Aldrich) and SN-38 (1mg/ml in dimethyl sulfoxide; Sigma Aldrich) were stored at -20°C. Clinical grade CPT-11 (20mg/ml; Princess Margaret Hospital Pharmacy, University Health Network) used in in vivo studies, was stored at 4°C and used within 3 weeks of dispensing.
Vaccinia virus. Vaccinia virus expressing the human somatostatin receptor (SR) and red fluorescent protein under control of the p7.5 and psel promoters, respectively, was generated as previously described (574, 600). Virus was propagated in 24-JK cells and purified by ultracentrifugation over a sucrose cushion. Virus was titered on CV-1 cells and stored at -80°C.

In vitro dose response. Cells were seeded in 96-well plates and incubated overnight. For simultaneous therapy, cells were pre-infected with vvDD-SR-RFP using low-serum DMEM (2.5% FBS) at low volume (25 μl). Plates were incubated for 2 h with shaking then DMEM (10% FBS) containing increasing drug concentrations was added and plates were incubated for 72 h. For virus pre-treatment experiments, pre-infection was performed as above, after which wells were suplemented with 75 μl DMEM (10% FBS) and incubated for 24 h prior to addition of drug diluted in DMEM (25 μl). In drug pre-treatment experiments, cells were treated with drug 24 h prior to infection. Drug media was removed and infections were performed in the absence of drug, as above, after which fresh drug media was added. All experiments used a fixed-ratio dose escalation. Seventy-two hours after the final treatment, cell viability was assessed by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter96® Aqueous One Solution, Promega, Madison, WI, USA) according to the manufacturers protocol. Experiments were performed in quadruplicate and three individual experiments were carried out for each cell line and drug.

Combination index. The Chou and Talalay method (467) for quantifying drug-drug interactions was used. Data from individual dose response experiments was used to calculate the combination index (CI) over a range of fraction-affected (Fa) values. The average CI values for three experiments were plotted in Fa-CI plots where CI = 1 is additive, CI < 1 is synergistic and CI > 1 is antagonistic.

Fluorescence microscopy. Infected cells were imaged using a Zeiss AxioObserver microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Series 120Q Fluorescence Illumination unit (EXFO, Quebec City, QC, Canada). Images were acquired with a Coolsnap HQ camera (Roper Scientific, Tucson, AZ, USA).
**Virus replication.** Cells were seeded in 6-well plates at 5 x 10^5/well and incubated overnight. Pre-infection was performed at a multiplicity of infection (MOI) of 0.1 for 2h in 0.5ml low-serum DMEM with shaking. Cells were supplemented with drug-containing media (at indicated doses) or media alone. Cells and supernatant were collected at indicated time points. Samples underwent three freeze-thaw cycles and sonication to release virus particles. Virus was quantified by plaque assay on CV-1 cells.

**Flow cytometry.** Treated cells were harvested, washed in PBS and stained with Annexin V-FITC (Biolegend, San Diego, CA, USA) and 7-Aminoactinomycin D (7-AAD; Biolegend) as per the manufacturer’s protocol. Triplicates for each treatment group were run and 20 000 events per sample were collected using a FACScan Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and analyzed (BD Cell QuestPro software). To analyze the cell-cycle, treated cells were harvested, washed in PBS and fixed in a 1% paraformaldehyde (PFA) solution on ice for 1h. PFA was removed and cells were washed with PBS, resuspended in ice cold 70% ethanol and incubated overnight or until further analysis. Ethanol was removed and cells were washed with PBS prior to staining with 4’6-diamidino-2-phenylindole (DAPI) solution (1ug/ml in 0.1% Triton-X-100, Bioshop, Burlington, ON, Canada). Cells were incubated for 30 minutes at room temperature and analyzed on a BD LSRFortessa cell analyzer (Becton, Dickinson and Company). Cell-cycle modeling was performed ModFit LT software (Verify Software House, Topsham, ME, USA).

**Mice.** Mice were housed under standard conditions and given food and water *ad lib*. Protocols were approved by the Animal Care Centre, UHN, Toronto, Canada. Female BALB/c Nu/Nu (Taconic Farms Inc., Hudson, NY, USA) mice were injected intraperitoneally (IP) with 5 x 10^6 DLD1 cells. CPT-11 (40 mg/kg) was delivered IP on days 10, 14, 18 and 22 and virus (10^9 plaque forming units; pfu) was given IP on day 12. Control mice received IP injections of Hanks Buffered Saline Solution (HBSS; Invitrogen, GIBCO) on the same schedule as combination treated mice. Mice were sacrificed at time points indicated and blood/tissues were collected for toxicology, biodistribution and immunohistochemistry (IHC) studies.

**Blood work.** Complete blood counts were performed using a HEMAVET® Multispecies Hematology Analyzer (Drew Scientific, Dallas, TX, USA). Liver enzyme biochemistry analysis
was performed on a VETSCAN VS2 (Abaxis, Union City, CA, USA) using the Comprehensive Diagnostic Profile (Abaxis).

**Virus biodistribution.** Tissues were collected 6 dpi and stored at -80°C in HBSS. Samples were homogenized using a TissueLyzer II (Qiagen, Hilden, Germany), underwent three freeze-thaw cycles and sonication prior to titering on CV-1 cells.

**Immunohistochemistry.** Tissues were fixed in 10% formalin for 72 h then transferred to 70% ethanol. Samples were paraffin embedded, sectioned and stained using the following primary antibodies: polyclonal goat anti-mouse CD31 (PECAM-1 M-20, SC-1506, 1/2000 dilution; Serotec, Raleigh, NC, USA), polyclonal rabbit anti-VV (ab35219, dilution 1/1000; Abcam, Cambridge, MA, USA), polyclonal rabbit anti-cleaved caspase 3 (cat# 9661, dilution 1/600, Cell Signaling, Danvers, MA, USA) and monoclonal rat anti-mouse F4/80 (MCA497GA clone A3.1, dilution 1/2000, Serotec). Slides were scanned using ScanScope XT (Aperio Technologies, Vista, CA, USA) and staining was quantified using ImageScope’s Positive Pixel algorithm (Aperio Technologies). Specifically, regions of interest were drawn around individual tumours and the software calculated the number of positive pixels relative to the total number of pixels per tumour section (% positive).

**Statistical Analysis.** Data were analyzed using the Student’s two-tailed t-test or one-way ANOVA were applicable. Survival curves were analyzed by OASIS (online application for survival analysis; [http://sbi.postech.ac.kr/oasis/surv](http://sbi.postech.ac.kr/oasis/surv)) (601) using a weighted log-rank test (rho = 0, gamma = 1). All other statistical analysis was performed using Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± SD or SEM and statistical significance was defined as $P < 0.05$.

### 3.4 Results

#### 3.4.1 Vaccinia virus synergizes with chemotherapy in CRC cell lines.

Cell viability was determined following treatment of human (HT29, DLD1) and mouse (MC38) CRC cell lines with vvDD and OX or SN-38 (active metabolite of CPT-11) alone or in combination. Cells were treated at increasing doses (fixed ratio) of virus and/or drug and assayed for cell viability at 72 h post treatment (Figure 3.1). Cell lines were differentially susceptible to
individual therapies, with MC38 and HT29 cells showing increased sensitivity to SN-38 (IC_{50} 0.4 µM ± 0.2 and 0.25 µM ± 0.06, respectively) and OX (23 µM ± 2 and 63 µM ± 18, respectively) compared to DLD1 cells (IC_{50} 8 µM ± 3 and 78 µM ± 27, respectively).

The Chou-Talalay method for determining drug-drug interactions (467) was used to analyze the data from three independent experiments (presented in Figure 3.1) the results of which are presented in Fa-CI plots (Figure 3.2). When administered simultaneously vvDD interacted synergistically with OX over a wide range of Fa values in MC38 cells [(CI)_{0.1} = 0.83 ± 0.06 to (CI)_{0.9} = 0.70 ± 0.07] (Figure 3.2A) while only displaying synergy at lower Fa values in HT29 [(CI)_{0.1} = 0.52 ± 0.09 to (CI)_{0.9} = 1.065 ± 0.002] (Figure 3.2C) and DLD1 [(CI)_{0.1} = 0.6 ± 0.3 to (CI)_{0.9} = 2.5 ± 0.9] (Figure 3.2E) cells. Pretreatment with either virus or OX had little effect on synergy relative to simultaneous treatment in both MC38 and HT29 cells. In DLD1 cells, pretreatment with either agent improved synergy at higher Fa values relative to simultaneous treatment. Combination therapy with SN-38 was extremely antagonistic in MC38 cells at low Fa values [(CI)_{0.1} not shown] but synergistic at higher values [(CI)_{0.1} = 27 ± 18 to (CI)_{0.9} = 0.30 ± 0.08] (Figure 3.2B). The opposite relationship between dose and synergy was observed in HT29 cells [(CI)_{0.1} = 0.3 ± 0.2 to (CI)_{0.9} = 17 ± 15] (Figure 3.2D). DLD1 cells showed consistently strong synergism between vvDD and SN-38 over all fractions affected following both simultaneous treatment [(CI)_{0.1} = 0.5 ± 0.3 to (CI)_{0.9} = 0.4 ± 0.2] and virus pretreatment [(CI)_{0.1} = 0.5 ± 0.1 to (CI)_{0.9} = 0.6 ± 0.1] (Figure 3.2F). Based on these data, the combination of vvDD and CPT-11 was selected for further study in vivo.

### 3.4.2 Combination therapy improves survival in vivo despite inhibition of virus replication.

BALB/c nu/nu mice bearing IP DLD1 tumours were treated with vvDD (10^9 pfu) and/or CPT-11 (40mg/mg) or buffer alone. Body weight was recorded as an indicator of toxicity for all groups until the first mouse was sacrificed (Figure 3.3A). No significant weight loss was observed with either vvDD or CPT-11 therapy alone. Transient weight loss was observed in combination treated mice relative to all other groups at day 14 but normalized by day 22. Complete blood counts and liver enzyme analysis were also performed to evaluate potential toxicity due to combination therapy (Table 1 and 2). Neutrophils and monocytes were elevated above the
Figure 3.1. Combination VV and chemotherapy in colorectal cancer cells. Cells were treated simultaneously with vvDD and OX (a, c, e) or SN-38 (b, d, f) alone or in combination at a fixed concentration ratio. Cell viability was assessed 72h post treatment by MTS assay. Shown is the average viability from three independent experiments ± SEM.
Figure 3.2. Characterization of VV and drug interactions. The combination index (CI) for each treatment combination was calculated from the dose response curves (Figure 1). Plotted are the average CI values from three independent experiments ± SEM. Extremely high CI values were omitted from the plots.
Figure 3.3. Combination therapy in in vivo CRC model. BALB/c nu/nu mice bearing intraperitoneal (IP) DLD1 tumours were treated with IP VV (day 12) and/or IP irinotecan (CPT-11; day 10, 14, 18, 22; 40 mg/kg) or vehicle alone. (a) Body weight was measured every 2-4 days up until day 30 as a measure of toxicity. (b) Biodistribution of live virus in tumours and normal organs was determined at 6 dpi. Shown is the mean plaque forming units (pfu) per mg of total protein ± SD (n=3). *P < 0.05 VV compared to VV + CPT-11 (two-way ANOVA with Bonferroni post test). (c) Kaplan-Meier survival curve for HBSS (n = 5), CPT-11 (n = 6), VV (n = 6) and VV + CPT-11 (n = 6) treated mice. Group-wise comparison shows median survival of combination treated mice (87.5 d) was significantly increased relative to either vvDD (57 days; p = 0.0179) or CPT-11 (48 d; p = 0.0190) (Weighted log-rank test, rho = 0, gamma = 1)
**Table 3.1 Complete blood count.** Blood collected 6 dpi from tumour-bearing mice treated as in Figure 3.3. Mean (SD)

<table>
<thead>
<tr>
<th></th>
<th>WBC</th>
<th>Neu</th>
<th>Lym</th>
<th>Mono</th>
<th>Eosin</th>
<th>Baso</th>
<th>RBC</th>
<th>Hb</th>
<th>HCT</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>RDW</th>
<th>PLT</th>
<th>MPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.23 (0.184)</td>
<td>0.96 (0.771)</td>
<td>2.89 (0.735)</td>
<td>0.33 (0.283)</td>
<td>0.05 (0.057)</td>
<td>0.01 (0.007)</td>
<td>8.28 (1.945)</td>
<td>11.75 (2.333)</td>
<td>45.25 (10.112)</td>
<td>54.75 (0.636)</td>
<td>14.25 (0.495)</td>
<td>26.05 (0.636)</td>
<td>17.8 (0.707)</td>
<td>High* (0.071)</td>
<td></td>
</tr>
<tr>
<td>vvDD</td>
<td>9.87 (2.351)</td>
<td>2.60* (1.049)</td>
<td>5.38 (0.988)</td>
<td>1.77* (0.662)</td>
<td>0.11 (0.086)</td>
<td>0.02 (0.021)</td>
<td>9.15 (0.715)</td>
<td>12.93 (1.060)</td>
<td>50.37* (5.824)</td>
<td>54.97 (2.515)</td>
<td>14.13 (0.153)</td>
<td>25.77 (0.981)</td>
<td>18.20 (0.361)</td>
<td>High* (0.058)</td>
<td></td>
</tr>
<tr>
<td>vvDD + CPT-11</td>
<td>8.07 (2.779)</td>
<td>1.97 (0.618)</td>
<td>5.15 (1.963)</td>
<td>0.90* (0.377)</td>
<td>0.04 (0.006)</td>
<td>0.02 (0.006)</td>
<td>9.77* (0.323)</td>
<td>13.67 (0.416)</td>
<td>55.03* (1.877)</td>
<td>43.00 (22.138)</td>
<td>13.97 (0.153)</td>
<td>24.87 (0.635)</td>
<td>18.53 (0.252)</td>
<td>3759.67* (0.058)</td>
<td></td>
</tr>
<tr>
<td>Normal Range</td>
<td>1.8 - 10.7</td>
<td>0.1 - 2.4</td>
<td>0.9 - 9.3</td>
<td>0.0 - 0.4</td>
<td>0.0 - 0.2</td>
<td>0 - 0.2</td>
<td>6.36 - 9.42</td>
<td>11.0 - 15.1</td>
<td>35.1 - 45.4</td>
<td>45.4 - 60.3</td>
<td>14.1 - 19.3</td>
<td>30.2 - 34.2</td>
<td>12.4 - 27.0</td>
<td>592 - 2972</td>
<td></td>
</tr>
</tbody>
</table>

WBC, white blood cell; Neu, neutrophil; Lym, lymphocyte; Mono, monocyte; Eosin, eosinophil; Baso, basophil; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet; MPV, mean platelet volume. *Mean outside normal range.

**Table 3.2 Blood biochemistry.** Blood collected 6 dpi from tumour-bearing mice treated as in Figure 3.3. Mean (SD)

<table>
<thead>
<tr>
<th></th>
<th>ALB</th>
<th>ALP</th>
<th>ALT</th>
<th>AMY</th>
<th>TBL</th>
<th>BUN</th>
<th>CA</th>
<th>PHOS</th>
<th>CRE</th>
<th>GLU</th>
<th>Na+</th>
<th>K+</th>
<th>TP</th>
<th>GLOB</th>
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<tbody>
<tr>
<td>Control</td>
<td>36.00* (4.243)</td>
<td>31.50 (9.192)</td>
<td>214.00* (237.588)</td>
<td>1226.50 (406.586)</td>
<td>4.50 (0.707)</td>
<td>7.40 (0.849)</td>
<td>2.76 (0.007)</td>
<td>2.96 (0.028)</td>
<td>&lt;18</td>
<td>8.80 (2.404)</td>
<td>152.50* (2.00)</td>
<td>55.00 (1.414)</td>
<td>19.00 (2.828)</td>
<td></td>
</tr>
<tr>
<td>vvDD</td>
<td>33.00 (2.00)</td>
<td>39.00 (7.00)</td>
<td>87.00 (82.286)</td>
<td>1208.67 (260.101)</td>
<td>4.33 (0.577)</td>
<td>6.20 (1.179)</td>
<td>2.65 (0.137)</td>
<td>3.28 (0.237)</td>
<td>&lt;18</td>
<td>11.73 (0.611)</td>
<td>150.33* (2.082)</td>
<td>8.17 (0.577)</td>
<td>51.00 (1.732)</td>
<td>18.00 (1.00)</td>
</tr>
<tr>
<td>vvDD + CPT-11</td>
<td>38.33* (1.5275)</td>
<td>38.67 (10.693)</td>
<td>38.33 (3.055)</td>
<td>981.00 (99.504)</td>
<td>4.00 (1.00)</td>
<td>5.83 (0.351)</td>
<td>2.79 (0.057)</td>
<td>3.23 (0.296)</td>
<td>&lt;18</td>
<td>12.20 (3.404)</td>
<td>151.33* (4.041)</td>
<td>8.30 (0.577)</td>
<td>53.67 (2.00)</td>
<td>16.00 (2.00)</td>
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<tr>
<td>Normal Range</td>
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<td>28 - 94</td>
<td>28 - 184</td>
<td>12.1 - 20.5</td>
<td>2.77 - 3.02</td>
<td>2.68 - 3.62</td>
<td>9.7 - 18.6</td>
<td>143 - 150</td>
<td>3.8 - 10.0</td>
<td>18 - 82</td>
<td></td>
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</tr>
</tbody>
</table>

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; TBL, total bilirubin; BUN, blood urea nitrogen; CA, calcium; PHOS, phosphorous; CRE, creatinine; GLU, glucose; TP, total protein; GLOB, globulin. * Mean outside normal range.
normal range in vvDD-treated mice while only monocytes were elevated in combination-treated mice. Nevertheless, there was no significant difference in any of the white blood cell compartments between treatment and control groups. Liver enzymes (albumin, alkaline phosphatase and alanine aminotransferase) were all within normal range for both vvDD vvDD + CPT-11 treated mice.

Six days post-infection tumour and normal tissues were harvested to evaluate the biodistribution of live virus particles (Figure 3.3B). Virus localized specifically to tumours in both virus alone and combination treated mice. Normal tissue mean titers were at least 2–4 log decreased relative to tumour tissue in both vvDD and vvDD + CPT-11 treated mice (with the exception of the ovaries in virus alone treated mice). While combination therapy resulted in virus being detected in the bowel (3 of 3 mice), ovary (2 of 3), spleen (2 of 3), brain (2 of 3), bone marrow (2 of 3), mean titers were only significantly decreased in the tumours (7 x 10^4 pfu/mg) relative to virus alone (3 x 10^6 ± 6 x 10^6 pfu/mg). Combination therapy significantly improved survival relative to either monotherapy (median survival vvDD + CPT-11, 87 d; vvDD, 57 d, p = 0.0179; and CPT-11, 48 d, p = 0.0190) (Figure 3.3C). These data are consistent with our in vitro results which showed a strong synergy between vvDD and SN-38 in DLD1 cells. In addition, they indicate that virus replication is not the mediator of improved anti-tumour efficacy.

3.4.3 Effect of chemotherapy on vvDD replication.

Given that SN-38 interacts with cellular DNA it is possible that it may also affect DNA synthesis. To determine whether drug treatment affected virus replication, the kinetics of viral RFP expression (Figure 3.4A) and live virus production (Figure 3.4B-D) were assessed. The presence or absence of SN-38. Viral RFP was slightly decreased in all cell lines when combined with SN-38 (0.1 – 5 µM; 5 µM not shown) relative to virus alone (MOI=0.1). Crystal violet staining of duplicate wells confirmed cell viability over all drug doses (with exception of HT29 cells); therefore, decreases in RFP expression were not due to a loss of target cell not shown). SN-38-induced cytotoxicity in HT29 cells may account for the observed decrease in RFP expression. Synergistic dose combinations were selected to assess the effect of drug on virus replication. Despite SN-38 and vvDD exhibiting a strongly synergistic effect in DLD1 cells, there was a significant inhibitory effect of drug on viral RFP expression (Figure 3.4A).
Figure 3.4. Effects of drug on virus gene expression and replication. Viral RFP expression was evaluated in the presence and absence of SN-38. Cells were infected at MOI 0.1 and treated with increasing concentrations of drug. Fluorescence microscopy was performed at 24 – 72h post treatment. Virus replication was determined in the presence and absence of SN-38 (b-d). Cells were treated at synergistic dose combinations and live virus particles were quantified at 2 – 72h post infection by plaque assay. Data represents the mean plaque forming units (pfu) of triplicate values ± SD. DMSO was used as a vehicle control. *P < 0.05.
replication (Figure 3.4D). SN-38 also inhibited virus replication at synergistic dose combinations in MC38 (Figure 3.4B) and HT29 (Figure 3.4C) cells. Similarly, OX did not improve virus replication at synergistic dose combinations (Appendix 3.1).

### 3.4.4 Combination therapy increases apoptosis in RFP-negative virus-treated cells.

Cells were infected (MOI = 1) and/or treated with SN-38 (1µM) using a ‘simultaneous’ treatment protocol. In the following flow cytometry experiments a higher dose ratio (MOI:µM) was used relative to the previous experiments, due to the low rate of infection in DLD1 cells. Synergy at this higher dose ratio was confirmed by Chou-Talalay’s combination index (Appendix 3.2). Controls were mock-infected and treated with DMSO. At 48 and 72 hpi a significant increase in apoptosis (Figure 5a, top) was observed in both drug- (8 % ± 1 and 31 % ± 7) and combination- (15 % ± 1 and 46 % ± 6) treated cells relative to virus (3.4 % ± 0.9 and 6.8 % ± 0.8; one-way ANOVA, p < 0.05). Combination therapy resulted in significantly higher levels of early apoptosis (Annexin V+/7AAD-) relative to drug and virus alone (9 % ± 1 vs. 5.8 % ± 0.3 and 4.6 % ± 0.4, respectively; one-way ANOVA, p <0.05). Representative scatterplots from 72 hpi are shown in Figure 3.5A.

VV efficiently replicates its DNA and produces thousands of viral progeny leading to host cell lysis within approximately 24 hpi (168). Given that that synergy was not due to an increase in viral production and that apoptosis – the primary mechanism of SN-38 induced cell death - was increased with combination therapy, we hypothesized that virus treatment sensitized cells to drug-induced cell death. To test this hypothesis, we determined whether the observed increase in apoptosis following combination treatment occurred preferentially in RFP-positive or RFP-negative cells. In these studies, RFP expression is under control of the early/late promoter, psel, and therefore served as a marker of both early viral gene expression, which occurs prior to viral DNA replication and late expression(602). Therefore, RFP-negative cells represent the population of cells that are either uninfected or in the initial stages of viral infection (i.e. prior to early gene expression). Cells were stratified based on RFP expression and the percentage of apoptotic cells (annexin-V+) in the RFP+ and RFP- compartments was compared between vvDD and vvDD + SN-38 treatments (Figure 3.5B). At 72 hpi virus alone resulted in 15.3 ± 0.8 %
**Figure 3.5 Induction of apoptosis in combination VV and SN-38 treated cells.** DLD1 cells treated with VV (MOI 0.1) in the presence and absence of SN-38 (1μM) were analyzed by flow cytometry. (a) Total (top graph) and early (bottom graph) apoptosis (Annexin-V positive) cells at 48 and 72 h post treatment. Representative dot plots (right) of 72 hpi data presented in graphs. Bars represent the mean percentage of triplicate values ± SEM. *p < 0.05; one-way ANOVA. (b) Proportion of uninfected cells (RFP-) and infected (RFP+) cells undergoing apoptosis in vvDD and vvDD + SN-38 treated cells at 48 hpi (left). Representative dot plots (right) of data presented in graphs. Bars represent the mean percentage of RFP+/AV+ and RFP-/AV+ cells of triplicates ± SEM. *p < 0.05; two-way ANOVA.
annexin-V+ staining, of which 60 ± 14% (10 ± 2% of total) was RFP+ and 40 ± 14% (5 ± 1% of total) was RFP-. Addition of SN-38 increased annexin V staining to 53 ± 2% of which 23.7 ± 0.8% (12.5 ± 0.9% of total) was RFP+ and 76.3 ± 0.8% (40 ± 2% of total) were RFP-. Therefore, in addition to significantly increasing the overall levels of apoptosis relative to either monotherapy, combination therapy increased apoptosis by approximately 1.2-fold in RFP+ cells and 7.9-fold in RFP- cells relative to virus alone. The disproportionate increase in apoptosis in RFP- virus-treated cells suggests that these cells are being sensitized to drug-induced cell death and this likely contributed to the observed synergy.

3.4.5 VV induced cell-cycle arrest may prime cells for SN-38 therapy.

The effect of VV on cell cycle has been well documented; shortly after infection (24h) VV induces S-phase arrest in order to promote virus replication (603). Additionally, cells in S-phase are known to show heightened susceptibility to SN-38-induced cell death (42). Taken together with our findings that SN-38 inhibits virus replication in addition to increasing apoptosis when given in combination we hypothesized that vvDD sensitizes cells to SN-38 treatment through a bystander effect on cell cycle. To test this hypothesis DLD1 (Figure 3.6) and HT29 (Appendix 3.3) cells treated at synergistic and non-synergistic dose combinations, respectively, and were analysed by flow cytometry following DAPI staining. Cells were sub-confluent at the time of plating and control cells were undergoing exponential growth throughout the course of the experiment. Following treatment of DLD1 cells, virus alone resulted in a significant increase in S-phase cells (38 ± 1% vs 16.1 ± 0.6%, p < 0.001, two-way ANOVA) and small but significant increase in G2/M phase cells (17 ± 2% vs 12.7 ± 0.9%, p < 0.05), while drug alone resulted in a significant increase in S- (45 ± 3% vs 16.1 ± 0.6%, p < 0.001) and G2/M-phase (35 ± 2% vs 12.7 ± 0.9%, p < 0.001) cells relative to control (Figure 3.6A). Combination therapy also resulted in a significant increase in S- (50 ± 2% vs 16.1 ± 0.6%, p < 0.001) but not G2/M- (12 ± 2% vs 12.7% ± 0.9, p > 0.05) phase cells relative to control (Figure 3.6A). Next we determined the percentage of RFP+ and RFP- virus-treated cells in S-phase alone and in combination with SN-38. vvDD alone resulted in a significant increase in both RFP+ (44 ± 3% vs 16.1 ± 0.6%, p < 0.001) and RFP- (32.4 ± 0.9% vs 16.1 ± 0.6%, p < 0.001) S-phase cells relative to control (Figure 3.6B). Similarly, the percentage of RFP- combination-treated cells in S-phase (59 ± 2%) was significantly higher than that observed with virus (32.4 ± 0.9%, p <
0.001), drug (45 ± 3%, p < 0.001) and control (16.1 ± 0.6 %, p < 0.001)-treated cells. The significant S-phase

Figure 3.6 Cell-cycle analyses of VV and SN-38 treated cells. DLD1 cells treated with VV (MOI 1) in the presence and absence of SN-38 (1μM) were stained with DAPI and analyzed by flow cytometry for DNA content at 72 h post treatment. (a) Representative cell-cycle histograms (top) and quantification of cell-cycle distribution (bottom). (b) Cell-cycle analysis of uninfected (gated RFP-) and infected (gated RFP+) virus-treated cells from (a) (left) and quantification of cells in S-phase (right). Bar graphs represent the mean of triplicates ± SD, * p < 0.05. Histograms are superimposed with a software-generated model of DNA distribution.
increase in RFP-, virus-treated cells combined with the disproportional increase in apoptosis in RFP- combination-treated cells supports our conclusion that VV sensitizes cells that are either uninfected or in the early stages of infection to SN-38-induced apoptosis.

In HT29 cells treated at a non-synergistic dose combination, virus alone had no effect on the overall proportion of S-phase cells compared to mock at 48 hpi (31 ± 5 % vs 29 ± 3%, p > 0.05) (Appendix 3.3). In contrast, SN-38 (0.02 µM) had a profound effect of cell cycle, causing a dramatic shift of cells into G2/M (90 ± 2%). Cell cycle distribution following combination therapy was indistinguishable from that of SN-38 alone. Comparison of cell cycle alterations in DLD1 and HT29 cells shows that whereas combination therapy in latter resulted an intermediate distribution between that of VV alone and SN-38 alone, HT29 cells were extremely sensitive to the effects of SN-38 on cell cycle in the presence or absence of vvDD. This suggests that the lack of synergy in HT29 cells may be due in part to the absence of a substantial VV-induced bystander effect and the high level of SN-38 sensitivity.

3.4.6 Apoptosis and immune cell infiltration in combination treated CRC tumours.

In order to determine whether vvDD interacted in a similar way with CPT-11 in vivo as it did with SN-38 in vitro, IHC studies were performed at various time points to look at virus, apoptosis and immune cell infiltration in tumours (Figure 3.7). Biodistribution data suggested that CPT-11 had an inhibitory effect on virus replication (Figure 3.3B) as was seen in vitro (Figure 3.4). The percentage of positively stained pixels relative to the total number of pixels was determined for multiple tumours per mouse using ImageScope’s Positive Pixel Algorithm. IHC staining 1 dpi showed no significant difference in the amount of virus in tumours following combination therapy compared to virus alone; whereas by 3 dpi VV staining was significantly increased in tumours from virus alone treated mice (15.4 ± 2.77% vs 3.10 ± 1.49%, p = 0.0175) (Figure 3.7A). This indicates that there is no significant difference in the initial infection of tumours and that virus replication and/or spread is impeded by CPT-11. Given the role of apoptosis in in vitro synergy we also looked at markers of apoptosis (Figure 3.7B and C). At 1 dpi combination treatment significantly increased activated caspase-3 staining relative to all
other groups (3.02 ± 0.308%, vvDD+CPT-11; vs 0.794 ± 0.120%, CPT-11; 0.638 ± 0.087%, vvDD; 0.439 ± 0.120%, HBSS; p < 0.0001). By 3 dpi caspase-3 activation

Figure 3.7 *In vivo* virus replication, apoptosis and immune infiltration is augmented by VV and CPT-11 combination therapy. Mice treated as in Figure 3.3 were sacrificed at 1, 3 and 6 dpi and all macroscopic tumours were removed, formalin-fixed and analyzed by IHC (n=3-4). (a) Representative images (left) and quantification (right) of VV staining of tumours from mice treated with vvDD or vvDD + CPT-11. Representative images (b) and quantification of IHC staining for activated caspase 3 (c) and F480 (d). Bars represent the average of all tumours from each mouse in each treatment group ± SEM. Mean [range] number of tumours analyzed per mouse: 3 [1-6], 1 dpi and 23.3 [15 – 29], 3 dpi (HBSS); 16.3 [13-20], 1dpi and 17.7 [13-22], 3 dpi (CPT-11); 20.8 [8-36], 1dpi and 15 [9-23], 3 dpi (vvDD); 14.25 [7-31], 1dpi and 5.7 [1-14], 3 dpi (vvDD+CPT-11). * p < 0.05
was increased in all treatment groups relative to control (4.004 ± 0.3254%, CPT-11; 6.60 ± 0.6757%, vvDD; 4.597 ± 0.6265%, vvDD+CPT-11; vs 1.52 ± 0.185%, HBSS). The early increase in apoptosis following combination therapy likely contributes to the improved treatment efficacy, however to sustain long-term inhibition of disease progression, as was seen in the survival studies, there are almost certainly other factors involved.

Oncolytic VV therapy is mediated at least in part through induction of an anti-tumour immune response (154, 604). Viral lysis of tumour cells releases cellular- and viral-associated danger signals as well as tumour-associated antigens. Resultant local inflammation can lead to cross priming of immune cells towards tumour antigens. Both VV and CPT-11 are known to increase tumour-infiltrating immune cells such as macrophages in CRC tumour models (604, 605) and increased tumour-infiltrating lymphocyte scores have been shown to improve the clinical efficacy of CPT-11 in primary and metastatic CRC (270, 606). Given this evidence we examined the effect of combination therapy on immune cell infiltration. The early increase in tumour apoptosis in combination treated mice was accompanied by an increase in macrophage infiltration (Figure 3.7B and D). At 1 dpi combination treatment resulted in significantly increased macrophage infiltration relative to control (20.56 ± 1.593% vs 9.526 ± 1.322%, p<0.05). By 3 dpi macrophage infiltration was still increased in vvDD+CPT-11 treated tumours (17.05 ± 1.647%) relative to control (9.602 ± 0.6868%, p<0.05) or drug alone (12.13 ± 0.7838%, p < 0.05).

Infiltration into tumours from vvDD treated mice was also significantly increased relative to control (14.82 ±1.265%, p<0.05). Increased macrophage infiltration in combination treated mice relative to all other groups was maintained at 6 dpi (p < 0.05). Staining for other immune cell infiltrates and stroma cells (B-cells and endothelial cells) was not significantly different between treatment groups. The early and sustained increase in macrophage infiltration following combination therapy likely contributed to improved anti-tumour efficacy, as seen in previous studies, while simultaneously limiting VV replication and spread.
3.4.7 Discussion

Metastatic CRC still represents a complex and challenging clinical problem. Unfortunately, the efficacy of current chemotherapy regimens is limited by the development of dose-limiting toxicities (607, 608) and chemo-resistance (609). Novel combination treatments that could effectively decrease tumour burden or decrease toxicity and chemo-resistance would be of clinical benefit.

OVs have unique characteristics that make them ideal candidates for the treatment of such locally advanced diseases. Owing to their multi-mechanistic mode of action the risk of acquired-resistance is low and potential to synergize with secondary therapies is high. Not surprisingly, the preclinical literature increasingly supports the concept that, like most other cancer therapies, OV therapy may be most effective when delivered as part of a rationally designed combination treatment strategy (466).

We and others have shown that vvDD can be used to significantly improve survival in murine models of peritoneally disseminated CRC (196, 600). Two early phase clinical trials are currently underway looking at IV (Jennerex, NCT01380600) and IP (Genelux, NCT01443260) delivery of VV in this patient population. Here we aimed to identify potential synergistic interactions between oncolytic VV and current CRC chemotherapy drugs such that future clinical trials may take advantage of existing therapies to maximize the therapeutic benefit OVs in these patients.

Analysis of virus-drug interactions using the Chou-Talaly method (467) demonstrated that vvDD interacts synergistically with OX and SN-38 in a dose-, cell line- and schedule-dependent manner. Previous work reported no synergy between a different oncolytic VV (vJS6) and OX in MC38 and DLD1 cells (507), however we observed synergistic interactions in all cell lines tested (at specific doses with different treatment schedules). Differences in the virus backbone (vJS6; contains a single attenuation deletion, tk-) as well as the selected dose combinations and experimental design may account for the divergent findings. Despite being the most sensitive to SN-38 induced cell death, HT29 cells showed the least amount of synergy when treated with vvDD + SN-38 (synergy observed only a low Fa values). SN-38 was strongly
synergistic in MC38 and DLD1 cells over a range of Fa values. In these cells, SN-38 synergistically improved cell killing when delivered as part of a simultaneous and virus pretreatment schedule; however, drug pretreatment was extremely antagonistic in MC38 cells (data not shown) and at most additive in DLD1 cells.

In vivo combination of vvDD with CPT-11 was well tolerated; no hematological or liver function abnormalities were attributed to combination treatment. Despite substantially decreased virus in the tumours, combination therapy significantly improved survival over either monotherapy. In vitro investigation of the mechanism of synergy confirmed that drug-induced enhancement of virus replication did not account for improved cell death with combination therapy. SN-38/CPT-11 act at the level of cellular DNA replication, binding to DNA and topoisomerase I to form irreversible double-strand DNA breaks when in contact with a replication fork (610) therefore it is conceivable that the drugs may also act on viral DNA replication. However, VV encodes its own viral topoisomerase I and previous work has shown it not to be susceptible to camptothecin (parent drug of irinotecan)-induced inhibition (611). Currently, it is not clear how SN-38/CPT-11 inhibit virus replication and given the added influence of the immune system the mechanism may differ between the in vivo and in vitro models.

The effect of treatment schedule on virus-drug interactions in DLD1 cells – namely that virus pretreatment or simultaneous schedules were generally more synergistic than drug pretreatment - points to virus-induced sensitization of cells to SN-38 toxicity as the likely mediator of synergy. Consistent with this hypothesis we show that combination treatment significantly increased apoptosis, the primary mechanism of SN-38- (610) but not VV-induced cell death. SN-38/CPT-11 are cell-cycle-specific drugs that induce apoptosis in cells undergoing active DNA replication(42). Effects of VV on cell-cycle have been previously reported for total cell populations exposed to virus (603). We show for the first time that VV-induced S-phase accumulation occurs in cells undergoing active viral gene expression as well as in cells that are either uninfected or in the very early stages of viral infection. Uninfected cells thereby become sensitized to SN-38 induced-apoptosis leading to synergistic cell death following combination therapy. VV has previously been shown to induce early activation of MEK and ERK1/2 with subsequent induction of c-fos (612). Activation of this mitogenic signal occurred in the absence
of virus replication even when using a vgf-deleted mutant. Activation of c-fos was shown to be essential in human CRC cells for downstream production of tumour growth factor (TGF)-β, which induced mitosis in a paracrine fashion (613).

Consistent with the results presented here, there have been several reports of synergistic interactions between OV's and chemotherapy drugs in the absence of enhanced viral production (495, 504). vvDD sensitizes cells to paclitaxel through induction of the danger-associated molecular pathogen molecule HMGB1 and interferon (IFN) -β (495). Sensitization through type-I IFN occurred despite VV’s expression of numerous anti-IFN proteins (155). Type I IFNs can also sensitize CRC cell lines to CPT-11 induced cell death (614), likely through accumulation of IFN-treated cells in S-phase. It is possible that IFN may play a role in the synergistic interactions observed in our studies.

Key limitations of this study and many others like it are the reliance on in vitro systems to identify synergy and the lack of confirmatory in vivo data. Indeed in vitro cultures allow focused dissection of individual pathways and proteins involved in virus-drug interactions. However, not all in vitro synergy translates into in vivo synergy. In our study, vvDD and SN-38 interacted synergistically in MC38 cells in vitro, however, combination therapy in MC38 tumour-bearing mice was not effective (Appendix Figure 3.4). In our DLD1 model, in vitro synergy did correspond to improved survival in vivo. In order to evaluate in vivo synergy, as defined by Chou and Talalay (467) a similar design to that used in vitro would be required. Understanding mechanisms of synergy are important as they allow us to better design combination treatment strategies to amplify synergistic interactions. Nevertheless, demonstrated in vitro synergy and in vivo survival improvement without toxicity warrants further investigation of such combinations in clinical trials.

The complex metabolic pathways responsible for the conversion CPT-11 to SN-38 and its subsequent inactivation may also contribute to the differences observed between in vitro and in vivo models. In mice serum carboxylesterases represent one of the primary means of CPT-11 metabolism (615). Metabolism in the gut (specifically duodenum) may also contribute SN-38 production (615). Tumour cells can also express CES, however their contribution to drug metabolism is in murine CRC models relatively minor (616). Overall, mice show very high
levels of metabolic conversion of CPT-11 relative to humans (617). For these reasons, we concluded that SN-38 was the appropriate choice for in vitro experimentation as it more accurately modeled the extra-tumoural conversion of CPT-11. Furthermore, it is interestingly to note that different mouse strains show different levels of SN-38 inactivation through glucuronidation, with C57BL/6 having an approximately 2-fold higher glucuronidation activity compared to BALB/c mice (618). Such differences may have contributed to the lack of efficacy observed in the C57BL/6 model (Appendix 3.4).

The data presented here provides strong evidence to support the clinical investigation of VV in combination with CPT-11. As the safety of VV for use in CRC patients is confirmed through phase I clinical trials, investigators may consider designing future clinical studies to examine the combination of VV with CPT-11 in patients that have failed second-line therapy. This strategy would be particularly pertinent given the bystander effect of VV on uninfected cells and the potential to reverse chemo-resistance to CPT-11.
Chapter 4

4 Vaccinia virus and peptide-receptor radiotherapy synergize to improve treatment of peritoneal carcinomatosis

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Contributions:

KOP, JAM and RR contributed to the overall study design. Individual experiments were devised by KOP with the assistance of JAM, DS and MJ. Experiments were performed by KOP with the assistance of CS, SAF, FAA, LL and DS. Peptide labeling was performed by MJ. Data analysis and manuscript preparation were performed by KOP with critical review by JAM and RR.
4.1 Abstract

**Introduction/Purpose:** Tumour-specific overexpression of receptors has formed the foundation of many targeted cancer therapies such as peptide-receptor radiotherapy (PRRT). PRRT has significantly improved outcomes in patients with somatostatin receptor (SSTR)-positive neuroendocrine tumours. This targeted treatment approach is particularly promising for patients with inoperable metastatic disease however it is currently restricted to use in tumours with specific SSTR overexpression. To overcome this limitation, we propose to use oncolytic vaccinia virus (vvDD)-mediated receptor gene transfer as a tool to permit molecular imaging and PRRT in colorectal cancer (CRC) tumours without endogenous tumour-specific receptor overexpression, a strategy termed radiovirotherapy. The objective of this study is to evaluate the feasibility and efficacy of using vvDD directed gene delivery to make SSTR2-negative tumours amenable to PRRT in an orthotopic peritoneal carcinomatosis (PC) model. **Hypothesis:** We hypothesize that vvDD-SSTR will enable imaging and improved treatment of intraperitoneal CRC tumours using a radiolabeled somatostatin analogue due to tumour-specific accumulation of the radiopeptide. **Methods:** Combination vvDD-SSTR and $^{177}$Lu-DOTATOC were evaluated in a human colorectal cancer cell line (DLD1). Effects of $^{177}$Lu-DOTATOC on vvDD-SSTR replication and cytotoxicity were first evaluated in vitro. Biodistribution of vvDD-SSTR ($10^9$ pfu on day 12) and $^{177}$Lu-DOTATOC (3.5 MBq on day 18) in tumour and normal tissues of an orthotopic CRC PC model were evaluated by plaque assay and gamma-counting, respectively, at several time points post radiopeptide delivery. Tumour uptake was also evaluated by microSPECT/CT imaging. The effect of vvDD-SSTR ($10^9$ pfu) and $^{177}$Lu-DOTATOC (7.5-15 MBq) on complete blood counts and kidney pathology as well as long-term survival in tumour-bearing mice were also investigated. **Results:** $^{177}$Lu-DOTATOC had no effect on virus replication and synergistically improved vvDD-SSTR induced cell killing in a receptor-dependent manner. $^{177}$Lu-DOTATOC did not affect in vivo vvDD-SSTR biodistribution while virus-directed SSTR2 expression significantly increased tumour-specific accumulation of $^{177}$Lu-DOTATOC and the tumour-to-blood ratio relative to controls which included mice treated with $^{177}$Lu-DOTATOC alone or in combination with a receptor-negative vvDD expressing enhanced green fluorescent protein (EGFP). Tumours in mice treated with vvDD-SSTR but not vvDD-EGFP were imageable by microSPECT/CT following $^{177}$Lu-DOTATOC. Radiovirotherapy did not result in any significant general toxicity (assessed by body weight) or hematological toxicity.
Addition of $^{177}\text{Lu}$-DOTATOC (15MBq) to vvDD-SSTR significantly improved survival over virus alone (p<0.05) whereas it did not improve survival when combined with a control virus. **Conclusion:** vvDD-SSTR can be used to convert receptor-negative tumours into receptor-positive tumours thereby making them amenable to molecular imaging and PRRT using radiolabeled somatostatin analogues. Radiovirotherapy represents a promising treatment strategy that could have applications in a wide range of cancer types.

### 4.2 Introduction

Tumour-specific overexpression of cell surface receptors is the foundation upon which many targeted cancer therapies, such as peptide-receptor radiotherapy (PRRT), have been developed. PRRT, which involves the systemic delivery of radiolabeled peptides, is most frequently used in patients with somatostatin receptor (SSTR)-positive neuroendocrine tumours (NETs) (368). The development of reliable radiolabeling methods that can stably link one of the many available somatostatin (SS) analogues (eg. pentetreotide, octreotide, octreotate, etc.) with one of a selection of radionuclides (eg. $^{177}\text{Lu}$, $^{90}\text{Y}$, $^{111}\text{In}$ and $^{68}\text{Ga}$) using a metal-chelator such as 1,4,7,10-tetraazacyclododecane-$N,N',N''$,$N'''$-tetraacetic acid (DOTA), has led to the use of these radiopeptides (RP) as both diagnostic and therapeutic agents. In patients with NETs, pretreatment molecular-imaging is used to identify SSTR-positive primary and metastatic tumours and sophisticated software can quantify the uptake in individual tissue compartments thereby permitting patient-specific dosimetric calculations that predict the toxicity to normal tissues as a result of PRRT.

This targeted treatment approach is particularly beneficial for patients with metastatic disease that is inoperable as evidenced by the significant survival benefit in NET patients following the introduction of PRRT to the clinic (465). Given the success of PRRT in this patient population, its investigational use has been extended to patients with other advanced and metastatic cancers (619), however it remains limited to use in those with tumour-specific SSTR overexpression. To overcome this limitation, oncolytic virus (OV)-mediated receptor gene transfer has been investigated as a tool to permit molecular imaging and targeted radiotherapy using radiopharmaceuticals that specifically bind to these receptors in tumours that do not have endogenous receptor overexpression. This multimodality treatment strategy, termed radiovirotherapy, has been studied using a variety OVs (eg. vaccinia virus, measles virus, herpes...
virus and adenovirus) and receptor/ligand pairs (241) including SSTR with radiolabeled SS-analogues (574, 620) and more frequently, human sodium iodide symporter (NIS) with radioactive iodide (e.g. $^{131}$I) (546, 548, 549, 566, 567, 571). This approach, which allows radioligand targeting of tumours with low endogenous receptor expression through virus-mediated receptor gene delivery, has diagnostic and therapeutic applications with the potential for synergistic enhancement in treatment efficacy through taking advantage of the oncolytic effects of viruses and the DNA-damaging effects of radiation (466).

Early studies designed to improve and expand the applicability of PRRT utilized a gene therapy approach involving non-replicating viruses as the receptor-delivery vector (621). In these studies, radiation was the sole therapeutic agent and tumour cell death was dependent on delivery of the radiation dose to both the transduced cell (to which the radioligand bound) as well as the uninfected neighboring cells. Transduction efficiency was a major limitation of these studies, as non-replicating viruses demonstrated very minimal spread (536) leading to similarly limited and heterogeneous radioligand accumulation throughout the tumour volume (622). Radiovirotherapy relies on the tumour killing capacity of both the virus and radioligand. OVs are highly cytolytic replicating viruses; therefore it is generally assumed that infected tumour cells will die as a result of virus. The benefit of the radioligand exists in the ability to deliver a radiation dose to uninfected neighbouring cells through the radiation cross-fire effect.

Vaccinia virus (VV) is a potent oncolytic vector capable of high levels of tumour-specific replication and transgene expression (181). Oncolytic VV has demonstrated excellent anti-tumour efficacy in numerous preclinical models (623) and is currently being investigated in clinical trials with promising results (217, 257, 314, 342, 624). We have previously demonstrated that an attenuated oncolytic VV with deletions in two viral genes (vaccinia growth factor and viral thymidine kinase) and encoding the human somatostatin receptor subtype 2A (vvDD-SSTR) results in specific accumulation of an $^{111}$In-labeled SS analogue in a subcutaneous colorectal cancer (CRC) tumour model (574). While this study demonstrated the in vivo RP-accumulating activity of vvDD-SSTR, it did not evaluate the therapeutic effect of combination therapy. Improved cell killing over virus alone was later demonstrated in an in vitro CRC tumour spheroid model using $^{111}$In- and $^{177}$Lu-DOTA-octreotide (DOTATOC).
To date, most radiovirotherapy studies have utilized subcutaneous tumour models (549, 550, 557-559, 571) with only a minority investigating efficacy in disseminated tumour models, which more closely resembles the type of disease (ie. unresectable) most likely to benefit from PRRT (555, 561, 565, 568). The objective of this study is to evaluate the therapeutic efficacy of SSTR-mediated radiovirotherapy using vvDD-SSTR in a disseminated CRC tumour model. vvDD has previously been reported to improve survival in disseminated ovarian (196), mesothelioma (199) and CRC carcinomatosis models (625) and we have recently demonstrated that vvDD-SSTR increases the median survival in both syngeneic and xenograft models of CRC carcinomatosis (600) (Chapter 2). We hypothesize that vvDD-SSTR will enable the imaging and improved treatment of intraperitoneal CRC tumours using $^{177}$Lu-DOTATOC due to virus-directed tumour-specific accumulation of the radiopeptides.

4.3 Materials and Methods

**Cell lines, virus and reagents.** Human colorectal adenocarcinoma (DLD1) and monkey kidney fibroblasts (CV-1) cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). 24-JK murine sarcoma cell lines were obtained from the National Institutes of Health (Bethesda, Maryland, USA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON, Canada) and 1% antibiotic-antimycotic (Invitrogen, GIBCO, Grand Island, NY, USA) at 37°C with 5% CO$_2$. The previously described thymidine kinase ($tk$) and vaccinia growth factor ($vgf$)–deleted VV (vvDD) expressing the human somatostatin receptor subtype 2A (SSTR) (574) and control vvDD expressing enhanced green fluorescent protein (EGFP) or red fluorescent protein (RFP) (123) were propagated in 24-JK cells and purified by ultracentrifugation over a sucrose cushion. Virus stocks were stored at -80 °C and titered on CV-1 cells (580). D-lysine (Sigma Aldrich) was dissolved in PBS to a concentration of 200 mg/ml and filter sterilized using a 0.22 µm pore syringe filter immediately prior to administration.

**Peptide Labeling.** All chemicals were obtained from commercial sources and used without further purification. 1,4,7,10-tetraazacyclododecane-N,N’,N”,N”’-tetraacetic acid (DOTA)-conjugated D-Phe$^1$-[Tyr$^3$]-Octreotide (DOTATOC; American Peptide Company, Sunnyvale, CA, USA) was radiolabeled with $^{177}$LuCl$_3$ (Perkin Elmer, Waltham, MA, USA) as previously
reported (626). The radioconjugate was obtained in >99% radiochemical purity at a specific activity of 42 – 44 MBq/nmol DOTA-peptide (29.5 – 31.0 MBq/µg).

**In vitro virus infections and plaque assays.** Cells were infected at a low volume in 2.5% DMEM for 2 h with shaking every 10 minutes after which DMEM with 10% FBS was added to the wells. Plaque assays were performed on harvested cells and tissues to quantify live virus. Samples were homogenized using a TissueLyzer II (Qiagen, Hilden, Germany), exposed to three freeze-thaw cycles and sonication prior to titering on CV-1 cells. Infected CV-1 cells were incubated for 48 h prior to staining with crystal violet and quantitation of the plaque forming units (PFU)/ml. For quantification of virus in tissues, titers were normalized to the total protein per sample as determined by a Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

**In vitro cell viability.** Cells were seeded at 1 x 10^4 per well in a 96-well plate and incubated overnight. Cells were infected with vvDD-SSTR or vvDD-RFP as above and incubated for 24 h. Supernatant was removed and cells were incubated with ^177^Lu-DOTATOC (37 kBq – 740 kBq; 1.2 ng – 25 ng) diluted in 10% DMEM for 4 h. Unbound ^177^Lu-DOTATOC was removed, wells were washed twice with PBS and media was added. Seventy-two hours later cell viability was assessed by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter96® Aqueous One Solution, Promega, Madison, WI, USA) according to the manufacturers protocol.

**Virus Replication in the presence of ^177^Lu-DOTATOC.** Cells were seeded at 5 x 10^5 per well (6-well plate) and incubated overnight. Cells were infected at an MOI of 0.1 for 24 h prior to a 4 h incubation with increasing activities of ^177^Lu-DOTATOC (1 – 20 µCi; 1.2 ng – 25 ng) diluted in 10% DMEM. Wells were washed twice with PBS to remove any unbound ^177^Lu-DOTATOC, supplemented with 10% DMEM and incubated for the indicated times. The entire content of wells (cells and supernatant) were collected by cell scraping every 24 h beginning at baseline (after RP incubation). Samples were titered on CV-1 cells immediately following their harvest.

**Combination Index.** Dose response curves generated from the cell viability data were analysed using the Chou and Talalay method for quantifying drug-drug interactions (467). Experiments were performed using non-fixed dose ratios. Data is presented in a normalized isobologram.
where \( (D)_{1,2} \) is the dose required to achieve a given fraction when drug 1 or 2 are administered separately and \( (Dx)_{1,2} \) is the dose required to achieve the same fraction affected when drugs 1 and 2 are administered in combination. On the \( (D)_{1}/(Dx)_{1} \) vs \( (D)_{2}/(Dx)_{2} \) plot (normalized isobologram) synergy is defined as data points falling below the line connecting the points \((0, 1)\) and \((1, 0)\). Data points falling on this line represent additive interactions while those above the line indicate synergism.

**Mice.** Six- to eight-week old female BALB/c nude mice (C.Cg/AnNTac-Foxn1nu NE9, Taconic Farms, NY, USA) were housed under standard conditions and given food and water *ad lib.* Experiment protocols were approved by the Animal Care Committee, UHN, Toronto, Canada. Survival endpoint criteria as defined by the Animal Care Committee included severe abdominal distension resulting in decreased mobility and/or cachexia. Mice were injected intraperitoneally (IP) with \(5 \times 10^6\) DLD1 on day 0 followed by virus \((10^9\) pfu IP) suspended in HBSS + 0.1% bovine serum albumin (BSA) or vehicle alone on day 12. To evaluate the expression SSTR in IP tumours groups of vvDD-SSTR-treated mice \((n = 3)\) were sacrificed at 3, 5, 7, and 9 days post infection (dpi) and tumours (up to 5 per mouse) were harvested, bisected and processed as described for immunohistochemistry (IHC) and titering. For the biodistribution study, tumour-bearing mice \((n = 3\) per time point) were treated with vvDD-SSTR, vvDD-EGFP or vehicle alone (day 12) followed by \(3.5\) MBq \(^{177}\)Lu-DOTATOC intravenously (IV) on day 18. Imaging was performed on mice \((n = 3)\) treated with vvDD-SSTR or vvDD-EGFP in combination with 37 MBq \(^{177}\)Lu-DOTATOC. For the treatment study mice were divided into 7 groups: vehicle alone, \(^{177}\)Lu-DOTATOC, vvDD-EGFP, vvDD-EGFP + \(^{177}\)Lu- DOTATOC, vvDD-SSTR + \(^{177}\)Lu-DOTATOC (low dose) and vvDD-SSTR + \(^{177}\)Lu-DOTATOC (high dose). Radiovirotherapy was evaluated at two \(^{177}\)Lu-DOTATOC doses \((7.5\) and 15 MBq) in the vvDD-SSTR treated mice and at the higher dose \((15\) MBq) in all control groups. For both the imaging and treatment studies D-lysine was used to minimize kidney uptake. Mice received 4 IP injections of D-lysine \((2000\) mg/kg per injection) once every hour beginning 30 minutes prior to RP injection.

**Immunohistochemistry and histopathology.** Staining was performed on tissues fixed in 10% buffered formalin for 72 h followed by 70% ethanol. Samples were paraffin embedded, sectioned and stained using rabbit monoclonal anti-SSTR subtype 2 [UMB1] \((ab134152, 1/800, \text{Abcam, Cambridge, MA, USA})\) or polyclonal rabbit anti-VV \((ab35219, \text{dilution } 1/1000; \text{Abcam,})\)
Cambridge, MA, USA) primary antibodies and HRP labeled secondary antibodies. Stained slides were scanned using ScanScope XT (Aperio Technologies, Vista, CA, USA) and staining was quantified using ImageScope’s Positive Pixel algorithm (Aperio Technologies) where % positivity represents the percentage of positively staining pixels relative to the total number of pixels in a defined area. Defined areas were manually drawn around each individual tumour. Kidneys from the treatment study were stained using a standard H & E protocol and evaluated for toxicity by a blinded veterinary pathologist.

**Biodistribution studies.** Mice were sacrificed at selected time points post RP injection and blood (collected via cardiac puncture) and tissues (tumour, heart, lung, liver, kidney, spleen, pancreas, adrenal gland, bowel, ovary, bone marrow and brain) were harvested. Up to five tumours per mouse were removed (depending on the number of tumours present). Individual tumours and normal tissues were bisected with one half stored in HBSS at -80°C for virus quantification by plaque assay (all tissues except blood) and the other half placed in pre-weighed scintillation vials for quantification of $^{177}$Lu activity.

**Gamma counting.** Tissues were weighed, and activity was measured in a gamma-counter (PerkinElmer Wizard 1480 Wizard 3”, Waltham, MA, USA) along with a standard of the injected dose, such that decay-corrected uptakes were calculated as the percentage of the injected dose per gram of tissue (%ID/g). The total injected dose per mouse was equal to the difference between the pre- and post-injection syringe radioactivity determined by a CRC-15R dose-calibrator (Caointec, Ramsay, NJ, USA).

**MicroSPECT/CT Imaging.** Mice were imaged 3-6 h post $^{177}$Lu-DOTATOC injection. Mice were anesthesized by inhalation of 2% isoflurane in O$_2$. Imaging was performed on a NanoSPECT/CT tomograph (BioScan, Washington, DC) equipped with 4NaI(Tl) detectors and fitted with 1.4 mm multipinhole collimators (resolution $<$ 1.2 mm at full width at half maximum). Photons were accepted from the 10% windows centered on lutetium’s three photopeaks at 208, 113 and 245 keV. Projections were acquired in a 256 x 256 acquisition matrix for a total of 60 minutes. Images were reconstructed using an ordered-subset expectation maximization (OSEM) algorithm (nine iterations). Cone beam CT images were acquired (180 projections, 1s/projection, 45 kVp) before microSPECT images. MicroSPECT and CT images
were co-registered using InVivoScope software (Bioscan/inviCRO, Boston, MA, USA). Mice were sacrificed immediately following imaging and necropsies were performed to confirm the location of IP tumours. Normal and tumour tissues were then harvested for gamma-counting as in biodistribution studies.

**Treatment and Toxicity Studies.** Tumour-bearing mice were treated with virus ($10^9$ PFU) and/or $^{177}$Lu-DOTATOC (7.5MBq or 15MBq). Cages were changed 24 h after RP administration. Mice were weighed every 2-3 d and followed for signs of toxicity or disease progression. Saphenous vein blood was collected from three mice per group at 8 d post-RP for analysis. Complete blood counts were performed using a HEMAVET® Multispecies Hematology Analyzer (Drew Scientific, Dallas, TX, USA). When mice reached the defined endpoint they were sacrificed and kidneys were collected and fixed in formalin for histopathology.

### 4.4 Results

#### 4.4.1 vvDD-SSTR and $^{177}$Lu-DOTATOC synergistically increase cytotoxicity towards CRC cells

DLD1 human CRC cells were treated with vvDD-SSTR and/or $^{177}$Lu-DOTATOC (37 – 740 kBq/well; 1.2 ng per 37 kBq) and assayed for virus replication and cell viability (**Figure 4.1**). $^{177}$Lu-DOTATOC had no effect on virus replication at all radioactivities tested (**Figure 4.1A**). The effect of combination therapy on cell viability was evaluated in cells infected with vvDD-SSTR (MOI 0.01-5) (**Figure 4.1B & C**) or a receptor-negative control virus, vvDD-RFP (MOI 0.1-1) (**Figure 4.1D**). When given alone both vvDD-SSTR (**Figure 4.1B**) and $^{177}$Lu-DOTATOC (**Figure 4.1C**) had a dose response effect. $^{177}$Lu-DOTATOC did not decrease cell viability at 37 K bq but showed significant cytotoxicity at 370 and 740 kBq in the absence of virus infection and therefore receptor expression. This indicates that at these high doses, $^{177}$Lu-DOTATOC induced loss of cell viability is mainly receptor-independent and a result of the non-specific irradiation of cells during the 4 h incubation period. At the 37 kBq dose of $^{177}$Lu-DOTATOC, cell killing was significantly improved over either monotherapy when cells were infected at an MOI ≥ 1 (**Figure 4.1C**). This enhanced cell killing was receptor-dependent, as the same dose of $^{177}$Lu-DOTATOC did not affect cell viability in cells infected with a receptor-negative control virus (**Figure 4.1D**). When treated at lower MOIs (0.01 and 0.1) of vvDD-SSTR, addition of high dose (370 and 740
Figure 4.1. $^{177}$Lu-DOTATOC synergistically improves vvDD-SSTR-induced cytotoxicity. DLD1 cells were infected with vvDD-SSTR for 24 h and incubated in the presence or absence of increasing activities of $^{177}$Lu-DOTATOC (37 – 740 kBq; 1.2 ng per 37 kBq) for 4 h. (A) vvDD-SSTR (MOI 0.1) replication was determined up to 96 h post $^{177}$Lu-DOTATOC treatment. (B-D) Cell viability was determined by MTS assay in cells infected with vvDD-SSTR (B & C; MOI 0.01 - 5) or vvDD-RFP (D; MOI 0.1-1) at 72 h post $^{177}$Lu-DOTATOC treatment. Data in B and C are from the same experiment. (E) Dose response data (from B & C) was analyzed by the Chou-Talalay method for determining drug-drug interactions and plotted on a normalized isobologram. Data points falling below the solid line represent synergistic interactions. All bars represents the mean ± SEM. *p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison test.
kBq) $^{177}$Lu-DOTATOC improved cell killing over virus alone but not $^{177}$Lu-DOTATOC alone whereas addition of low dose (37 kBq) $^{177}$Lu-DOTATOC had no effect on cell viability relative to either monotherapy (Figure 4.1B & C). Taken together, these data suggest there is likely a threshold of receptor expression required to see a therapeutic benefit in vitro following combination therapy at doses of $^{177}$Lu-DOTATOC.

Dose response data (Figure 4.1B & C) were analyzed using the Chou-Talalay method for quantifying drug-drug interactions (467). Synergy was observed at MOIs 1 and 5 when combined with 37 MBq as well as at MOIs 0.01-1 when combine with 370 or 740 MBq of $^{177}$Lu-DOTATOC (Figure 4.1C). The synergy observed at the higher doses of $^{177}$Lu-DOTATOC was likely receptor expression-independent as there was minimal effect of virus at those highly cytotoxic doses (Figure 4.1B).

**4.4.2 vvDD-SSTR results in expression of SSTR in intraperitoneal colorectal cancer tumours with low endogenous receptor expression**

*In vivo* virus-directed expression of SSTR in peritoneally disseminated CRC tumours was confirmed by IHC. DLD1 tumour-bearing mice were treated with $10^9$ PFU of vvDD-SSTR and sacrificed at 3, 5, 7, and 9 dpi. Multiple tumours per mouse were harvested and divided into two pieces for either IHC or titering. Staining for VV and SSTR (subtype 2) on serial sections showed expression of SSTR in vvDD-SSTR-infected tumours but not HBSS-treated control tumours (Figure 4.2A). Quantification of staining expressed as the percent positive pixels relative to total pixels showed that both VV and SSTR2 staining peaked on day 5 (Figure 4.2B & C), which was consistent with the tumour titers (Figure 4.2E). Staining for both VV and SSTR as well as virus titers declined by 9 dpi (data not shown). SSTR expression localized specifically to areas of VV staining within the tumours (Figure 4.2A) and there was a strong positive correlation between VV and SSTR staining among all the tumours harvested (Figure 4.2D).
Figure 4.2 SSTR expression in vvDD-SSTR treated tumour-bearing mice. Tumours (3-4 per mouse) from vvDD-SSTR treated mice (10⁹ PFU; n = 3 per time point) were harvested 3, 5, 7, and 9 dpi and bisected for IHC and virus titering. Formalin-fixed tumour sections were stained with anti-VV and anti-SSTR2 antibodies while corresponding specimens suspended in HBSS were processed for titering. (A) Representative images of the tumours from vvDD-SSTR and HBSS treated mice. (B-C) Quantification of IHC staining expressed as the percent positive pixels relative to total pixels per tumour. (D) Linear correlation between VV and SSTR staining in individual tumours over all time points. (E) Tumour titers as determined by plaque assay. Data points represent the results for individual tumours harvested from each mouse (M1-3) with the overall mean represented by the solid line. Scale bar = 100 µm (10x) or 500 µm (1-4x).
4.4.3 Virus mediated SSTR expression results in specific uptake of $^{177}$Lu-DOTATOC in CRC tumours

Tumour bearing mice were treated with $10^9$ PFU of vvDD-SSTR or vvDD-EGFP, a receptor negative control virus, followed by 3.5 MBq (29.5 – 31 MBq/µg) $^{177}$Lu-DOTATOC (IV) 6 dpi. The effect of virus-mediated SSTR expression on $^{177}$Lu-DOTATOC uptake in tumours and normal tissues was evaluated at 6, 24 and 72 h post-RP delivery (Figure 4.3). Tumour uptake (Figure 4.3A, left) was increased at all time points in vvDD-SSTR treated mice compared to control virus or $^{177}$Lu-DOTATOC alone and showed statistical significance compared to both control groups at 6 h (1.0 ± 0.13 %ID/g vs 0.1 ± 0.05 %ID/g, p < 0.01 and 0.3 ± 0.1 %ID/g, p < 0.05, respectively, one-way ANOVA) and $^{177}$Lu-DOTATOC at 72 h (0.2 ± 0.04 %ID/g vs 0.0 ± 0.0 %ID/g, p < 0.05, one-way ANOVA). Over the time points evaluated vvDD-SSTR resulted in an approximately 2.2 – 8.4 fold-increase in the mean tumour uptake of radioactivity relative to vvDD-EGFP and a 3.8 – 5.5 fold increase relative to $^{177}$Lu-DOTATOC alone. The tumour-to-blood ratio for vvDD-SSTR treated mice was 37.9 ± 0.6, 143.0 ± 85.4 and 101.6 ± 6.5 at 6 h, 24 h and 72 h, respectively (Figure 4.3A, right). This was significantly increased relative to vvDD-EFGP and $^{177}$Lu-DOTATOC alone treated mice at 6 h (7.6 ± 4.0 and 5.6 ± 3.6, p < 0.05, one-way ANOVA) and 72 h (45.7 ± 11.2 and 22.0 ± 15.5, p < 0.05, one-way ANOVA). Normal tissue uptake was generally unaffected by vvDD-directed expression of SSTR (Figure 4.3B-D) with a few exceptions. Uptake in the kidneys and ovaries was significantly increased at various time points in mice treated with vvDD-SSTR relative to controls and was likely due to virus infection in these organs (Figure 4.4). The ovaries are known to support relatively high levels of vvDD replication in mice. Non-human primate studies have demonstrated no specific ovary tropism with vvDD (194) therefore it is unlikely that this off target effect represents a clinically relevant toxicity concern. While our data showed low levels of virus in the kidneys of vvDD-treated mice (Figure 4.4B-D), previous preclinical investigations in immunocompetent murine, rabbit and non-human primate models have not identified the kidneys as a site of vvDD replication or toxicity (194, 600, 627). Therefore, it seems unlikely that the slight increase in kidney radioactivity observed in the vvDD-SSTR + $^{177}$Lu-DOTATOC treated-mice would be a toxicity concern in immunocompetent models. Consistent with vVDDS highly tumour-specific replication (Figure 4.4), uptake of radioactivity in all other organs was not significantly affected by virus-directed SSTR expression (Figure 4.3B-D).
Figure 4.3 Effect of vvDD-directed SSTR expression on $^{177}$Lu-DOTATOC biodistribution. Tumour-bearing mice were treated with vvDD-SSTR or vvDD-EGFP (10$^9$ PFU) in combination with $^{177}$Lu-DOTATOC (3.5 MBq, 29.5 – 31 MBq/µg) 6 dpi. Tumours (A) and normal tissues (B-D) were harvest at several time points post RP delivery and analyzed by gamma counting. (A) Tumour uptake and tumour-to-blood ratio. *p < 0.05, one-way ANOVA. (B-D) Normal tissue uptake at 6 h (B), 24 h (C) and 72 h (D). *p < 0.05, two-way ANOVA. All data are presented as the mean %ID/g ± SEM (n = 3 per time point).
4.4.4 Specific uptake of $^{177}$Lu-DOTATOC in vvDD-SSTR infected CRC tumours does not effect virus biodistribution

Tumours and tissues harvested from mice in 4.3 were also used to evaluate virus biodistribution. The effect of $^{177}$Lu-DOTATOC on virus in the tumours (Figure 4.4A) as well as normal tissues (Figure 4.4B-D) was evaluated at 24 h (7 dpi), 72 h (9 dpi) and 7 d (13 dpi) post-RP delivery. Consistent with our in vitro data, $^{177}$Lu-DOTATOC did not decrease virus titers in the tumours or most normal tissues. Interestingly, addition of the RP resulted in a significant increase in tumour titers at 72 h, which was lost by 7 d. Overall, tumour titers were high relative to normal organs (mean titer ± SEM at 24h: 9.6 x $10^6$ ± 4.4 x $10^6$ PFU/mg; 72h: 2.1 x $10^7$ ± 6.4 x $10^6$ PFU/mg; 7 d: 7.8 x $10^6$ ± 1.6 x $10^6$ PFU/mg). The ovaries, which are known to support VV replication, showed some of the highest normal tissue titers (mean titer ± SEM at 24 h: 1.4 x $10^6$ ± 1.1 x $10^4$ PFU/mg; 72 h: 3.1 x $10^6$ ± 1.9 x $10^6$ PFU/mg; 7 d: 4.8 x $10^5$ ± 4.4 PFU/mg) however were still up to 2-logs lower than tumour titers. Interestingly, a statistically significant decrease in mean virus titer was observed in the ovaries of mice treated with $^{177}$Lu-DOTATOC relative to virus alone at 24 h (Figure 4.4B). This difference was not observed at the later time points and therefore may not reflect a true inhibition of virus replication in ovaries. Alternatively, it may represent a transient decrease in virus replication related to the increased uptake of $^{177}$Lu-DOTATOC in the ovaries early after radiopeptide injection (Figure 4.3B). Given the established connection between PRRT and renal toxicity, it is important to evaluate off target virus replication in the kidneys. Virus was present in the kidneys of all vvDD-SSTR + $^{177}$Lu-DOTATOC treated mice at all time points (mean titer ± SEM at 24 h: 9.1 x $10^4$ ± 9.1 x $10^4$ PFU/mg; 72 h: 5.0 x $10^3$ ± 4.0 x $10^3$ PFU/mg; 7 d: 6.9 x $10^3$ ± 6.3 x $10^3$ PFU/mg). This low level of virus may account for the slightly increased radiopeptide uptake observed in the mice treated with vvDD-SSTR + $^{177}$Lu-DOTATOC (Figure 4.3).

4.4.5 Virus-directed expression of SSTR allows for molecular imaging of intraperitoneal CRC tumours with low endogenous receptor expression

One of the many advantages of using γ-ray emitting radionuclides is that they can be imaged using standard nuclear imaging modalities such whole body planar imaging using a
Figure 4.4 Effect of $^{177}\text{Lu}$-DOTATOC on vvDD-SSTR2 biodistribution. DLD1 tumour bearing mice (n = 3 per time point) were treated with vvDD-SSTR ($10^9$ PFU IP) following by $^{177}\text{Lu}$-DOTATOC (3.5MBq IV) 6 d later. Tumours (A) and normal tissues (B-D) were harvested and titered by plaque assay. (A) Titors in tumours at 24 h, 72 h and 7 d post-radiopptide. *p < 0.05, t-test (n = 3 per time point). (B-D) Normal tissue titers at 24 h (B), 72 h (C) and 7 d (D) post-radiopptide administration. *p < 0.05, two-way ANOVA with Bonferroni’s post-test. All bars represent the mean ± SEM.

gamma-camera or 3-dimensional imaging by SPECT/CT. Radionuclides like $^{177}\text{Lu}$ that emit both $\gamma$-rays and particulate radiation ($\beta^-$-particles) are therefore valuable for both diagnostic and post-therapy imaging as well as in the actual treatment course. We evaluated the ability of vvDD-directed SSTR expression to lead to sufficient tumour-specific uptake of $^{177}\text{Lu}$-DOTATOC such that IP tumours were imageable by SPECT/CT (Figure 4.5A). Six hours post $^{177}\text{Lu}$-DOTATOC
Figure 4.5
Figure 4.5 MicroSPECT/CT imaging of intraperitoneal CRC tumours following vvDD-SSTR directed tumour-specific receptor expression. Tumour bearing mice were treated with vvDD-SSTR or vvDD-EGFP (10^9 PFU) followed by 37 MBq (29.5 – 31 MBq/µg) ^177^Lu-DOTATOC 6 d later. Mice were co-administered D-lysine (2000 mg/kg) to decrease kidney uptake. (A) Mice were imaged at 6 h post RP delivery and sacrificed immediately thereafter. White light images were taken at necropsy to confirm tumour locations. The locations of the kidneys (K), tumours (T) and bladder (Bl) are indicated in both the white light and SPECT/CT images. (B) Uptake in tumours and normal tissues immediately after imaging was quantified by gamma-counting. Data represent the mean %ID/g ± SEM (n = 3). * p < 0.05 two-way ANOVA with Bonferroni’s post-test.
Figure 4.6 Radiovirotherapy toxicity and efficacy in an orthotopic model of metastatic colorectal cancer. Tumour-bearing mice were treated with virus (10^9 PFU IP) on day 12 followed by ^177^Lu-DOTATOC (7.5 or 15 MBq IV) 6 d later. (A) Total body weight over time was monitored for signs of toxicity. (B) Complete blood counts were performed 8 d post-RP. *p < 0.05 relative to HBSS, one-way ANOVA (n = 3). Horizontal lines represent the lower and upper threshold of the normal range for each measurement. (C) Kaplan-Meier survival curves were compared to evaluate the efficacy of treatment. Median survivals were 38 d (HBSS, n = 3), 47 d (vvDD-EGFP, n = 6), 40 d (vvDD-SSTR, n = 6), 40 d (177^Lu-DOTATOC 15 MBq, n = 3), 55 d (vvDD-EGFP + 177^Lu-DOTATOC 15 MBq, n = 6), 51 d (vvDD-SSTR + 177^Lu-DOTATOC 7.5 MBq) and 70 d (vvDD-SSTR + 177^Lu-DOTATOC 15 MBq). *p < 0.05 compared to HBSS group, *p < 0.05 compared to vvDD-SSTR + 177^Lu-DOTATOC (15 MBq), log-rank test.
administration tumours from vvDD-SSTR treated mice were clearly visible by SPECT/CT imaging whereas no signal was observed in the tumours of vvDD-EGFP treated mice. Necropsies were performed immediately following imaging and white light images were taken to confirm the location of the tumours. Increased uptake in the tumours of mice treated with vvDD-SSTR was confirmed by gamma-counting (Figure 4.5B). As expected, kidney uptake was high in both groups, although gamma counting showed it to be statistically decreased in mice treated with vvDD-SSTR (Figure 4.5B). This is the opposite effect to that observed in the biodistribution studies (Figure 4.3) where $^{177}$Lu-DOTATOC was delivered at approximately 1/10 of the imaging activity but with the same specific activity (29.5 – 31 MBq/µg). It is possible that kidney uptake was saturated at the higher dose therefore the small effect attributed to virus directed SSTR expression observed in the biodistribution studies may be negligible in the context of this high dose. Additionally the tumour-specific expression of SSTR appears to be acting as a tumour sink leading to decreased kidney uptake in vvDD-SSTR treated mice.

4.4.6 Radiovirotherapy improves survival without toxicity in an orthotopic model of CRC peritoneal carcinomatosis

Tumour bearing mice were treated with vvDD-SSTR or a receptor-negative control virus (vvDD-EGFP) and/or $^{177}$Lu-DOTATOC 6 d later. Radiovirotherapy with vvDD-SSTR was performed at two doses (7.5 MBq and 15 MBq) of $^{177}$Lu-DOTATOC while all control groups received the higher dose (15 MBq). All groups also received the kidney protector D-lysine (2000 mg/kg). Radiovirotherapy was not associated with any generalized toxicity as determined by total body weight recorded every 1-3 d following $^{177}$Lu-DOTATOC up to day 38, at which point all HBSS-treated mice had reached endpoint (Figure 4.6A). Given that bone marrow toxicity is one of the major concerns associated with cancer therapy, complete blood counts were performed at 8 d post-RP delivery to evaluate the effect of radiovirotherapy on different blood cell compartments (Figure 4.6B). Radiovirotherapy did not have any myelosuppressive effects at this time point, which corresponds approximately to the maximum drop in WBC reported in rats treated with a $^{177}$Lu-DOTATATE (628). Given that radiovirotherapy was well tolerated in this model, we then evaluated the effect of treatment on overall survival (Figure 4.6). The median survival in all treatment groups was increased relative to the HBSS-treated controls (38 d median survival) and was statistically significant in all cases with the exception of vvDD-EGFP + $^{177}$Lu-DOTATOC (15MBq) (55 d median survival) which showed a trend towards improvement.
Addition of $^{177}$Lu-DOTATOC to vvDD-SSTR significantly improved the median survival relative to virus alone when administered at 15 MBq (median survival 70 d vs 40 d, $p = 0.0498$) but not at 7.5 MBq (median survival 51 d vs 40 d, $p = 0.365$). This shows that there is a dose effect wherein the high dose (15 MBq) $^{177}$Lu-DOTATOC treatment was significantly more effective than the low dose (7.5 MBq) when given in combination with the SSTR-expressing virus (median survival 70 d vs 51 d, $p = 0.0467$). There was no significant improvement in survival between mice treated with vvDD-EGFP alone or in combination with 15 MBq $^{177}$Lu-DOTATOC (median survival 47.5 d vs 55 d, $p = 0.256$) demonstrating that radiovirotherapy was receptor expression-specific.

4.5 Discussion

PRRT using $^{177}$Lu-labeled SS analogues is an effective treatment option for patients with SSTR-positive NETs. In patients with inoperable disease, PRRT has resulted in a 3-6 y increased survival benefit from the time of diagnosis compared to historically reported data (465). Currently, radiolabeled somatostatin analogues represent the only class of RPs approved for use in North America. Metastatic NETs, particularly carcinoid malignancies, often occur in some of the same anatomical locations common to metastasized CRC, including the peritoneum, liver and lungs (629). In both patient populations complete surgical resection is one of the best prognostic indicators (17, 630), unfortunately for a significant population of patients with metastatic disease complete resection is not an option. One of the major factors precluding patients from surgery is the proximity of a tumour to critical organs and structures; similarly, this makes external beam radiation therapy and brachytherapy mostly untenable in these patients due to significant toxicity concerns. In the case of SSTR-expressing NETs, inoperable tumours can be effectively controlled by targeted radiotherapy using systemically administered radiolabeled SS analogues. Given the success of PRRT in controlling advanced and metastatic disease we and others have proposed the use of receptor-encoding OVs to direct tumour-specific receptor expression thereby making cancers with low endogenous receptor expression amenable to PRRT (522, 545, 547-552, 554, 558, 559, 563, 566-568, 571, 573, 574, 631).

In this study we hypothesized that vvDD expressing the human SSTR (subtype 2A) would lead to specific accumulation of the radiolabeled SS-analogue $^{177}$Lu-DOTATOC in receptor-negative CRC tumours leading to improved survival in an orthotopic xenograft model.
Our results confirmed that virus-directed receptor expression led to specific uptake of the radiopeptide in tumours with minimal effect on the normal tissue biodistribution as determined by both ex vivo quantification of tissue radioactivity and non-invasive SPECT/CT imaging. Furthermore, we report a ≥ 20 d improvement in the median survival of mice treated with vvDD-SSTR and $^{177}$Lu-DOTATOC (15 MBq) compared to all other groups. The only other study investigating OV delivery of SSTR combined with a radiolabeled SS analogue ($^{90}$Y-DOTATOC) with the intent to treat was performed in nude mice bearing subcutaneous non-small cell lung tumours (620). In that study, mice were treated intratumourally with a SSTR2-expressing adenovirus followed by RP 2 dpi at radioactivities (400-500 µCi; 14.8-18.5 MBq) comparable to that used in our treatment studies. The treatment course differed in that their mice received two injections of the RP 2 d apart followed by a second treatment cycle repeated 7 d later. The tumour uptake observed in our biodistribution study following vvDD-SSTR treatment (1.03 ± 0.12% ID/g 24 h post-RP) was similar to that reported by Rogers et al. following $^{111}$In-DTPA-D-Phe$^1$-octreotide administration in AdSSTR2 treated mice (1.3 ± 0.7% ID/g 48 h post-RP) (620). They also reported a significant delay in the time to tumour quadrupling in mice treated with radiovirotherapy compared to $^{90}$Y-DOTATOC alone and untreated controls however the effect of virus alone was not evaluated. Studies that look at $^{177}$Lu-DOTATE uptake in mouse models of subcutaneous SSTR-positive small cell lung or carcinoid tumours have reported moderately (3.7 ± 0.99 %ID/g) (448) or substantially (17 ± 3% ID/g) (632) increased uptake, respectively, compared to that reported here. This likely has to do with differences in the density of receptor expression on the surface of the various cell lines relative to that achieved by virus infection.

Despite the clear benefit of combination vvDD-SSTR and $^{177}$Lu-DOTATOC radiovirotherapy, all mice were eventually sacrificed as a result of tumour growth. This indicates that there is still room for improvement in the design and implementation of this radiovirotherapy strategy. There are several modifications to the treatment regimen that warrant further investigation, the first being to increase the activity of $^{177}$Lu-DOTATOC administered. Previous studies in xenograft models of SSTR-positive NETs have demonstrated efficacy and safety with doses up to 30 MBq (632). Furthermore, nephrotoxicity profiling in nude mice receiving $^{177}$Lu-DOTATATE suggests that we may be able to administer doses much higher than 30 MBq (633) particularly considering that $^{177}$Lu-DOTATOC appears to have slightly lower renal uptake relative to $^{177}$Lu-DOTATATE (438).
While it is the case that replicating OVs are capable of much greater spread than their non-replicating counterparts and therefore result in significantly increased tumour uptake of radioligands (536), incomplete tumour infection remains a challenge to effective OV monotherapy. Low density and heterogeneity of receptor expression have been identified as a critical limitation to effective PRRT of SSTR-positive NETs, due to non-homogenous delivery and insufficient uptake of the RP throughout the tumour volume (634). The IHC analysis presented here revealed heterogeneous SSTR expression corresponding to areas of VV-staining throughout the tumours of vvDD-SSTR treated mice (Figure 4.2). Furthermore, there was substantial variation in the extent of infection and therefore receptor expression between individual tumours harvested from the same mouse, as has previously been reported with vvDD in similar mouse models (600). The heterogeneous distribution of virus-mediated receptor expression in these studies may have limited the maximum effect achievable with 15 MBq of $^{177}$Lu-DOTATOC had receptor expression been more homogenous. Accordingly, strategies to specifically increase the uniformity of receptor expression and overall uptake in the tumours (but not normal tissues) would likely improve the therapeutic efficacy of radiovirotherapy. A previous study using a NIS-expressing adenovirus combined with radioiodide demonstrated that this could be achieved through increasing the virus dose administrated (551), which presumably increased the level of receptor expression in the tumour. However, in the context of VV OV therapy, doses above $10^9$ PFU (the dose used in our studies) may not be tolerable in immunosuppressed mice, and are near the top threshold of achievable titers under clinical manufacturing conditions. Therefore, increasing virus-mediated receptor expression through other means such as improving virus delivery and/or spread in tumours could have a meaningful impact on the density and distribution of receptor expression within the tumour. For example, cell-carriers have been used to mask systemically delivered (IP or IV) virus from immune neutralization and thereby improve virus delivery (635), while modified OVs encoding proteins involved in extracellular matrix (ECM) degradation or enhancing cell-cell fusion demonstrate more efficient spread through tumours (636). It is likely that cell carrier would improve delivery of vvDD-SSTR in our model as they have been shown to significantly increase vvDD delivery to IP CRC tumours, an effect that was further enhanced by addition of immunosuppressive drugs (579). Strategies to improve spread have also proved effective with VV, wherein a recombinant Lister strain virus encoding the ECM degrading enzyme, metalloproteinase (MMP)-9, resulted in
increased virus spread and improved tumour growth inhibition in a subcutaneous tumour model (244).

Translation of preclinical radiovirotherapy studies to the clinic demands careful consideration of issues related to potential toxicity. The primary organs of concern in radiotherapy in general and PRRT specifically are the kidneys, bone marrow (373, 447) and ovaries (637). Liver toxicity has been observed in some PRRT clinical trials although this is generally attributed to the presence of liver lesions (465). In addition to the expected off-target sites of radiation toxicity, it is important to also consider the off-target sites of virus replication, as virus-directed receptor expression may lead to RP uptake in these tissues. The biodistribution studies presented here demonstrate receptor-dependent increases in $^{177}$Lu-DOTATOC uptake in the kidneys and ovaries of vvDD-SSTR treated mice (Figure 4.3). Our virus biodistribution data, which are consistent with previously published results in similar models (475, 600), showed low levels of virus in the kidneys at all time points and ovary titers approximately 10-fold lower than tumour titers. When the kidney protectant D-lysine was co-administered with $^{177}$Lu-DOTATOC (37 MBq) for the imaging studies we observed a decrease in the mean kidney %ID/g uptake from mice treated with vvDD-SSTR relative to the receptor-negative control virus (Figure 4.5B). It is widely recommended that patients undergoing SSTR-directed PRRT also receive some sort of kidney protecting agent (eg. lysine, arginine or an amino acid mixture) therefore based on our data it seems unlikely that virus-directed receptor expression would lead to any increased risk of renal toxicity. Mouse ovaries are consistently shown to be an off-target site of VV replication for mechanisms that are not entirely understood (123). Nevertheless, it is important to be aware that this organ tropism has not been observed in non-human primate studies (194) nor has ovarian toxicity been reported in any clinical trials (although to date it has not been looked at specifically). Therefore, this likely represents a murine-specific phenomenon and consequently any virus-receptor driven ovarian toxicity is unlikely to extend beyond preclinical mouse models. That being said, the ovaries are a common site of implantation in patients with colorectal PC and virus-directed uptake in this organ could be beneficial to therapy.

SS analogues have the advantage of being amenable to labeling with a variety of radionuclides with different physical and biological properties. In the context of radiovirotherapy, which relies primarily on the cross-fire effect, the radionuclide must have sufficient tissue penetrating capacity to deposit its energy multiple cell diameters from its source,
as is the case of $\beta^+$ emitters such as $^{177}\text{Lu}$, $^{90}\text{Y}$ or $^{188}\text{Re}$, which have a maximum range of 2 mm, 3 mm and 12 mm in tissue, respectively. Weighing the benefits of the desired cross-fire effect within the tumour against the risks associated with the undesired cross-fire effect in normal tissues has led to more frequent use of $^{177}\text{Lu}$- and $^{90}\text{Y}$-labeled SS analogues (although others have also been investigated). Previous work performed in tumour-bearing rats suggests using a $^{90}\text{Y}/^{177}\text{Lu}$-DOTATATE cocktail may have better tumour control activity in disease models with multiple tumours of varying sizes (638). Recent clinical studies provide further evidence that a $^{90}\text{Y}/^{177}\text{Lu}$-DOTATATE cocktail may be more efficacious (461) and less toxic (639) than a single RP. Radionuclide cocktails may be similarly beneficial to radiovirotherapy of peritoneally disseminated CRC, which is characterized by many tumours over a range of sizes (microscopic to > 5 cm in diameter) in a single patient.

In this study, SPECT imaging was feasible due to the $\gamma$-photons emitted during $^{177}\text{Lu}$ decay. That said, relative to other $\gamma$-emitting radionuclides, $^{177}\text{Lu}$ is not the best suited for SPECT imaging due to the low abundance of imageable $\gamma$-photons. $^{111}\text{In}$-DOTATOC represents an ideal agent for SPECT imaging as a result of the two high abundance $\gamma$-photons emitted during $^{111}\text{In}$ decay. Alternatively, imaging of vvDD-SSTR-directed DOTATOC uptake could also be achieved using a $^{68}\text{Ga}$ labeled peptide in conjunction with PET imaging. This has the advantage of allowing for much higher imaging sensitivity and therefore a more accurate representation of the tissue uptake biodistribution.

In addition to the cross-fire effect, both targeted radiotherapy and VV therapy have been shown to induce biological bystander effects (381, 397, 495), which are thought to be influential in mediating their respective anti-tumour activities. There is a growing body of evidence indicating that immunological mechanisms underlie the abscopal effects observed in radiation therapy of metastatic disease and that this effect can be bolstered by pretreatment with immunologic preparations (eg. activated DCs, IL-2, active macrophage inflammatory protein 1$\alpha$ (MIP-1$\alpha$), Toll-like receptor (TLR)) (640). For example, studies have shown that type I IFNs can have a radiosensitizing effect in various cancer cell lines including human CRC cells (641, 642). As well, cytokines produced by activated CD4$^+$ T cells were also found to sensitize tumour cells to $\gamma$-irradiation (643). OVs are in part a type of immunotherapy and the biological bystander effect induced by OVs, which consists primarily of intracellular and secreted ‘danger signals’, anti-viral cytokines, chemokines and activated immune cells (239), closely resembles the tumour
microenvironment that has been shown to improve the anti-tumour effects of radiation therapy. Therefore it is perhaps not surprising, that therapeutic efficacy has been improved upon combination of many OVs (466) including VV (524-526), with radiation therapy. This bystander effect could be further exploited in the radiovirotherapy approach by using a virus expressing an immunomodulatory protein that has been shown to sensitize cells to radiation.

To date, the vast majority of radiovirotherapy studies have looked at virus delivery of human NIS with subsequent $^{131}$I treatment (631). Currently, there are several early phase (I/II) clinical trials designed to evaluate the safety of an NIS-encoding measles virus as well as our ability to non-invasively track virus gene expression by SPECT/CT imaging. The results of these studies, most of which are still in the recruiting stage, will determine whether future trials will include a radiovirotherapy study arm.

Oncolytic VV shows specific and robust replication in a broad range of tumour cell types (181), therefore oncolytic VV-directed SSTR expression combined with PRRT could have widespread applicability beyond CRC. The results presented here support the investigation of this treatment approach in other models with the objective of clinical translation. Specifically, a more detailed examination of the long-term effect(s) of vvDD-SSTR and targeted radiotherapy on the kidneys is necessary to confirm there is no reasonable expectation of increased renal toxicity risk in patients. The results of a recently published phase I clinical trial examining the safety of western reserve vvDD in 17 patients (10 with CRC) (342), represents a promising step forwards towards the future clinical translation of its SSTR-expressing counterpart. This trial demonstrated safe intratumoural administration of vvDD at doses up to $3 \times 10^9$ PFU and further reported clinical benefit in one melanoma patient leading to subsequent complete surgical resection of their tumour with negative margins. In future trials, use of vvDD-SSTR would have the advantage of allowing for non-invasive monitoring of virus biodistribution through molecular imaging and would pave the way for later investigation of radiovirotherapy with radiolabeled somatostatin peptides.
5 Conclusions and Future Directions

5.1 Project Summary

The overall objective of this project was to investigate novel oncolytic virus-based combination therapies for the treatment of colorectal PC. We first explored the use of vvDD therapy alone in syngeneic and xenograft murine models of colorectal PC. In Chapter 2 we demonstrated that a single IP injection of vvDD ($10^9$ pfu) led to tumour-specific virus replication (with the exception of the ovaries in the xenograft model) and significantly improved survival in both models. In the syngeneic model, treatment efficacy was associated with a well-distributed vascular network and increased cellular proliferation in the tumours at the time of treatment. We then investigated two multi-modality treatment strategies to improve virus monotherapy: (1) VV combined with current first-line chemotherapy drugs, OX and CPT-11; and (2) SSTR2-expressing VV combined with PRRT. In Chapter 3 we identified synergistic interactions between VV and the active CPT-11 metabolite, SN-38, as well as demonstrated improved survival in a xenograft model of colorectal PC following combination therapy. Lastly, in Chapter 4 we provide evidence to support that vvDD-SSTR can render receptor-negative tumour s amenable to molecular imaging and PRRT using radiolabeled somatostatin analogues.

5.2 Study limitations and challenges

5.2.1 Considerations of the animal model

The use of animal models is critical to the study of novel cancer therapies; the commercial availability of inbred and transgenic mouse strains as well as hundreds of human- and animal-derived tumour cell lines has made selecting an appropriate model increasingly feasible. Even so, no tumour model is perfect, and each comes with its own set of advantages and disadvantages (Table 5.1). The primary variables that must be considered are the tumour origin, the site of implantation, and the immune status of the model. Tumours may be derived from cell lines, patient samples, or grown spontaneously in transgenic or chemically-induced mice. Additionally the site of implantation, for cell line or patient derived tumours, can be orthotopic (in the environment from which it was derived) or ectopic (e.g. subcutaneous). It was with careful consideration of the benefits and challenges of each model that we opted to use
orthotopic implantation of tumour cell lines in syngeneic and xenograft models. It remains important to acknowledge the limitations associated with these models, the most pertinent of which are discussed below.

**Table 5.1 Comparison of Different Animal Tumour Models**

<table>
<thead>
<tr>
<th>Tumor origin</th>
<th>Site of implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines vs. Fresh tumor tissue vs. Transgenic</td>
<td>Ectopic vs. Orthotopic</td>
</tr>
<tr>
<td>Heterogeneity</td>
<td>More homogeneous vs. Different cell populations vs. Depend on tumor origin</td>
</tr>
<tr>
<td>Microenvironment</td>
<td>Murine stroma vs. % Human stroma vs. Murine stroma</td>
</tr>
<tr>
<td>Metastatic potential</td>
<td>Depends on site of implantation vs. Depends on site of implantation vs. Metastasis occurs infrequently</td>
</tr>
<tr>
<td>Time required</td>
<td>Short, often only several weeks vs. Several weeks or months vs. Long, often requiring ≥ 1 year</td>
</tr>
<tr>
<td>Cost</td>
<td>Very inexpensive vs. Expensive, based on labor vs. More expensive, based on labor and housing</td>
</tr>
<tr>
<td>Tumor burden assessment</td>
<td>Depends on the site of implantation vs. Depends on the site of implantation vs. More difficult, because of orthotopic growth</td>
</tr>
</tbody>
</table>

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### 5.2.1.1 Orthotopic vs Subcutaneous Tumours

One of the primary objectives of this thesis is to investigate the use of VV as a potential treatment for colorectal PC. With this in mind, we chose to employ orthotopic models, which more closely resemble the clinical disease we aimed to treat than do the more frequently used subcutaneous models. Traditional orthotopic models of CRC involve implantation of tumour cells or tumour explants into the cecum wall. This results in growth of a primary tumour, spread to the regional lymph nodes and peritoneal cavity, as well as metastasis to the liver and lung (645). While patients presenting with colorectal PC may also have metastases in the liver and/or lung, in the majority of patients (approximately 60%) disease is limited to the peritoneal cavity (597). Therefore, in order to mimic the latter clinical presentation we chose to implant cells directly in the peritoneal cavity. In the models used in our studies, the primary sites of tumour growth were the peritoneum, mesentery (particularly near vessels supplying the intestines and colon) and omentum, as well as the diaphragm, adjacent to the pancreas and on the serous membrane covering the liver, intestines, ovaries and stomach. Each model showed a slightly different pattern of implantation; there was also some degree of variability between mice of the same model. Nevertheless, this distribution is generally representative of the implantation sites...
observed in humans. In patients, implantation is related to several factors including: whether the adenocarcinoma is mucinous or non-mucinous; the dynamic flow and absorption of ascitic fluid in the peritoneal cavity; sites of trauma (a result of surgical intervention); as well as organ movement and gravitational forces (646-649). Generally, the major sites of implantation comprise the omentum, mesentery, bowel surface, rectouterine pouch, right paracolic gutter and diaphragm.

The environment of implantation not only alters the properties of the tumour, it can also affect its sensitivity to anticancer treatments. In a study of xenograft models of pancreatic cancer, implantation site (orthotopic vs. subcutaneous vs. metastatic) was found to alter signaling pathways involved in apoptosis and cell-cycle regulation in a cell line dependent manner (650). In a different study, Lovo cells were shown to be more resistant to 5-FU treatment when implanted subcutaneously compared to intra-colonic (651). Similar results have been shown with murine CRC cells implanted at different sites, wherein varying sensitivity to chemotherapy was attributed, in part, to differences in signaling transduction pathways as opposed to drug concentrations (652).

For the reasons outlined above we determined IP models to be more representative of the human disease than other implantation sites. The use of this type of model did, however, create additional challenges when endeavoring to understand the MOA of various treatments. Whereas tumour growth in a subcutaneous model can be readily compared to baseline tumour size and/or treatment can be started when tumours reach a specific size, such normalization to account for differences between mice is significantly more challenging with IP models. Furthermore, tumour response over time is much more difficult to track compared to caliper measurement of subcutaneous tumours. One option for longitudinal monitoring of tumour response is non-invasive imaging of fluorescently labeled or luciferase-expressing tumour cells. For deep tissue penetration, bioluminescence imaging is generally recommended over fluorescence imaging (653); in PC models, however, accurate quantitation of tumour growth over time is subject to many challenges, including: a requirement for precise identical positioning of the animal and camera over repeated imaging sessions (654); dampening of signal from tumours shielded by large pigmented organs such as the liver (655); and, misrepresentation of the true tumour size based on the depth of the signal source as well as differences in the fatty tissue density in healthy relative to tumour-burdened animals both of which affect light penetration (655). For these
reasons, we did not employ non-invasive imaging to track tumour burden, as we were not convinced it would provide additional useful information to our investigation.

In subcutaneous tumour models, where response is measured in 1, or sometimes 2, tumour(s) per mouse, it is comparatively simple to quantify and draw conclusions about the role of various factors, such as virus infection, tumour vasculature/perfusion, apoptosis, necrosis and immune response in mediating tumour response. When working with an IP model, there can be upwards of 50-100 tumour nodules of varying sizes in a single animal; this can complicate analysis, as there can be significant variability in the level of infection, and therefore response, between tumours in the same mouse. For example, tumours from an individual untreated mouse generally have similar levels of apoptosis, whereas a treated mouse might show increased apoptosis in 50% of the tumours, but not in the other half. The unresponsive tumours in a treated mouse lead to averages with large deviations. This can make identifying statistically significant differences between treated and untreated mice problematic, particularly if the changes are expected to be on a small scale (e.g. changes in CD31 staining). Finding the best method to evaluate tumour response is also a major challenge in clinical studies, at least in part due to the nature of tumour dissemination. Response evaluation criteria in solid tumours (RECIST) and modified RECIST (mRECIST) are used to evaluate tumour shrinkage, typically on CT or MRI scans. These criteria, though, they can only be applied to tumours greater than 1 cm in diameter and requires reliable identification of all individual tumours. In patients with diffuse spread of hundreds of tumours, some less than 1 cm, it is very challenging to accurately evaluate tumour response.

Finally, OV delivery continues to be a major challenge to effective treatment, and represents an area of intense research. To properly assess the root causes of inadequate virus delivery it is useful to use models where the route from syringe to tumour mimics that which would take place within a clinical setting. Therefore, by using orthotopic implantation combined with IP delivery of virus – which we propose is likely to represent the ideal route of treatment for PC patients (discussed in 2.5) – we gain a more accurate picture of the extent of virus delivery and a better model for evaluating improved delivery strategies.
5.2.1.2 Immunocompetent vs. immunosuppressed host

In our studies we make use of both immunocompetent (syngeneic) and immunosuppressed (xenograft) models. Each provides important and unique information to our understanding of OV efficacy in vivo. Human tumour cells will only grow inside an immunosuppressed host, therefore xenograft models represent the only way to evaluate the preclinical in vivo antitumour efficacy of an OV in a human tumour. The use of these models does still attract criticism, and rightly so, particularly when evaluating biological therapeutics. For better or for worse, interactions with host immune system occur at many stages throughout the course of OV therapy, therefore in many ways these models represent a false system for evaluating efficacy and MOA. In the absence of a complete immune response, the mechanisms of tumour cell killing are likely to shift from potentially immune-mediated towards virus oncolysis-dependent. This may lead investigators to conclude that strategies to improve replication will improve therapy, when in reality such efforts may be futile if the virus is incapable of colonizing tumours due to rapid immune clearance. Immunocompetent models allow for evaluation of efficacy in the context of an intact (albeit murine) immune system; the use of mouse rather than human tumour cells does however limit the clinical applicability of these models.

In Chapter 2 we demonstrated efficacy in both immunocompetent and immunosuppressed models. In Chapters 3 and 4 we presented data in only one immunosuppressed model. In investigating both combination therapy strategies we aimed to demonstrate efficacy in the immunocompetent MC38 model. Repeated studies were performed combining vvDD with OX or CPT-11 in MC38 tumour bearing mice. While either monotherapy improved survival, combination therapy did not. Similar results were seen when vvDD-SSTR was combined with $^{177}$Lu-DOTATOC in this model, despite confirmed expression of SSTR in the tumours (Appendix 4.1). There are likely 2 primary factors contributing to the lack of efficacy: (1) this is a rapidly progressing model that displays a huge tumour burden at the time of sacrifice; and (2) virus is cleared from tumours more rapidly in the presence of an intact immune system. Based on our data, it is not clear that either combination treatment approach would work in immunocompetent models of colorectal PC. Furthermore, the potential MOAs attributed to combination therapy (particularly pertaining to the CPT-11 studies) may or may not be applicable in the presence of an intact immune system. Investigation of these combination
strategies in less aggressive immunocompetent CRC PC models would provide insight into whether they are viable options for hosts with intact immune systems. Ultimately, these model systems should be employed in concert, and conclusions regarding applicability to patients should be drawn with caution, as the true test of efficacy will be borne out in clinical trials.

5.2.1.3 Tumour cell lines vs patient-derived tumours

In our studies we employed syngeneic and xenograft tumour models derived from tumour cell lines. This is an attractive approach because models are reliable and reproducible, but there are significant criticisms to be made of the use of cell line-derived tumour models. The techniques used to generate tumour cell lines as well as the conditions in which they are propagated cause cells to undergo changes that make them fundamentally distinct from the tumours from which they were derived. In culture, selective pressures promote the survival of the least differentiated cells and the tumour cell population can show reduced complexity of gene expression patterns (656). Cell lines also lack the many ‘non-tumour’ stromal cells that make up the tumour microenvironment and play a critical role in tumour growth, invasion and metastasis (657). Patient tumours are a heterogeneous population of tumour cells displaying significant genetic clonal diversity as well as stromal cells. The loss of heterogeneity may be one of the primary reasons so many cell line-derived tumour models are poorly predictive of treatment efficacy in the clinic (658).

One way investigators have attempted to overcome these limitations is to create patient-derived xenograft models, wherein primary tumour explants are implanted subcutaneously or orthotopically into immunosuppressed mice. In traditional xenografts, stromal components, including vascular endothelial cells, immune and inflammatory cells, fibroblasts and fat cells are exclusively mouse-derived. In patient-derived xenografts, some stromal components are transplanted from the patient into the mouse (659). Patient-derived xenografts have been performed for numerous tumour types (660), including CRC tumour explants (661, 662). Recent studies have demonstrated that patient-derived xenografts accurately recapitulate the genetic and phenotypic profile of the tumour from which they were derived (659, 663). Furthermore, response to therapy correlates well with responses in the donor patients or tumour-type matched clinical data (661, 662). While these models have many advantages over traditional xenografts, they have not been widely adopted among the research community. This is because they are performed at significant cost (both financial and time-based) require a higher degree of technical
skill and necessitate access to fresh patient tumours. While the upfront cost may be greater the more widespread use of these models may help in the long run to prevent development of therapies that are likely to fail efficacy testing in phase II trials. As an alternative to the generally prohibitive use of patient-derived xenografts, we instead opted for the use of ex vivo cultured patient explants, which allowed us to demonstrate the tumour-specificity of VV infection in patient tumours (Figure 2.2). We continue to work with patient samples as they become available to better distinguish the salient tumour characteristics that lead to improved virus infection.

5.2.2 Understanding the tumour microenvironment

5.2.2.1 Dissecting the role of tumour vascularization and vascular disruption in OV therapy

In Chapter 2 we identified the tumour vasculature as both an important tumour characteristic at the time of treatment as well as a target of vvDD’s anti-tumour effect (the latter being consistent with previously published results (154, 198, 213, 216, 217)). Our data suggests that vessel distribution at the time of treatment was important to therapy success. One limitation of the vasculature data presented in this chapter is that we looked only at a single marker of angiogenesis at a particular time point, and did not evaluate vessel function. CD31 is widely viewed as a reliable marker of endothelial cells and angiogenesis (664), but it is also expressed on hematopoietic cells, specifically hematopoietic stem cells (HSC) (665), T-cell subsets (666), dendritic cells (DCs) (667), macrophages (668) and neutrophils (669). We used visual confirmation to ensure that the staining was specific to vessels; another strategy would be to also include other markers of endothelial cells such as CD34, von Willebrand factor (vWF) or Ulex europaeus lectin (664). Importantly, not all vessels identified by CD31, CD34 or vWF staining are necessarily functional vessels. To determine whether tumour vessels are actually supplying blood, tracers such as fluorescent lectin can be injected just prior to sacrifice followed by histological examination using fluorescent microscopy to identify active blood circulation in the tumour.

Based on our CD31 data, we suspect that tumours in earlier stages of growth (day 8) were less perfused, specifically in the tumour core, relative to more established tumours (day 12), and
as a result there was decreased vvDD delivery. This was supported by the fact that tumours with more widespread vessel distribution also showed increased VV staining in the tumour core shortly after delivery. To confirm our assumption about CD31 staining and perfusion, it would be necessary to evaluate blood flow in the tumours. One of the confounding factors in this experiment (with the potential to complicate the results) is that virus was delivered intraperitoneally. If we solely intended to prove that day 8 tumours were less perfused, and therefore vascular delivery of the virus was diminished, then proof would have been more easily established delivering the virus IV. It was our objective, however, to understand the mechanism of IP virus treatment of IP tumours. Clinical pharmacokinetic studies demonstrate that direct delivery of drugs into the peritoneal cavity leads to rapid uptake of a fraction of the dose into the systemic circulation (670). Consistent with what has been reported for IP chemotherapy in patients, we also observed rapid uptake of vvDD into the blood of mice, suggesting the potential for bidirectional infection of tumours. To address the question of whether tumour perfusion affects tumour delivery after IP virus administration, a constitutively fluorescent VV, similar to the one developed by Katsafanas and Moss (671) could be used to track the distribution of virus particles prior to active infection. Katsafanas and Moss describe a recombinant virus encoding a virus core protein fused to yellow fluorescent protein (YFP) that allows for visualization of individual virus particles using confocal fluorescence microscopy. This virus could be used in conjunction with fluorescently labeled lectin to determine approximately what proportion of IP VV particles are absorbed and delivered to the tumours via functional tumour vessels compared to that delivered directly from the peritoneal cavity, as well as whether the increased vessel distribution truly corresponded to increased perfusion and vascular delivery.

There is ample evidence that VV has vascular disrupting effects in both preclinical models and patient tumours (reviewed in (240); see Appendix 1.2). Based on the existing data, there are 2 mechanism by which VV is proposed to exert its vascular disrupting effect: (1) through direct infection of tumour endothelial cells, as demonstrated by this study (Figure 2.6) and others (154, 217), and (2) through indirect action, likely mediated by neutrophil-induced clot formation and a subsequent loss of tumour perfusion (198). With the exception of the data presented in Figure 2.6, all other preclinical data supporting these MOAs have been derived from subcutaneous tumour models. While we provide evidence for direct infection and loss of tumour endothelial cells in an IP tumour model, we did not extend our findings to look at effects of VV
on tumour perfusion, nor did we evaluate the proposed indirect MOA (although we did observe an influx of neutrophils). As described above, fluorescently labeled lectin could be used to answer the question of tumour perfusion post-VV treatment, while experiments similar to those performed with VSV by Breitbach et al. (198, 358) could delineate the role of the indirect mechanism of vascular disruption in our model. Specifically, such work could demonstrate whether neutrophil depletion and/or abrogation of clot formation would lead to a loss of treatment efficacy.

It has been widely stated that systemically delivered VV extravasates into tumours through characteristically hyperpermeable vessels, and that this is one of the initial mechanisms of VV tumour-specificity. Given the more recent evidence that VV specifically infects tumour vessels over normal vessels (217; unpublished data from our lab), investigators have now begun to question whether there is an alternative mechanism by which systemically delivered VV gains access to tumours; namely, through specific infection of tumour endothelial cells and subsequent release into the tumour either through budding or endothelial cell lysis (240). These mechanisms of exiting the circulation are not mutually exclusive and it is possible that VV may employ both or either under different conditions. The existing evidence gathered from VV pathogenesis and OV therapy studies suggests that tumour endothelial infection likely occurs secondary to tumour cell infection, as opposed to the reverse. The endothelium is a specialized structure composed of tightly connected polarized cells with apical (luminal) and basolateral (abluminal) membranes with distinct properties, including differential trafficking of cell surface receptors (e.g. apical-specific CD34) and structural proteins (672). In order for VV to exit the vasculature secondary to endothelial cell infection, it must be capable of entering polarized endothelial cells through the apical membrane. Data currently exists showing preferential entry and egress of several viruses – including VV – through either the basolateral or apical membranes of epithelial cells (673), but there is little data that looks at entry and egress through polarized endothelial cells. For example, VV has been shown to preferentially infect through the basolateral membrane of the polarized MDCK epithelial cell line (674). Despite important differences, polarized epithelial and endothelial cells do share many common features, one of which, the density of heparin sulfate (HS), may suggest a similar preference for basolateral entry into endothelial cells. HS has been identified as essential in the binding of VV to cells prior to infection (675, 676) as well as having differential distribution between the apical and basolateral
surfaces of both polarized epithelial and endothelial cells. In simple cuboidal/columnar epithelial cells, such as the tissue from which MDCK were derived, HS is found almost exclusively on the basolateral aspect (677). This may account at least in part for the improved infection efficiency through the basolateral membrane of polarized MDCKs. A recent study found there is also a high-to-low gradient of HS density between the basolateral and apical membranes of resting and inflamed skin vessels in mice (678). Therefore, similarly restricted basolateral infection mediated by HS density may occur in polarized endothelial cells. If this were the case, it would suggest that VV infection of tumour endothelial cells in vivo occurs primary through the basolateral surface secondary to tumour infection as opposed to through the apical surface prior to tumour infection. This hypothesis is supported by in vivo data from our window chamber model wherein viral RFP was detected in tumour cells prior to detection in tumour endothelial cells (unpublished data). Further investigation of the window chamber model using a VV expressing a fluorescent core fusion protein (described above) (671) could help elucidate the mechanism of VV’s exit from the tumour vasculature and the directionality of endothelial cell infection.

5.2.2.2 What is the role of the immune response in mediating treatment efficacy in our models of PC?

In Chapter 2, we discussed the role of tumour vascularization and proliferative rate on the outcome of vvDD treatment. We found that tumours in earlier stage of development (day 8) had lower proliferation rates, poorly distributed vascular networks and decreased tumour-associated macrophages (TAMs). We did not, however, determine to what extent these individual properties were responsible for the difference in tumour response observed between treatment at early stage compared to the later stage (day 12) tumour development. In particular, for uninfected tumours we measured a difference in the presence of TAMs between day 8 and day 12 tumours (Figure 2.5), orthogonally, we also observed differences between controls and infected tumours (Figure 3.7) in a different tumour model, it is not clear however, whether the TAMs possess tumour-promoting or tumour-inhibiting properties.

Macrophages can be classified into 2 broadly defined subsets: classically-activated (M1) macrophages and alternatively-activated (M2) macrophages (reviewed in (679)). M1-type macrophages are efficient antigen-presenting cells (APCs) that produce high levels of T-cell stimulating cytokines and are capable of killing microorganisms and tumour cells. Conversely,
M2-type macrophages are poor antigen presenters and have wound-healing properties such as secretion of T-cell suppressing factors and promotion of angiogenesis and tissue repair and remodeling. Clinical data have generated some controversy over the role of TAMs in tumour progression; while in many cancer types (e.g. breast, prostate, bladder, cervical and kidney) TAMs appear to be predominately M2 and associated with a poor prognosis (reviewed in (680)), data suggest that CRC TAMs tend to be more M1-type and their presence is associated with a better prognosis (681). Adding to the complexity is the fact that, unlike the largely fixed nature of lymphocyte polarization, macrophage polarization exhibits a great deal of plasticity that is highly dependent on its local microenvironment. In vitro data clearly shows that transition between the 2 subsets occurs in the presence of specific stimuli. For example, treatment with IFN-γ and/or exposure to Th1 cells effectively repolarizes TAMs from the M2 to M1 phenotype (682, 683). It is therefore postulated that TAM polarization may vary between different areas of the same tumour and that it may shift over the course of tumour growth.

Existing data suggest that the TAM phenotype and their potential role in VV treatment likely differs between our syngeneic and xenograft models. Whereas human HT29 CRC cells induce differentiation of human monocytes into M1-macrophages with T-cell activating properties (684) and DLD1 cells are killed in the presence of conditioned media from activated M1-macrophages (685), the opposite appears to be the case for mouse MC38 CRC cells. TAMs are abundant in in vivo MC38 tumours and they contribute to tumour growth (686). Taking together our knowledge of macrophage polarization plasticity and the similarity between the stimuli required to repolarize M2 and/or activate M1 macrophages with the environment created by VV vaccination (687) or infection in preclinical PC models (688), leads to several interesting and critical questions that warrant further investigation. In general, is vvDD therapy efficacy dependent, at least in part, on effective M1 repolarization and/or activation? More specifically, in our MC38 model where TAMs are likely M2, does vvDD treatment repolarize them towards the anti-tumour M1 phenotype, and could treatment be improved by augmenting this effect? Additionally, in our DLD1 model (where TAMs may be M1) does vvDD treatment amplify their anti-tumour effect and is this effect critical to treatment efficacy? Finally, if TAMs are critical to therapy, could modification of vvDD to encode M1 polarizing cytokines improve its overall efficacy?
To test the ability of vvDD to induce M2 repolarization and/or M1 activation in our models, *in vitro* conditioned media experiments could be performed using monocytes isolated from C57BL/6 or BALB/c nude mice PBMCs. Monocytes would be induced to undergo either M1 or M2 polarization by treatment with GM-CSF or M-CSF, respectively (689). Polarized macrophages would then be incubated with supernatant from virus infected tumour cells and phenotypic and functional analysis could be performed. To understand the role TAMs and other immune infiltrates play in *in vivo* tumour response, further IHC analysis as well as depletion studies could be performed. Both approaches are associated with certain limitations. Use of IHC to distinguish macrophage subtypes is somewhat controversial as there is not a consensus on what markers reliably differentiate M1 and M2 macrophages. Instead, changes in metabolic activity have been widely looked to, as increased arginase and iNOS are associated with activated M2 and M1 macrophages, respectively (690).

Another potential role for macrophages in mediating oncolytic VV therapy can be delineated from the results of a recent study by Byrd et al. (691). It has previously been fairly widely accepted that monocytes, macrophages and DCs are mostly resistant to productive VV infection (692-697). Byrd et al., however, demonstrate that primary human monocyte-derived M1 or M2-polarized macrophages were susceptible to productive VV infection, with M2-polarized macrophages supporting higher levels of virus replication (691). Furthermore, they found that virus released from M2 cells was predominately EEV, which is in contrast to the mostly IMV that is released during CV-1 infection. This novel finding opens a new line of questioning that requires further investigation – namely whether macrophages play a role in propagating and disseminating VV within a host. It is possible that they serve as virus reservoirs, which could help explain the long-term persistence sometimes observed in VV-treated tumour models (698). The window chamber model, described in Chapter 2, could provide a useful tool for exploring this potential role of macrophages. Using the recently generated transgenic MacGreen mice, which express EGFP under the control of the macrophage colony-stimulating factor (CSF-1) promoter (699), along with a constitutively fluorescent VV (671), viral infection and macrophage trafficking in the tumour could be monitored over time.

Depletion studies would provide critical insight into the role of both resident TAMs as well as post-therapy tumour-infiltrating macrophages. Examining depletion of macrophages at the time of implantation up until the time of treatment would allow us to determine the importance
of resident TAMs in the tumour, and differences that may have contributed to the variable treatment efficacy in day 8 (low TAMs) versus day 12 (high TAMs) tumours (Figure 2.4 and 2.5). On the other hand, depletion performed immediately prior to treatment would provide information about the role of infiltrating macrophages. There are many factors and potential challenges associated with macrophage depletion studies. First, the method of depletion must be considered carefully. The 3 primary options are clodronate liposome-mediated depletion, antibody-mediated depletion (e.g. anti-CD115) or CD11b-Diptheria Toxin Receptor (DTR) transgenic mice. Clodronate-containing liposomes work by delivering apoptosis-inducing clodronate into the cytoplasm of phagocytic cells. This results in efficient elimination of all phagocytic cells, including DCs. To look specifically at the role of macrophages, it is recommended that investigators perform additional studies where macrophages are replenished by isogenic transplant following liposome depletion. Transgenic mouse models use the CD11b-DTR construct where human DTR is under the control of CD11b regulatory elements, thereby allowing for conditional ablation of macrophages following administration of DT. More recently, anti-CD115 (CSF-1R) antibodies have also been used as a means of depleting macrophages although their specific effects in vivo have not been fully examined. No matter which method is used, these studies would be subject to several limitations. Firstly, none of these methods allow for selective depletion of a single subset, although mice could be replenished with ex vivo polarized macrophages following depletion (700). Secondly, TAMs play a fundamental role in the development and progression of tumours, therefore it would be necessary to characterize any changes to the natural progression of disease in our models prior to investigating the effect of macrophage depletion on treatment efficacy. Lastly, all depletion methods result in pan elimination of macrophages (and other phagocytes in the case of liposomal clodronate) and this could have serious implications for vvDD toxicity (701).

In our MC38 model, we also observed an increase in Ly6B.2-positive cells, which we identified as neutrophils. We were not able to assess the infiltration of neutrophils in the DLD1 model using this antibody because BALB/c mice express the Ly6B.1 haplotype. The Ly6B.2 antigen is expressed on neutrophils, bone marrow progenitors and monocytes but is absent on tissue resident macrophages and DCs (reviewed in (702)). Neutrophils have previously been implicated in the efficacy of VSV therapy in studies where the anti-Ly6G (Gr-1) mAb RB6-8C5, which has been used extensively as a means of neutrophil depletion, resulted in a loss of virus-
induced vasculature disruption and apoptosis in murine CRC tumours (198, 358). While neutrophils were identified as the cells responsible for mediating this effect, Gr-1 is also a marker of monocytes, therefore it is not possible to be absolutely clear which cell-type was necessary to induce the downstream events. Indeed, others have since reported on the controversy pertaining to in vivo neutrophil depletion using this antibody and concurrently developed more specific antibody clones (703). Use of these improved antibodies could help to confirm the conclusions of previous studies and determine if neutrophils are similarly critical to vvDD treatment of IP tumours.

5.2.3 Understanding the mechanism of vvDD and SN-38 interaction

In Chapter 3 we hypothesize that vvDD has a bystander effect on the cell-cycle of uninfected virus-treated DLD1 cells. We propose that this sensitizes uninfected cells to SN-38-mediated apoptosis. Unfortunately, our efforts to demonstrate the presence of a soluble factor in conditioned media from virus infected cells that is capable of either sensitizing cells to SN-38 cytotoxicity or inducing S-phase accumulation, proved inconclusive. Based on the preliminary studies performed it seems likely that either (1) the method(s) used to remove virus from the conditioned media somehow negated or failed to capture the effect of the soluble protein or (2) the bystander effect was not mediated by a soluble factor.

Several different methods for generating virus-free conditioned media were tested. The first involved centrifugal filtration of supernatants from virus infected cells with a 100 kDa exclusion limit, thereby excluding all particles greater than 100 kDa. Plaque assays confirmed the absence of virus however the conditioned media had no sensitizing effect when combined with SN-38. We hypothesized that the lack of effect was perhaps due to issues of concentration, and therefore added a concentration step, using a centrifugal filter with a 10 kDa exclusion limit, which allowed us to filter 15 ml of conditioned media and concentrate it to a total volume of approximately 200 µl while retaining all proteins >10 but <100 kDa. In these experiments we found that both virus conditioned media as well as conditioned media from mock-infected cells sensitized cells to SN-38-induced cell death. If the factor mediating the bystander effect is a soluble cellular factor, as we have hypothesized, it is possible it is a stress protein that is also secreted by mock-infected cells at a lesser concentration, the difference of which was lost when subjected to the concentrating step. Another possible explanation is that the time at which the
conditioned media was collected may have failed to capture the timing of maximum secretion of this soluble factor. In a study describing the sensitization of tumour cells to paclitaxel following VV infection, IFN-β, one of the mediators of the effect peaked at 6 hpi (495), which is much earlier than the 24 hpi time point used in our experiments. Alternative methods, including simple filtration through a 0.2 µm pore filter as well as coincubation with neutralizing serum were found to be ineffective at completely eliminating infectious particles.

Given the inconclusive results of our conditioned media experiments, we must also consider the possibility that the bystander effect is mediated by a mechanism other than paracrine signaling by a soluble factor. In a recent study, the transmission of innate anti-viral signals between cells was demonstrated to occur through gap-junction signaling in response to cytosolic dsDNA (704). Mammalian cells synthesize cyclic guanosine monophosphate-adenosine monophosphate(2’-5’) (cGAMP(2’-5’)) in response to DNA virus infection, which in turn activates the endoplasmic reticulum protein stimulator of interferon genes (STING) protein (705-707). STING activation leads to induction of type-I IFNs through activation of interferon response factor (IRF)-3 (706). In murine and human cells, cGAMP(2’-5’) produced in response to dsDNA binding to cGAMP synthase (cGAS), leads to activation and perinuclear relocalization of STING in neighbouring non-cGAS-expressing cells in a gap junction-dependent manner (704). This provides a mechanism by which uninfected cells are rapidly induced to produce type-I IFNs, which themselves have been shown to prolong S-phase in human HT29 CRC cells through induction of p21^WAF1/CIP1 (708). Furthermore, p21 activation is also propagated in a gap-junction dependent manner (378), which could directly impact cell-cycle progression in uninfected cells. To test the role of gap-junction mediated signaling in sensitizing uninfected cells to SN-38-induced cell death, vvDD infection could be performed in the presence of specific gap junction inhibitors and the effects on cell-cycle as well as combination therapy synergy could be evaluated. If gap-junction signaling is found to be critical, then it is possible that the differential response to combination therapy between cell lines is related to variable expression and/or trafficking of important connexins (Cx), such as Cx43, as has been seen in CRC cell lines (709). Further supporting this line of reasoning is the fact that HT29 cells, which we found to be more resistant to combination therapy synergy (Figure 3.1 & 3.2) do not express Cx43, whereas MC38 cells, which supported combination therapy synergy, are capable of Cx43-mediated intercellular communication (710).
5.2.4 Methods for determining synergy

5.2.4.1 *In vitro* vs. *in vivo* combination therapy

As demonstrated in Chapter 2 *in vitro* synergy does not always translate to improved *in vivo* therapy. Whereas vvDD synergized with both OX and SN-38 in MC38 cells *in vitro*, neither combination led to improved survival over monotherapy in tumour-bearing mice (data not shown). The reasons for this discrepancy between *in vitro* and *in vivo* results as well as the response to combination with CPT-11 between the syngeneic (MC38) and xenograft (DLD1) tumour models, are likely many-fold. Firstly, the syngeneic model is a particularly aggressive tumour model that develops rapidly. The disease progresses from small tumour nodules, appearing between 8-12 d post implantation, to end point tumour burden within approximately 10-15 d. Our treatment schedule with chemotherapy given once every 4 d beginning on day 10, was effective in the more slowly progressing xenograft tumour model, but seems to have been too protracted to enhance tumour growth inhibition in the syngeneic model. Additionally, rapid drug inactivation and poor penetration/deposition into the large tumours may have also affected CPT-11 treatment in C57BL/6 mice (discussed below).

In the *in vitro* experiments, we used SN-38 as opposed to CPT-11. This was decided because *in vivo* metabolic conversion of CPT-11 to SN-38 occurs primarily in the liver and we were interested in the effect of the active drug and rather than on the expression level of CPT-11 metabolizing CES in the different tumour cell lines. Differences in tumour response between the same cells culture *in vitro* or grown *in vivo* may therefore be attributed to the complex nature of CPT-11 metabolism (discussed in Section 1.1.3.3). Polymorphisms in enzymes involved in CPT-11 metabolism have been correlated with efficacy and toxicity in patients and it is possible similar variations in response may occur across mouse strains. In silico comparison of the metabolic conversion of SN-38 to SN-38-G between 16 inbred mouse strains found that liver enzymes from C57BL/6 mice displayed the second highest rate of glucuronidation. C57BL/6 mice, therefore, may be less susceptible to CPT-11 therapy compared to BALB/c mice with a glucuronidation rate approximately half that of C57BL/6 mice (618). There are also documented differences in the CES activity in different mouse strains (711), but to the best of our knowledge a direct comparison of CES activity between C57BL/6 and BALB/c mice has not been performed.
Other factors that likely contribute to the lack of efficacy of combination therapy in our syngeneic MC38 model are an insufficient concentration of CPT-11 and/or vvDD delivery and replication in the tumours. These same issues likely explain, at least in part, the failure of vvDD-SSTR + $^{177}$Lu-DOTATOC treatment to improve survival in this model (data not shown). As was shown in the in vitro isobolograms (Figure 3.2), synergy was only observed at the highest dose combinations in MC38 cells. In fact, the lower dose combinations resulted in an antagonistic interaction. In vivo, we saw that the average tumour titer was approximately 1-log lower in the C57BL/6 compared BALB/c models at 6 dpi (Figure 2.4 vs Figure 3.3), likely due primarily to the presence of an intact immune system and perhaps to a lesser extent due to the difference in tumour burden. Similarly, drug penetration and deposition is also a major challenge for the treatment of solid tumours and is thought to contribute significantly to resistance in patients (712). In a CRC xenograft model, CPT-11 concentrations, determined by drug autofluorescence, were substantially elevated at 1-4 h post injection in areas up to 50 µm away from the nearest blood vessel (713). At distances greater than 50 µm from the nearest blood vessel and time points between 8-72 h post injection CPT-11 concentrations tapered off significantly. While RPs have better tumour penetrating properties than radiolabeled mAb, they are still susceptible to many of the penetration barriers that apply to traditional chemotherapy drugs. In the context of the large and abundant peritoneal MC38 tumours, it is possible that insufficient drug/RP concentrations, along with lower VV titers throughout the tumours, resulted in a loss of their synergistic potential. Additionally, lower virus titers corresponds to less expression of SSTR, which likely led to lower RP uptake. Drug delivery systems are a major area of research and development, for example a new formulation of lipid encapsulated CPT-11 is currently under investigation in a clinical trial (714). In the future, use of improved drug and OV delivery systems (discussed in Section 5.3.1) will increase the amount of each agent reaching the tumours and therefore improve both their individual anti-tumour effects as well as their potential for synergistic interaction.

5.3 Future Directions: towards improving OV therapy and its combination with chemo- and/or radiotherapy

5.3.1 Improving virus delivery systems

One of the major challenges facing OV therapy remains virus delivery. Achieving adequate levels of infection with sufficiently widespread distribution within a tumour is critical
to achieving complete response (236, 242). This obstacle is magnified when confronting treatment of a disease with a heavy tumour burden, characterized by many dispersed tumours of varying sizes and potentially different characteristics (e.g. vascularization, proliferation, immune infiltration etc.). Our IHC analysis of tumours across studies and tumour models consistently show substantial intra- and inter-mouse variability in tumour infection levels, with some tumours showing substantial infection and others showing little to no infection (Figures 2.3, 2.4, 3.7 and 4.2). These data indicate that there is room to improve virus delivery in these models. This would not only yield improved outcomes following monotherapy but would increase the opportunity for interaction with additional treatment modalities such as chemotherapy or PRRT.

Our use of IP virus delivery was based on both preclinical studies showing equivalent or improved virus delivery to tumours following IP vs. IV delivery (475, 591) as well as clinical data demonstrating decreased toxicity following IP chemotherapy in PC patients (90, 92). Furthermore, by delivering the virus IP we aimed to overcome one of the initial barriers to virus delivery – mediators of the systemic cellular and humoral innate immune system. Components of the complement system are very effective at neutralizing virus and inhibiting infection even in the absence of preexisting anti-vaccinia antibodies (715). Complement inhibitors could be used to improve systemic delivery (715), but non-human primate studies suggest this could have major toxicity ramifications (unpublished data). Furthermore, a recent study showed that complement-dependent cytotoxicity (CDC) of cancer cells is a major effector of the anti-tumour effect in both preclinical tumour models and patients (314). As it stands, IP delivery of virus to IP tumours appears to be the optimal route, although if issues surrounding virus inactivation in the systemic circulation are overcome and lead to improved outcomes with IV therapy, this could be reconsidered.

5.3.1.1 Cell carriers for improved OV delivery

Research into improving OV delivery has explored many different strategies that could be applied to virus delivered both IV and IP. The traditional approach has been to combine viruses with immunosuppressive therapies so as to temper the anti-viral immune response (reviewed in (466); see Appendix 1.1). More recent efforts have looked towards strategies of hiding the virus from the immune system through use of cell carriers or polymer-based coatings. One challenge faced in employing virus-masking strategies is maintaining the tumour-targeting
capacity of the therapy which, in the case of cell carriers, is no longer mediated by the virus – at least during the initial round of infection. This may, though, not be as critical of an issue as originally proposed, as data shows that OVs rapidly (within 30 mins) associate with cells in the plasma following intravenous delivery (635); this suggests that OVs are already using endogenous cells as a means of trafficking to tumours. Selection of the appropriate cell carrier is therefore paramount to ensuring maximum delivery. Studies have looked primarily at the use of immune cells (e.g. DCs, T-cells) and stem cells, which show innate tumour-homing properties (344, 716-719), as well as cancer cells (579, 625, 720); the clinical application of the latter strategy, however, remains tenuous.

5.3.1.2 Polymer-coated OVs

Polymer coating of virus particles has been performed almost exclusively with Ad, a non-enveloped virus, (721-725), although there is no reason why such a strategy could not be applied to enveloped OVs such as VV. Polyethylene glycol (PEG)-coated liposomes have been used extensively as drug delivery systems, demonstrating the feasibility of inserting polymers into a lipid membrane (726). A recent study described the construction and characterization of the first polyethylene glycol (PEG) coated-VSV (enveloped virus) and found that the coated virus persisted at significantly higher titers and for longer periods in the systemic circulation of immunized mice (727). Uptake of polymer-coated liposomes occurs primarily via endocytosis and is dependent on the polymer properties (e.g. charge, size, etc.) and particle size. Given that uptake is passive and non-specific, differences in microvascular permeability between tumours and normal tissues are thought to be the main mediator of tumour-specificity. This could be viewed as an impediment to polymer-coated OVs, as it could affect tumour diffusion properties. Polymer coating would likely increase the size of the particle; copolymer coating of Ad resulted in an approximately 20% increase in particle size (728). Given the rather large size of the VV particle, a 20% increase could have a significant effect of its ability to permeate vessels and enter the intercellular spaces of tumours. Another question that arises is whether polymer coating would decrease infectivity. Polymer coating-induced steric hindrance of the viral proteins normally involved in binding of cell surface receptors/proteins would invariably alter the mechanism of viral entry. At the same time, it could provide a unique opportunity to retarget VV, a feat that has not been possible through genetic modification. Targeting ligands can be
chemically conjugated to the polymers such that the particles will be actively taken up following specific binding to a cell surface receptor. Polymer-coated Ad was retargeted to FGFR- and VEGFR- expressing tumour cells by incorporation of bFGF and VEGF<sub>165</sub> onto the polymer (728). Consideration must be taken when picking a ligand: it should target an appropriate receptor for the selected tumour type and induce cell internalization upon receptor binding, and it should not result in any deleterious downstream signaling. Another confounding variable specific to VV is the presence of multiple virion forms (e.g. IMV, IEV and CEV/EEV) in a single virus preparation. Given that the majority of virus particles are IMV following standard amplification and purification procedures, it would be logical to focus attention on coating this particle form. Significant work would be required to develop the procedures and assays necessary to produce, characterize and test polymer-coated and retargeted VVs – however it has the potential to profoundly improve the systemic delivery of virus.

5.3.2 Understanding and exploiting the immune response

5.3.2.1 Effect of VV pre-existing immunity on anti-tumour efficacy

A reoccurring concern in the field of OV therapy is the potential negative impact that pre-existing anti-viral immunity may have on the efficacy of virotherapy. Significant proportions of the general population have been exposed to various strains/serotypes of many of the viruses currently under investigation as oncolytic agents either through natural exposure (e.g. HSV, Ad, etc.) or vaccination (e.g. VV). Furthermore, it is likely that as with other therapies, OVs may require repeated administrations over a period of time, which could lead to the development of immunity even in patients without pre-existing immunity. While this is an active area of investigation, it is still not clear whether pre-existing immunity will affect treatment outcomes and more specifically how the nature of that immunity (e.g. cellular vs. humoral) will impact OV efficacy. A better understanding of this issue could lead to the development of eligibility criteria wherein patients with specific markers of pre-existing immunity are excluded from receiving a specific virus and must consider other OVs. Alternatively, it could lead to more targeted approaches for improving VV delivery that work with the nature of immunity the individual patient displays.
Tumour models in C57BL/6 mice represent an ideal system for investigating the effect of preexisting VV-immunity and potential strategies to overcome it, as much is already known about the nature of the anti-VV immune response in these mice (205). During an acute IP infection with WR VV, antibody-mediated clearance is the dominant means of controlling virus replication. Although there is an increased presence of highly activated CD8 T cells 7 dpi, these cells have no effect on VV clearance following a primary infection in the context of a fully immunocompetent host. In the absence of humoral or CD4 T-cell mediated immunity, a protective effect by CD8 T-cells was observed. In this model, immunological memory to VV is attributed to both CD8 T-cells as well as persistent humoral immunity. This suggests that in hosts without previous exposure to VV, evasion of Ab-mediated clearance would significantly improve virus delivery to and persistence in tumours, whereas in pre-immune hosts both humoral and CD8 T-cell meditated immune responses must be overcome.

Not surprisingly, preclinical studies have demonstrated that preexisting immunity to VV, through vaccination of mice prior to implantation of tumours, can significantly inhibit vvDD recovery from subcutaneous and IP MC38 tumours (579, 625). In these studies cell carriers (discussed in 5.3.1.1) and/or immunosuppressive drugs were used as a means to bypass humoral immunity and/or eliminate innate and adaptive cellular immune responses, respectively. Consistent with our understanding of anti-VV immunity in C57BL/6 mice (205), elimination of circulating antibodies was shown to result in the greatest increase in VV recovery from subcutaneous tumours in preimmunized hosts. Furthermore, while both immunosuppressive drugs and cell carriers delivered individually increased virus titers in IP tumours relative to vvDD alone (no virus recovered), a combination of both resulted in a further 2- to 4-log increase (579). It also seems that circumventing preexisting immunity through improved delivery techniques such as carrier cells may in fact improve the safety of therapy, as virus appears to be more efficiently cleared from normal tissues (625). Ultimately, the true test of VV efficacy in the face of preexisting immunity will come from its performance in clinical trials, which have thus far reported no relationship between the levels of preexisting neutralizing antibodies and VV replication in tumours (179).
5.3.2.2 Harmonizing the immunomodulatory effects OV and radiation therapy to achieve greater in vivo efficacy

The results of our studies demonstrate that macrophages are affected by treatment with vvDD, CPT-11 and the combination of both. The exact role of TAMs in treatment efficacy has yet to be determined (discussed in Section 5.2.2.2). While there is little known about the interaction between CPT-11 and macrophages, there is substantive evidence suggesting that TAMs (as well as other immune cells) play a critical role in the outcome of RT. Appreciation for the role played by an ionizing radiation induced-proinflammatory microenvironment in mediating the tumour response has only recently been established (729). Whereas tumours are generally considered to have escaped immunosurveillance and exhibit an immunoregulatory phenotype (730), ionizing radiation has the potential to break this immune silence through induction of immunogenic tumour cell death (390), stimulation of chemokines/cytokines that recruit effector T-cells and upregulation of cell surface molecules (e.g. MHC-1) that promote effector cell-mediated cell death (731). Evidence of this has existed in the clinic for some time, where patients undergoing radiation therapy show responses in tumours outside the irradiated field (732). This phenomenon, called the abscopal effect, has recently been linked to radiation-induced activation of anti-tumour immunity in preclinical models (733) and in patients (734, 735).

An improved understanding of the abscopal effect has led to active investigation of immunotherapy as a compliment to RT. In preclinical murine tumour models, administration of exogenous factors that activate TLRs, increase DC proliferation and activity or activate effector T-cells improve responses to radiation, both at the site of irradiation as well as in distant metastasis (736). Similar results were found when local RT was combined with systemic autologous tumour cell vaccination in an orthotopic glioma model (737). These studies provide a very strong rationale for the combination of RT with oncolytic virotherapy and suggest potential for synergistic interactions by mechanisms similar to those described for the combination of RT with immunotherapy.

OVs are increasingly being thought of as immunotherapeutics in addition to cytolytic agents, although the extent to which each plays a role in the tumour response is not fully understood (738). Many of the immunogenic properties of OVs are inherent to the virus and
reflect the host’s natural response to an invading foreign entity (739); at the same time, they can also be enhanced and redirected towards the tumour through the incorporation of immunoregulatory genes into the virus backbone (discussed in Section 1.2.5). Whether by innate mechanisms or through the assistance of immunomodulatory ‘arming’, OVs have been shown to induce many of the same host immune responses that are known to sensitize cells to radiation. TLRs (3 and 9), which recognize various virus associated PAMPs, are activated in oncolytic parvovirus H-1 (H-1PV) infected cells and human DCs exposed to virus-infected tumour cell lysates (740). An oncolytic Ad that has been been modified to enhance its TLR stimulating capacity (741) may be particularly effective in combination with RT. Host expression of type-I and -II IFNs is commonly upregulated in response to many different OVs (211, 212, 263, 337, 495, 742-749) as are other proinflammatory cytokines such as TNF-α (746, 750) and IL-12 (203, 750), and chemokines such as MIG (742, 750) and IP-10 (203, 742). All of these contribute to increase the activation of effector T cells. Concordantly, tumour infiltration and/or activation of immune cells, among them DCs and T-cells (203, 211, 212, 585, 742, 751-756), have also been widely reported in response to OV therapy. For example, VSV-treated tumour-bearing mice showed significant increases in the number of tumour-infiltrating T-cells an effect that was both virus-replication dependent and necessary for treatment efficacy (752). Similar induction of tumour-specific T-cell responses have been identified in models of oncolytic HSV virotherapy (757, 758) including a murine model of ovarian PC where virus treatment led to significant production of MIG, IP-10 and IFN-γ by tumour-associated monocyte-derived DCs and subsequent infiltration of NK- and T-cells (742). Based on studies of radiosensitization, these OV-induced proinflammatory microenvironments would likely be increasingly susceptible to RT.

The immunologic ‘bystander effect’ induced by OVs has been shown to affect both directly infected as well as uninfected (e.g. contralateral or metastatic) tumours (755, 757, 759, 760). Capitalizing on this systemic immunologic ‘bystander effect’ has obvious therapeutic benefits for OV monotherapy; it could also have a profound impact on efficacy when combined with RT. Very little, if anything, is known about what role the immune response is playing in the interaction of these 2 treatment modalities. A simple way to test whether OV-induced radiosensitization is mediated by induction of a systemic immune response would be to use a model, such as that described by Toda et al., where there is substantial anti-tumour immune
response in subcutaneous tumours on the contralateral flank to the OV-treated tumour (757). The effects of subsequent irradiation of both tumours compared to appropriate controls would reveal whether the uninfected tumours of virus-treated mice are more susceptible to RT. Further dissection of the specific immune-mediators of this effect could then be performed.

Abscopal effects of radiation therapy, as well as the radiosensitizing effects of immunotherapy, have been described exclusively in the setting of EBRT. It is not clear, therefore, what role the radiation- and virus-induced immunologic bystander effects might play in fostering the anti-tumour response following radiovirotherapy. In this setting, radiation is delivered specifically to infected tumours, rendering radiosensitization of distant uninfected tumours is inapplicable. It still seems reasonable to hypothesize that, at least on a local level, uninfected cells of an incompletely infected tumour may be more susceptible to radiation-induced death than those of an uninfected tumour. Additionally, the combination of OV and PRRT may lead to a more robust anti-tumour immune response than either monotherapy, and this could affect response in distant uninfected, non-irradiated tumours.

Dissecting the immunologic component of radiovirotherapy remains somewhat of a challenge. The PRRT-sensitizing effect of virus-induced secreted proteins on uninfected cells could be examined in vitro using conditioned media experiments, wherein virus-free media from infected cells is added to uninfected receptor-positive cells prior to treatment with RP. This would not, however, capture the role of APCs and T-cells, which are important in mediating responses to EBRT in combination with immunotherapy (736). Characterization of the tumour-infiltrating immune cells, as well as those in the spleens, would indicate whether the local and/or systemic anti-tumour immune response is significantly altered by combination therapy.

5.3.3 Chemo-radiovirotherapy

Going forward, it may be interesting to explore the combination of all 3 treatment modalities presented above. Many chemotherapy drugs have radiosensitizing effects (761) and their use in combination with PRRT for the treatment of NETs has been the focus of recently reported retrospective studies (762-764) and phase I-II clinical trials (765, 766). In the phase I-II trials, patients receiving $^{177}$Lu-DOTATATE were pretreated with a cocktail of radiosensitizing capecitabine (prodrug of 5-FU) (761) and temozolomide, which synergizes with capecitabine.
In mCRC patients, the addition of 5-FU to anti-CEA RIT was well tolerated and permitted more frequent use of multiple treatment cycles (768). The CRC chemotherapy drugs OX and CPT-11, which we investigated in combination with VV (Chapter 2), also have radiosensitizing potential. In vitro, sublethal doses of SN-38 sensitized HT29 CRC spheroids to EBRT (769) and concurrent CPT-11 and EBRT therapy led to significant tumour growth reduction in 2 lung carcinoma xenografts (770) and a syngeneic breast carcinoma model (771). Clinically, chemoradiotherapy with combinations of 5-FU and CPT-11 have been investigated for the treatment of locally advanced rectal adenocarcinoma (772) as well as other cancers. The preclinical and clinical experience with OX as a radiosensitizer is much more extensive; it has been investigated in models of CRC, glioblastoma, pancreatic cancer, HNSCC, cervical cancer, lung cancer and breast cancer, as well as been the focus of more than 30 phase I-II and 3 phase III clinical trials (reviewed in (773)). Taking together the evidence that (1) chemosensitization is feasible in the context of PRRT for metastatic disease; (2) CRC chemotherapy drugs can synergize with RT; and (3) combination of either CPT-11 (Chapter 3) or PRRT (Chapter 4) with VV improves survival in a mCRC model, there is a sound rationale to support the investigation of VV-mediated PRRT with chemotherapy (chemo-radiovirotherapy) for mCRC.

5.3.4 Clinical Translation

Both the successes and challenges of OV clinical translation to date have been previously presented (see Section 1.2.6), but there are specific considerations that warrant discussion surrounding the translation of an SSTR-expressing Western Reserve (WR) strain VV for the treatment of patients with PC. Encouragingly, the results of the first clinical trial using WR vvDD in patients have recently published (342). This study demonstrated safe intratumoural administration in 17 patients as well as regression leading to subsequent complete surgical-resection in 1 melanoma patient. Given that SSTR2 is an endogenously expressed cell surface receptor, there may be reason for concern regarding the potential for an autoimmune response directed towards normal SSTR2-expressing cells. Development of antibodies against foreign virus-encoded transgenes, such as β-gal, has been reported in patients treated with JX-594 (257). This notion of virus-induced autoimmunity has been most closely considered in the field of cancer vaccines, where the ultimate goal is to produce a robust immune response against a ‘self’-albeit tumour-specific, antigen (774). Furthermore, preclinical and clinical data have shown that vaccine-induced autoimmunity is a real risk with potentially catastrophic consequences (775,
As a result, extensive study of the autoimmunity-inducing potential of specific tumour antigens is always a part of cancer vaccine development. A classic example of this type of reaction is therapy-induced vitiligo in melanoma patients, which has been reported both in cancer vaccine studies (776) as well as following treatment with an armed oncolytic VV (275, 777).

To date, clinical experience with the use of MV-NIS, an OV encoding a cell surface protein that is also endogenously expressed in specific normal tissues, has not identified induction of an NIS-directed autoimmune response as a source for concern. To our knowledge, there have been no studies designed specifically to address this issue. To mitigate against potential toxicities, future clinical trials could examine the development of SSTR-directed antibodies in the serum of patients treated with vvDD-SSTR and preclinical studies could be performed to determine the pathological significance of an SSTR-directed immune response.

As OVs progress further through clinical development for the treatment of patients with PC, it is important to consider how they can be most effectively incorporated into existing treatment regimes. Namely, should OVs be delivered as a neoadjuvant therapy prior to surgery, at the time of cytoreduction as part of HIPEC, or as an adjuvant treatment post-surgery to target MRD? Preclinical data addressing VV’s MOA in the context of peritoneal disease will likely guide this decision however all 3 options should be evaluated in clinical trial to determine which provides the best outcome. For example, our data suggests that while an undefined proportion of human CRCs may respond well to combination vvDD and CPT-11 or OX therapy, vvDD may not be suitable for adjuvant treatment of MRD given that mice with small avascular tumours with low proliferative indices did not respond to treatment (Figure 2.4). Similar results were seen with adjuvant vvDD treatment in a mesothelioma PC model of post-surgery MRD where combination therapy did not improve survival over virus alone (199). Provided that initial clinical investigations of combination VV and chemotherapy reveal no serious toxicities, future studies designed to evaluate the comparative effectiveness of different treatment schedules will allow for empirical determination of the optimal method of incorporation into existing treatment plans. Ideally these studies would also be designed to investigate relevant patient and tumour characteristics that correlate with response to combination therapy. Once established as a viable treatment option for PC patients, clinical application will also depend on defined patient characteristics, such as immune status and extent of metastasis. Patients with inadequate immune function would likely be excluded from any form of OV therapy due to the risk of toxicity.
Additionally, patients with extra-peritoneal disease are typically not candidates for cytoreductive surgery or HIPEC leaving only the question of timing and route of virus administration relative to systemic chemotherapy.

The notion of combining VV with HIPEC is appealing and existing data on the favourable effects of hyperthermia on VV efficacy (210) provide rationale for incorporation of virus into the HIPEC delivery system. Additionally, concomitant administration of the virus with HIPEC means fewer visits to the hospital, which offers significant benefits from the perspective of patient experience and cost of treatment. Alternatively, there are arguments against the use of VV during HIPEC, including physiologic factors that could negatively affect VV efficacy, and obstacles that may limit the uptake of this approach by the surgical oncologists. Firstly, it is not clear how the acute inflammation caused by aggressive cytoreductive surgery would affect VV efficacy. Secondly, although decreased relative to tumour tropism, VV does display tropism for sites of wound healing over normal tissues (unpublished data from our lab) suggesting there could be an increased risk of post-surgical complications in patients. Encouragingly, data from both preclinical and clinical studies provide evidence to dispel such concerns. In IP tumour-bearing mice that had undergone cytoreductive surgery treatment, healing of the surgical incision was unaffected by IP treatment with VV(199). Similarly, no signs of impaired wound healing were observed along the needle track of patients undergoing liver biopsy following treatment with JX-594 (217). Lastly, OVs as a drug class are still quite new and even after clinical efficacy is established, significant knowledge translation and dissemination efforts will be required to ensure the widespread uptake of this treatment option amongst surgical oncologists.

5.4 Concluding remarks

OV therapy is a rapidly growing area of clinical research and development. The more we learn about OVs the more it becomes apparent that they fulfill the many criteria of an ideal cancer therapeutic. Broadly speaking, OVs are amenable to multiple routes of administration, have a high therapeutic index, are effective in a broad range of cancer types, kill through multiple non-overlapping mechanisms of action and have the potential to induce long-term immunologic resistance to recurrence. Clinical experience with OV therapy has been widely encouraging, with limited reports of serious toxicities and many examples of clinical responses. While many OVs are capable of effectively resolving single solid tumours, as has been seen in
preclinical models as well as in patients following VV therapy, curative treatment of advanced metastatic disease with OV monotherapy remains challenging. Over the past few decades, modifications to the vectors themselves have greatly improved their efficacy; it has also been widely acknowledged, however, that the greatest clinical benefit will likely be achieved through the rationale combination of OVs with other targeted therapies (466, 510, 511, 631).

The research presented in this thesis provides 2 examples of novel combination treatment strategies for improving oncolytic VV treatment of colorectal PC. The findings from investigation of the first strategy, combination oncolytic VV and chemotherapy, could have important applications for the design of future VV clinical trials and potentially in the treatment of mCRC. Importantly, the proposed mechanism of synergy – OV-sensitization of tumour cells to chemotherapy – suggests that this combination therapy could represent a much needed treatment option for patients with chemoresistant disease. The second strategy, radiovirotherapy has even wider potential applicability; any tumour that is susceptible to VV infection could be made a target of SSTR-directed PRRT. Continued investigation of the mechanisms of interaction between these treatment modalities across different tumour models will invariably lead to a better understanding of how to further improve these combination strategies as well as the clinical setting in which they are likely to have the greatest impact.
References


41. Strumberg D, Pilon AA, Smith M, Hickey R, Malkas L, Pommier Y. Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5'-


143. Miller CR, Buchsbaum DJ, Reynolds PN, et al. Differential susceptibility of primary and established human glioma cells to adenovirus infection: Targeting via the epidermal growth


221. Friedman GK, Haas MC, Kelly VM, Markert JM, Gillespie GY, Cassady KA. Hypoxia moderates gamma(1)34.5-deleted herpes simplex virus oncolytic activity in human glioma xenoline primary cultures. Transl Oncol 2012;5(3):200-7.


437. Schuchardt C, Kulkarni HR, Prasad V, Zachert C, Muller D, Baum RP. The bad berka dose protocol: Comparative results of dosimetry in peptide receptor radionuclide therapy using (177)lu-DOTATATE, (177)lu-DOTANOC, and (177)lu-DOTATOC. Recent Results Cancer Res 2013;194:519-36.


529. Chou J, Roizman B. Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. Proc Natl Acad Sci U S A 1994;91(12):5247-51.


698. Weibel S, Raab V, Yu YA, et al. Viral-mediated oncolysis is the most critical factor in the late-phase of the tumour regression process upon vaccinia virus infection. BMC Cancer 2011;11:68.


Appendices
Appendix 1.1. Intelligent Design: Combination Therapy with Oncolytic Viruses

Intelligent Design: Combination Therapy With Oncolytic Viruses

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Metastatic cancer remains an incurable disease in the majority of cases and thus novel treatment strategies such as oncolytic virotherapy are rapidly advancing toward clinical use. In order to be successful, it is likely that some type of combination therapy will be necessary to have a meaningful impact on this disease. Although it may be tempting to simply combine an oncolytic virus with the existing standard radiation or chemotherapeutics, the long-term goal of such treatments must be to have a rational, potentially synergistic combination strategy that can be safely and easily used in the clinical setting. The combination of oncolytic virotherapy with existing radiotherapy and chemotherapy modalities is reviewed along with novel biologic therapies including immunotherapies, in order to help investigators make intelligent decisions during the clinical development of these products.

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Introduction

Oncolytic viruses (OVs) are biological machines that kill cancer cells while sparing normal cells. Often they utilize sophisticated gene products to facilitate immune evasion, allow recognition and penetration of cells, co-opt cellular biosynthetic machinery and ultimately manipulate cell death programs. Interestingly, many of the biological pathways that viruses manipulate are the same ones that tumor cells deregulate during their malignant evolution and, as a consequence, these same pathways have become the targets for anticancer drug development. It seems reasonable to expect that certain kinds of chemical, radiological or biological therapy could be used to synergize with OVs and enhance tumor killing.

Broadly speaking, there have been three strategies for the creation of combination therapy approaches. The first is to simply combine an oncolytic virus with the current standard of care therapies, an approach which one could argue is the most likely to have immediate clinical relevance. The second strategy is to identify barriers that are limiting to oncolytic virus activity and select therapies that target that barrier. The third approach is to combine OVs, which may act to induce some level of antitumoral immunity as a byproduct of oncolysis, with some form of immunotherapy to achieve a synergistic immune response against the tumor.

Although the quickest route to the clinic may be to combine oncolytic therapy with the existing standard treatments, we know that in some cases certain chemotherapeutics and radiation modalities may have a negative influence on viral replication. Hence, this review will attempt to provide some insight into the types of combinations that rationally should be chosen for further development. These combinations will be discussed in detail.

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The prostate-specific adenovirus CV706 combined with XRT resulted in synergistic inhibition of tumor growth in a prostate cancer xenograft model at all time points from 7 to 42 days post-treatment. Furthermore, it reduced the prostate-specific antigen levels at 6 weeks to 1% of baseline which was significantly better than virus alone (86% of baseline) or radiation alone (139% of baseline). XRT combined with CV787 (also prostate specific) resulted in significant mean tumor volume regression (34% of baseline), complete regression (CR) in 80% of mice (at 8 weeks postinfection) and a significant reduction in serum prostate-specific antigen (11% of baseline) relative to either single therapy.

Significant improvements in disease outcomes have also been observed with combination herpes simplex virus (HSV) virotherapy and XRT in preclinical models. In two different studies, NV1086 (ICP0/ICP4 y34.5 deletions) combined with irradiation was shown to significantly reduce tumor volume compared to either treatment alone for nonsmall cell lung cancer and malignant mesothelioma. Complete eradication of cervical cancer, determined by histology, was achieved by 30 days post-treatment in 42% of mice treated with G207 (ICP6/y34.5 deletions) and XRT compared to 0% in all other treatment groups. Comparison of single versus multiple doses of R3616 (inactivated y34.5) for glioblastoma found that virus administered on three consecutive days and combined with fractionated XRT over 2 days resulted in a greater number of CRs (90%) compared to XRT combined with a single virus dose (56.5%). Furthermore, variance modeling showed that the effects of combination R3616 and XRT were greater than the additive effects of the individual therapies. The relationship of NV1023 (y34.5/UL24/UL56/US11/ICP47 deletions) in combination with XRT was investigated in three models of cholangiocarcinoma generated using different cell lines. Combination therapy showed a synergistic reduction in tumor volume in one model (at two different virus doses), whereas the effect was additive or not significantly better than individual therapies in the other models. Despite the promising results described above, the potential for combination therapy remains to be seen in a variety of other cancers. For example in the only study to look at herpes virotherapy and XRT in prostate cancer models, combination therapy was not significantly better then virus therapy alone in both immunocompetent and immunocompromised models.

With the exception of a handful of studies the majority of in vivo studies discussed above use s.c. tumor models combined with intratumoral OV delivery. This approach is simple and practical from an experimental point of view however perhaps less pragmatic in a clinical setting. Indeed, OV clinical trials have for the most part been restricted to intratumoral virus administration (largely due to safety concerns) however the focus should be to demonstrate safety and efficacy following systemic delivery. This will allow treatment of solid tumors inaccessible by a needle and targeting of metastases. Similarly, fractionated XRT is used widely in the clinic however used much less frequently in preclinical studies. Investigators would do well to strongly consider the value of the knowledge gained through use of orthotopic models combined with clinically relevant methods of OV and XRT delivery.

Mechanisms of synergy. The relationship between herpesvirus and radiation is perhaps the most well studied and well characterized. Radiation exposure increases HSV titers in a variety of different types of cancer cell lines in vitro and in vivo. Evidence suggests that the increase seen in viral titers is both virus and radiation dose-dependent in some but not all cell lines. The underlying mechanism of increased viral replication in the presence of XRT is widely hypothesized due to a radiation-mediated increase in cellular GADD43 expression (Figure 1). GADD34 is a DNA damage- and growth arrest-inducible gene that helps protect cells against genetic insults such as those caused by radiation. A region of the GADD34 protein shows significant structural homology to the HSV-1 y34.5 protein. In combination with other cellular proteins, dephosphorylates the cellular translational initiation factor eIF-2a leading to continued protein synthesis and cell survival. Deletion of y34.5 (a common modification in many oncolytic herpes viruses) significantly reduces virus-induced neurotoxicity; however, it also significantly attenuates viral replication and cytotoxicity in tumor cells. GADD34 also acts to prevent phosphorylation of eIF-2a. Therefore, radiation-mediated upregulation of cellular GADD43 can functionally replace y34.5 resulting in increased viral replication without the risk of neurovirulence. Exposure to XRT resulted in a 1.12- to 5.04-fold increase in cellular GADD43 mRNA by real-time reverse transcriptase PCR in human nonsmall cell lung cancer cell lines in vitro. Similar results were observed in human cholangiocarcinoma cells and confirmed by western blot in human malignant mesothelioma cells. In all cases, upregulation of GADD43 was associated with increased viral titers and improved cytotoxicity.

Many studies looking at combination adenovirus and XRT therapy have hypothesized that the radiation-mediated increase in oncolysis is due in part to an increase in viral replication rates. Unfortunately, little information about the underlying molecular mechanism has been uncovered. When compared to cells treated with virus alone, viral titers were significantly increased 24 hours postinfection in human prostate cancer cells treated with the prostate-specific adenoviruses CV787 (ref. 9) or CV706 (ref. 8) followed by XRT. Increased viral titers correlated with a synergistic cytotoxic effect in vitro. In vivo combination therapy significantly inhibited tumor growth relative to individual therapies. Histological analysis of prostate cancer xenografts found a significant decrease in the number of CD31+ cells (a marker of angiogenesis) and/or blood vessels in mice treated with combination CV706 (ref. 8) or CV787 (ref. 9) and XRT, relative to either therapy alone. Decreased blood flow to the tumor, resulting from combination therapy, likely contributed to the high levels of necrosis and eventual scar formation found at the tumor site. In another study, two out of three human glioblastoma cell lines supported increased viral replication and release when irradiated 6 hours prior to infection with ONYX-015 (ref. 7). However, in vivo there was no significant difference in ONYX-015 replication in tumors in the presence or absence of total body irradiation.

An increase in adenovirus uptake, due to upregulated CAR (coxsackie adenovirus receptor) and/or integrin expression levels following radiation exposure, has widely been postulated as one of the mechanisms that leads to increased viral titers and oncolysis but there are conflicting reports. Flow cytometry of human glioblastoma and malignant glioma cell lines 24 hours after exposure to radiation showed no significant increase in expression of
CAR and/or αβ, and αβ integrins. Conversely, CAR expression levels were found to be increased 24 and 48 hours following irradiation of a head and neck cancer and a colorectal cancer cell line. It has also been shown that a radiation-mediated increase in dynamin 2, a GTPase required for endocytosis of the virus, can act in a CAR-independent manner to increase viral uptake in colon, brain, breast, and pancreatic cell lines.

Depending on the mechanism of synergy, the timing and order of the treatment regime could have significant effects on treatment efficacy. If viral oncolysis is enhanced through radiation-induced changes in the host cell then it would be beneficial to deliver the virus after radiation therapy, at a time when such cellular changes are maximized. Conversely, if radiation is acting on the virus directly, therapy would be most effective when the virus is delivered prior to radiation. Evidence from a CV706 study suggests that in the long-term (8 weeks) the difference in tumor volume between groups receiving radiation 24 hours prior to or 24 hours after virus was negligible. However, a decreased antitumor effect was observed when radiotherapy was administered 7 days postinfection compared to 1 or 4 days postinfection. This supports the notion that the relative timing could strongly impact therapy efficacy.

Genetic modifications to enhance synergy. In order to increase the combinatorial effects of oncolytic virotherapy and radiotherapy, many viruses have been modified to include promoters that are activated by exposure to radiation or genes that sensitize cells to radiation-induced cell death. Nandi et al. tested the response of several mammalian promoters to XRT and found marked increases in survivin mRNA. When this promoter was used to drive adenovirus E1 expression, as with the CRAd-S-pk7 virus, combination with XRT in vivo significantly delayed glioma tumor growth at 6 days postinfection compared to either single treatment or a combination of XRT and wild-type adenovirus. Furthermore, viral titers in tumors 6 days postinfection were increased by 100-fold when combined with XRT. This strategy may prove useful in overcoming one of the major challenges facing current clinical usage of OVs: inadequate viral replication and spread.

In another attempt to improve the radiosensitivity of infected cells, the tumor-specific adenovirus, AdΔ24 (E1A-deleted), was modified to express the tumor suppressor gene p53 (ref. 4). Studies have shown that adenovirus-mediated cell lysis is more effective in cells that express p53 (ref. 25), however lack of functional p53 expression is common in malignancy. Also, introduction of functional p53 into p53-negative cells resulted in increased sensitivity to radiation-induced cell death. In vitro, AdΔ24-p53 was significantly more cytotoxic than AdΔ24 in human glioma cell lines and synergistic cytotoxicity was observed for both viruses when combined with XRT at viral doses as low as MOI 0.001 (ref. 4). Higher levels of apoptosis were observed in cells treated with AdΔ24-p53 plus XRT relative to those treated with XRT alone or in combination with AdΔ24. Considering that infection alone resulted in minimal levels of apoptosis, it is likely that the increased apoptosis is due to radiosensitization of AdΔ24-p53 infected cells. In immunocompromised s.c. glioma models, AdΔ24 + XRT and AdΔ24-p53 + XRT each resulted in 50% long-term survival which was significantly improved relative to either virus alone (11 and 22% for AdΔ24 and AdΔ24-p53, respectively).
OVs and targeted radionuclide therapy

Targeted radionuclide therapy is a treatment used in a subset of cancers that are known to over-express specific cell-surface receptors. Radiolabeled iodine is used to treat thyroid tumors which express the sodium iodide symporter (NIS), and radiolabeled somatostatin ligands are used in patients with somatostatin receptor-positive neuroendocrine tumors. Previously, targeted radiotherapy was limited to receptor-positive tumors. Today oncolytic virotherapy with viral-mediated delivery of specific receptors makes targeted radiotherapy possible, irrespective of the endogenous receptor status. With this approach, damaging radiation can be delivered specifically to infected cells (as well as neighboring cells) (Figure 2) thereby sparing the collateral damage typically sustained by normal cells during XRT.

A tumor-specific vaccinia virus (VV) expressing the human somatostatin receptor (vVDD-SSTR2) resulted in specific uptake of the radiolabeled somatostatin analogue $^{111}$In-pentetreotide in vivo.$^{35}$ $^{111}$In-pentetreotide delivered systemically to mice bearing s.c. colon cancer tumors localized specifically to the tumors of vVDD-SSTR2 but not vVDD-GFP treated mice.

Several OVs have been designed to encode the hNIS gene. Ad5-yCD/mutTK<sub>rep</sub>-hNIS is an oncolytic adenovirus vector that is currently being investigated in clinical trials.$^{36}$ In addition to hNIS, it expresses a highly efficient fusion protein of the catalytic domains of yeast cytosine deaminase (yCD) and herpes virus thymidine kinase (mutTK<sub>rep</sub>) that activate the prodrugs 5-fluorocytosine (5-FC) and ganciclovir (GCV), respectively. This virus was demonstrated to result in specific accumulation of $^{99m}$Tc in a canine model of spontaneous soft tissue sarcoma.$^{37}$ NanoSPECT/CT imaging was used to demonstrate dose-dependent tumor accumulation of $^{99m}$Tc in mice bearing s.c. colon cancer tumors treated with the hNIS-expressing oncolytic adenovirus AdAM6 (ref. 30). Accumulation was no longer detectable at 5–6 days postinfection, however it was not shown whether this was due to loss of functional NIS expression or loss of virus replication. Treatment of mice bearing s.c. and intraperitoneal ovarian cancers with a measles virus expressing NIS significantly decreased the tumor burden and increased survival, respectively, relative to saline treated controls.$^{38}$ Expression of NIS was used to image infected tumors by gamma camera imaging using $^{99m}$Tc. The efficacy of combination therapy was investigated in both s.c. and orthotopic models of multiple myeloma.$^{39}$ Combining an attenuated vesicular stomatitis virus (VSV) lacking the ability to block interferon (IFN) production and expressing NIS (VSV(Δ51)-NIS) with $^{131}$I radiotherapy prolonged survival relative to treatment with virus alone in a multiple myeloma model.$^{40}$ These studies are proof of principle that OVs encoding receptors result in tumor-specific accumulation of radiolabeled ligands. Further studies will be required to determine the efficacy of this combination therapy.

OVs and chemotherapy

Although many believe that OVs have the potential to be used as frontline therapies, immediate clinical applications will require that they are at least compatible with current chemotherapeutics. OVs have been investigated in combination with various standard chemotherapeutics that can be organized based on their mechanism of action. Some of the most common drugs fall into the categories of alkylating agents [cisplatin, cyclophosphamide (CPA), and mitomycin C (MMC)], DNA intercalators (doxorubicin), nucleotide analogues (5-fluorouracil (5-FU) and GCV), modifiers of the cellular cytoskeleton (paclitaxel and docetaxel) and cytostatic agents (rapamycin). Regardless of their mechanism of action, the effects of chemotherapy drugs are not specific to tumor cells but instead to all rapidly dividing cells. Consequently, chemotherapy is often associated with high levels of toxicity and significant side effects. OVs have a higher level of tumor-specificity relative to chemotherapy drugs due to both an innate preference for tumor cells and specificity-enhancing genetic modifications. Given that the antitumor effects of OVs and chemotherapy drugs are mediated by different pathways, many investigators have hypothesized that in combination they may act synergistically. Although this is not the case for all chemotherapy and virus combinations, many combinations do exert synergistic cytotoxicity, typically in a cell line-dependent manner.

Alkylating agents. CPA is a common chemotherapy drug used primarily for the treatment of lymphoma, chronic lymphocytic leukemia and breast, ovarian and bladder cancers. CPA is converted into its active metabolites, 4-hydroxyaclyophosphamide and aldophosphamide by liver oxidases. Only a small fraction of aldophosphamide is converted into the toxic metabolite phosphoramido mustard that causes DNA cross-linking leading to apoptosis. There have been two main strategies for combining
CPA with OVs. First, CPA is used as an immunosuppressant to enhance viral infectivity, replication and spread. The exact nature of the immunosuppressive effects of CPA leading to enhanced OV efficacy is not entirely clear however they have been shown to be both global (nonspecific) and in some cases virus-specific. Second, viruses engineered to encode cytochrome P450 (CYP2B1), which converts CPA to its active metabolites, are used to concentrate the toxic metabolites in virus-infected cells. Both strategies have proven to be very successful at enhancing the antitumor effects of OVs.

Use of CPA as an immunosuppressant to enhance viral oncolysis has improved virotherapy efficacy in combination with HSV-1,25-29 adenoviruses,30 measles virus,31 reovirus,32,33 and VV.34 Reovirus virotherapy of a melanoma lung metastasis model resulted in CR in 5/8 animals treated with combination therapy35 and survival was further enhanced with interleukin-2 treatment.36 In a syngeneic model of murine colon cancer, intratumoral injection of measles virus combined with CPA resulted in 100% survival at 90 days post-treatment and CR in 9/10 animals compared to 30% CR with either therapy individually.37

The immune-modulating effects of CPA are complex, affecting humoral and cellular mediators of both the innate and acquired immune responses. Initial infection, particularly for systemically delivered viruses, requires that the virus evade innate antiviral factors present in the serum. Serum complement and immunoglobulin M have been shown to significantly decrease the infectivity of the herpes virus hr3 (inactivated ribonucleotide reductase); whereas serum from animals treated with CPA show neutralizing antibody titers below the limit of detection and reduced inhibition of viral infection.38 Furthermore, in vivo depletion of complement significantly improved survival of HSV and CPA treated tumor-bearing rats.39 CPA was also reported to result in global immunosuppression, including significant decreases in total white blood cell, lymphocyte, neutrophil, and monocyte counts in tumor-bearing mice.40 This was accompanied by significantly improved survival and decreased tumor volume in mice treated with both adenovirus and CPA relative to treatment with either therapy alone. Numerous studies have shown that CPA significantly reduces virus-induced infiltration of immune cells into tumors. Infiltration by hematopoietic cells10-42 and macrophages42-45 as well as levels of phagocytosis46 were all decreased with CPA treatment relative to virus alone. The combined immunosuppressive effects of CPA correlated with increased viral transgene expression,40,42 replication10,46-48 and spread10 in a variety of tumor models. To further support immune modulation as a key mechanism by which CPA enhances oncolytic virus therapy, experiments performed in nonobese diabetes/severe combined immunodeficient mice (lacking functional T and B lymphocytes) or in vitro showed no effect of CPA on immune cell infiltrates, viral replication or viral transgene expression.42,43

Although high-dose CPA can cause widespread immune suppression in humans, administration of low-dose CPA (<100 mg/kg) to mice resulted in a significant reduction in regulatory T cell (Treg) frequency and function.49 In a tumor vaccine study, tumor cells infected ex vivo with an oncolytic adenovirus did not induce a significant antitumor immune response.50 Low-dose CPA significantly reduced the percent of splenic and tumor Tregs and resulted in a significant delay in tumor growth, prolonged survival and increased tumor-specific T-cell responses when combined with the infected tumor cell vaccine. Oncolytic reovirus in combination with low-dose CPA had numerous immune-modulating effects. CPA decreased the function of Tregs, induced natural killer cell expression of matrix metalloproteinase-2 when combined with interleukin-2, and significantly decreased the level of circulating neutralizing antibodies.51 Decreases in neutralizing antibodies as a result of CPA treatment have also been reported as early as 8 days and as late as 41 days post-treatment with herpes virus,52 adenovirus,53 measles virus,54 or reovirus.55 Combined, these effects are hypothesized to decrease immune sensitization to tumor cells, increase viral spread through tumors due to degradation of the extracellular matrix, and decrease antibody-mediated inhibition of viral infection.

One of the potential pitfalls of CPA-mediated immune suppression is that in addition to promoting tumor oncolysis, it may also lead to increased virus dissemination throughout the body. Immunocompetent hamsters with s.c. renal cell tumors treated with intratumoral adenovirus showed CPA-induced increases in blood and liver titers that ultimately led to viremia in several animals.56 CPA also induced the spread of HSV into normal brain tissue following treatment of an orthotopic glioma tumor.57 When combined with intravenous reovirus, CPA increased viral titers in the lung, blood, liver, spleen, intestine, brain, heart and bone marrow, and induced cardiac toxicity.58 Interestingly, when metronomic dosing was used (CPA given 1 day prior to virus with a total of three doses) the survival benefit of CPA was maintained whereas the virus titer in the heart and associated toxicity was significantly reduced.59 These data suggest that a balance must be achieved wherein the immune response is suppressed sufficiently to allow enhanced viral oncolysis but not to the point of widespread viral dissemination and toxicity.

Insertion of the CYP2B1 gene into the UL39 locus of herpes virus hr3 resulted in a virus (rPr450) which can convert CPA into its active metabolites. In the absence of viral replication, treatment of rPr450-infected glioma cells with GCV and CPA resulted in synergistic cytotoxicity.60 This is thought to be due to GCV-mediated inhibition of DNA repair following CPA-induced DNA damage. Furthermore, extracellular accumulation of the toxic CPA metabolites mediated bystander killing of uninfected colon cancer cells.61 Treatment of s.c. tumors with rPr450 combined with CPA significantly reduced tumor growth relative to virus alone62,63,64 and addition of GCV resulted in a further reduction in tumor volume.65

Cisplatin is another alkylating agent that binds and cross-links cellular DNA leading to apoptosis when DNA is not repaired. Cisplatin has been investigated in combination with oncolytic adenovirus,32,46 HSV,65-67 parvovirus68 VV69 and VSV.70 As with radiation therapy, viruses have been genetically modified in order maximize the combinatorial effects with cisplatin. Comparison of adenoviruses Ad-ΔE1B55 and Ad-ΔE1B19/55 showed that deletion of the E1B 19KD protein significantly increased cell susceptibility to cisplatin in vitro and in vivo.71 This is not surprising given that the E1B 19KD protein is a BCL-2-related apoptosis inhibitor homolog. Mice treated with Ad-ΔE1B19/55 and cisplatin showed 96% reduction in s.c. tumor volume relative...
to PBS treated mice and CR without recurrence at 6 months in two of six mice. Two other E1B 55KD-deleted adenoviruses, encoding activators of apoptosis (ZD55-TRAIL and ZD55-SMAC), also showed increased cytotoxicity in tumor cells when combined with cisplatin.\textsuperscript{5-14} The combination therapy showed dose-dependent cytotoxicity in normal cell lines; however it was not significantly greater than the drug-induced cytotoxicity. Comparison of several E3B-deleted adenoviruses in a panel of different tumor cell lines found that dl309 (E3B) and dl704 (E3Egp34kD) resulted in synergistic cytotoxicity in combination with cisplatin or paclitaxel in 4/7 cell lines and antagonistic effects in the remaining three cell lines.\textsuperscript{15-17} Replication of wild-type adenovirus or dl309 in tumors was significantly increased by cisplatin relative to virus alone in an immunocompetent model of nonsmall cell lung cancer but not in an immunocompromised model. Increased viral replication was accompanied by a synergistic reduction in tumor volume. Interestingly, an increase in \textit{in vitro} E1A protein expression was detected following combination treatment relative to virus alone. Previous studies have reported that upregulation of E1A may sensitize cells to cytotoxic drugs.\textsuperscript{18}

Three adenoviruses derived from the same backbone but encoding antisense cDNA for cell cycle regulating proteins (chk1, chk2, pkl-1), showed a significant increase in \textit{in vitro} apoptosis of tumor cells but not normal cells when combined with cisplatin, compared to the parental virus with cisplatin or either treatment alone.\textsuperscript{19,20,21} When combined with cisplatin \textit{in vivo} these viruses showed significantly improved tumor regression, reduction of metastases and increased survival relative to parental virus plus cisplatin or cisplatin alone in s.c. and orthotopic tumor models.

In an important controlled phase 2 clinical trial combining ONYX-015 with cisplatin and 5-FU for treatment of recurrent head and neck cancer patients, a clear benefit was observed in tumors receiving virus injections compared to those treated with chemotherapy alone.\textsuperscript{22} Objective (>50% reduction in tumor volume) responses in virus injected tumors occurred (>50% reduction in tumor volume) in 63% of patients (19 out of 30) including 8 complete responses and 11 partial responses. Furthermore, the time to progression in the virus injected tumors was significantly greater than that in the un.injected tumors.

The role of p53-status in cisplatin combination therapy was examined in a study using the wild-type H-1 parovirus.\textsuperscript{23} A human hepatocellular carcinoma cell line with wild-type p53 was transduced with a stable dominant-negative p53 mutant. Combination treatment was significantly better than individual therapies only in the p53-negative cell line. These findings are particularly interesting given the evidence that cisplatin induces apoptosis or cell cycle arrest through p53-dependent mechanisms.\textsuperscript{24} Therefore, this study suggests parovirus can sensitize p53-negative cells to the cytotoxic effects of these drugs.

In a comprehensive study of the interactions between the herpes virus NV1066 and cisplatin, moderate to strong synergy was observed in 7 out of 10 tumor cell lines tested.\textsuperscript{25} The mechanism of synergy between herpes virus and cisplatin may be similar to that with radiation. Cisplatin significantly increased \textit{in vitro} viral titers and resulted in a marked increase in GADD34 mRNA and protein expression. Inhibition of GADD34 using siRNA resulted in a loss of combination therapy cytotoxicity.\textsuperscript{26} Cisplatin has also been shown to improve VV oncolytic virotherapy.\textsuperscript{27} In a s.c. pancreatic tumor model, intravenous GLV-1hsh8 (F14.5L/J2R/A56R deleted, Lister strain vaccinia virus) combined with cisplatin resulted in faster growth inhibition, significant reduction in tumor volume and CR in seven of eight mice (compared to CR in one of eight virus alone-treated mice).

MMC is a DNA cross-linking antibiotic with antineoplastic properties. MMC and the p34.5-deleted HSV-1716 showed synergistic cytotoxicity in two of five nonsmall cell lung cancer cell lines and additive effects in the remaining three.\textsuperscript{28} Combined with NV1066 there was synergistic cytotoxicity in two human transitional cell carcinoma cell lines allowing for dose reductions of up to 10.4-fold and 156-fold for the virus and drug, respectively.\textsuperscript{29} MMC had no effect on viral replication in nonsmall cell lung cancer cells\textsuperscript{30} however it was shown to increase both GADD34 mRNA levels and viral titers in a human gastric cancer cell line.\textsuperscript{31} Similarly, temozolomide (TMZ), another DNA alkylating agent, was shown to synergistically enhance cytotoxicity when combined with G207 (p34.5-deleted HSV) in glioma cell lines.\textsuperscript{32} In this study, synergy was dependent on TMZ-induced upregulation of GADD34 and ribonuclease reductase, with significantly higher levels of virus found in GADD34 expressing cells. As with radiation and cisplatin, upregulation of GADD34 can functionally replace the deleted p34.5 causing increased cytotoxicity (Figure 1). To further support this mechanism, siRNA inhibition of GADD34 reduced viral titers and cytotoxic synergy in gastric cancer combined with MMC\textsuperscript{33} and glioma cells combined with TMZ.\textsuperscript{34} In vivo, combination herpes virus and MMC significantly improved therapeutic effects in models of gastric carcinomatosis\textsuperscript{34} and nonsmall cell lung cancer\textsuperscript{36} whereas combination with TMZ improved survival in immunosuppressed models of glioma.\textsuperscript{37}

Oncolytic viral therapy combined with TMZ for the treatment of glioma is particularly attractive as some viruses have been shown to downregulate DNA repair proteins, in particular O’-methylguanine DNA methyl transferase (MGMT) which is involved in glioma resistance to TMZ therapy.\textsuperscript{38} In human glioma cells, Ad-\textDelta 24RGD downregulated TMZ-induced MGMT expression and synergistically enhanced \textit{in vitro} cytotoxicity and \textit{in vivo} survival in glioma xenograft models.\textsuperscript{39} Similarly, inhibition of MGMT expression resulted in a loss of synergy with G207.\textsuperscript{40} TMZ has also been shown to significantly improve survival when combined with the adenovirus ICOVIR-5 in glioma xenografts.\textsuperscript{41}

DNA intercalating agents. Doxorubicin is an anthracycline antibiotic that intercalates into DNA and prevents the action of topoisomerase II. Doxorubicin was synergistically cytotoxic when combined with an oncolytic adenovirus in several osteosarcoma cell lines and one patient sample in a viral replication-independent mechanism.\textsuperscript{42} In cells where synergism was observed, a concomitant increase in G2/M phase arrest was also detected. Given that adenovirus infection is enhanced by an increased percentage of cells in G2 (ref. 77), this provides a possible mechanism through which synergy is achieved. Combination of adenovirus and doxorubicin resulted in synergistic \textit{in vitro} cytotoxicity\textsuperscript{43} and significantly reduced \textit{in vivo} tumor growth\textsuperscript{44} relative to either therapy alone in hepatocellular carcinoma models. ONYX-015 was successfully combined with MAP (MMC, doxorubicin, and cisplatin) chemotherapy in a phase 1–2 clinical trial for treatment
of advanced sarcomas. Therapy was well tolerated in all six patients and a partial response occurred in one patient.

Nucleotide analogues. GCV is a widely used antiviral agent originally developed for the treatment of cytomegalovirus infections. GCV is a guanosine analogue prodrug that upon phosphorylation by herpes virus thymidine kinase (TK) competes with cellular deoxyguanosine 5’- triphosphate for incorporation into DNA resulting in elongation termination. Viruses encoding the HSV TK gene lead to an accumulation of toxic GCV metabolites in tumor cells which interfere with cellular DNA synthesis leading to apoptosis. Early studies combining viruses with GCV focused on the use of viruses solely as vectors for gene therapy; therefore these vectors were predominately nonreplicating. There has been a shift toward using replicating oncolytic viruses as they have the potential to improve therapy both due to direct oncolysis and a more disseminated distribution of gene delivery. Targeted oncolytic HSVs in combination with GCV significantly improved survival in models of human ovarian cancer and rat gliosarcoma. Bystander killing of uninjected cells has been reported and is likely mediated by upregulation of gap junctions through which triphosphorylated GCV can travel. Adenoviruses and sindbis viruses, engineered to express the HSV TK gene, also show enhanced antitumor activity when combined with GCV. Intraperitoneal delivery of sindbis virus combined with GCV significantly reduced the peritoneal ovarian tumor burden compared to virus alone. In addition to its function as an enzyme for prodrug therapy, TK can also be a target for labeled fluoro-ethyl-18-arabinosyluridine, which can be detected using PET imaging. Tseng et al., used this noninvasive and clinically relevant approach to confirm tumor-specific localization of TK activity following treatment with a TK-expressing sindbis virus.

In addition to TK, Ad5-γCD/mutTKrep-ADP also encodes γCD which converts the prodrug 5-FC into 5-FU. Combination of this virus with radiation and dual prodrug therapy significantly improved the survival in a model of human pancreatic cancer. In a phase 1 trial for the treatment of prostate cancer, Ad5-γCD/mutTKrep-ADP in combination with radiation had limited toxicity and showed some therapeutic effect in intermediate risk patients. In contrast, Ad5/3-D24-TK-GFP which was highly cytotoxic in ovarian cancer cells, showed decreased viral replication in vitro and no improved therapeutic effect in vivo when combined with GCV.

CD/5-FC enzyme/prodrug therapy has also proven successful in combination with oncolytic virotherapy. 5-FU is a pyrimidine analogue that inhibits the synthesis of thymidine. The antitumor activity of two different VVs expressing CD was significantly enhanced when combined with 5-FC therapy in immunocompetent ovarian cancer and immunosuppressed colon cancer models. Interestingly, 5-FU also showed antiviral activity, and may also provide a safety mechanism for uncontrolled viral replication. M012, a recently described HSV expressing CD and designed for treatment of primary brain tumors showed little neurotoxicity and significantly inhibited growth of s.c. neuroblastoma tumors when combined with 5-FC.

Cytoskeleton modifiers. Taxanes are a class of chemotherapy drugs, including paclitaxel and docetaxel, which cause stabilization of cellular microtubules thereby preventing function of the cellular cytoskeleton, a requirement for mitosis. Combination of docetaxel or paclitaxel with an urothelium- or prostate-targeted adenovirus significantly reduced in vivo tumor volume and resulted in synergistic in vitro cytotoxicity. One effect of paclitaxel is an upregulation of TRAIL receptors which sensitize cells to TRAIL-mediated apoptosis. Pretreatment of tumor-bearing mice with paclitaxel and TRAIL prior to HSV injection significantly retarded tumor growth and increased viral spread in the tumors. Taxanes combined with other HSV-recombinants, demonstrated synergistic cytotoxicity in prostate cancer cells and significantly improved survival in a colon carcinomatosis model and lung cancer xenograft model relative to either therapy alone.

Cytostatic agents. Rapamycin (sirolimus) is an immunosuppressant commonly used in transplant patients, however it has also been shown to significantly enhance the oncolytic effects of the poxviruses myxoma and VV. Rapamycin inhibits the cellular serine/threonine kinase mTOR (mammalian target of rapamycin) which is critical to numerous pathways contributing to cell growth, proliferation, differentiation and survival. Myxoma virus infection is permissive in a limited range of cells, dependent on the cells endogenous activation levels the cellular serine/threonine kinase Akt. Cells with high levels of Akt (type I) are permissive to infection whereas cells with very low levels of Akt activation are not permissive to infection (type III). In cells with moderate levels of Akt (type II), myxoma virus-induced Akt phosphorylation through a mechanism dependent on the viral M-T5 protein. mTOR is a downstream mediator of Akt activation and although rapamycin alone decreased mTOR phosphorylation, it also resulted in increased Akt activation likely due to a positive feedback loop. When combined with myxoma virus, rapamycin-induced increases in Akt phosphorylation relative to virus alone correlated with increases in viral replication rates and cell-to-cell spread in type II cells. The in vitro mechanisms behind the increased activity of combination rapamycin and VV therapy are under investigation, however in vivo, rapamycin appears to influence the antiviral immune response with a decrease of tumor infiltrating natural killer cells. In preclinical models of medulloblastoma and melanoma, rapamycin has significantly improved virotherapy resulting in increased survival rates and decreased tumor burdens. The rapamycin analogue RAD001 has also been shown to significantly improve adenovirus virotherapy in models of glioblastoma. In vitro it was demonstrated that RAD001 (refs. 75,101,102) as well as temozolomide significantly increased virus-induced autophagy and resulted in synergistic cytotoxicity. The prototypical proteasome inhibitor MG-132 enhanced cellular CAR expression in Lovo colon carcinoma cells, which was accompanied with enhanced adenovirus target gene expression and oncolysis.

OVS COMBINED WTH BIOLOGIC THERAPY

Small molecules

Overcoming innate immune barriers to tumor infection. Mammals have evolved a variety of natural or innate barriers to rampant virus spread throughout the body and many of these
pose an impediment to virus infection of tumors, particularly when these have been purposely attenuated for the purpose of virotherapy. Of notable importance are barriers such as the cellular antiviral response (e.g., IFN signaling), the innate immune response (e.g., neutralizing antibodies, complement, scavenging macrophages), and physico-chemical barriers (e.g., blood flow, hypoxia/pH). Although many have tried to tackle these problems through viral engineering, others are finding success in combining both experimental and well established chemotherapeutics that target one or more of these barriers.

HDAC inhibitors and drugs that target the innate antiviral response. Histone deacetylase (HDAC) inhibitors (HDIs) have been explored for their use as anticancer drugs since the 1990s and have been recently approved for the treatment of lymphoma. Although their immediate targets are known (HDACs), HDI effects are pleiotropic because HDACs are prime controllers of transcriptional regulation. HDIs are known to both up- and downregulate a panoply of genes (up to 10% of the transcriptome), which typically leads to cell cycle arrest and apoptosis preferentially in cancer cells. HDIs have also been reported to have antiangiogenic and immuno-modulatory properties (reviewed in refs. 105-106).

Valproic acid, a low potency HDAC inhibitor was found to enhance CAR expression in cervix, breast, and bladder cancer cells in vitro and in ex vivo cervical cancer samples. This was accompanied by increased infection by adenoviral vectors.107 HDIs have been shown by several groups to suppress the innate cellular antiviral response, at least in part by downregulating IFN and the IFN-stimulated genes.108-111 This has led to the combination of HDIs with various OVs. Trichostatin A, a pan-HDAC inhibitor was found to modestly enhance HSV oncolysis in squamous cell carcinoma cells. In this study, it was proposed that the modest enhancement of HSV replication may be due to effects on viral replication induced by NF-kB activation and cell cycle inhibition.112 However, in a more recent study, valproic acid convincingly enhanced HSV oncolysis in human glioma cells, suggesting the effects on HSV replication may be cell or tissue specific.112 In another study, the antitumor effect of a telomerase-specific, replication-selective adenovirus (OBP-301) in human lung cancer cells was enhanced by the lesser known HDI FR901228 (ref. 113). Nguyen et al, have shown that several HDIs can synergize with the oncolytic VSV-Δ51, an attenuated oncolytic VSV-mutant which is incapable of blocking IFN production.109 Combination treatment with HDIs resulted in synergistic cell killing, likely due to both enhanced induction of cell death and increased viral output (typically over 100-fold). Enhanced spread of VV and semliki forest virus was also observed using HDIs. Perhaps most interestingly, the replication of VSV in SW620 colon carcinoma xenografts in vivo could be halted by interrupting treatment with HDIs and resumed once HDIs were resupplied. This brings forth the interesting possibility that HDIs can be used as molecular switches to control viral replication.109

Other drugs that may target the cellular antiviral response include Jun N-terminal kinase inhibitors. In one study, Jun N-terminal kinase-deficiency (genetic or induced by Jun N-terminal kinase inhibitors) could enhance oncolytic VV replication. This was suggested to occur by preventing the activation of double-stranded RNA-dependent protein kinase.114

Modulators of cell death and other oncolytic virus barriers. There are likely to be many barriers to oncolytic virotherapy, some of which have yet to be discovered. In parallel to the cellular antiviral responses and innate immune barriers, the ability of OVs to reach cancer cells and to kill them efficiently can likely be manipulated by small molecules. Tumilasci et al., were able to enhance the efficacy of oncolytic VSV against chronic lymphocytic leukemia cells by combination therapy with the BCL-2 inhibitor EM20-25 (ref. 115). Other less specific modulators of cell death have also been shown to enhance oncolysis. As mentioned previously, HDIs may function at least partially by increasing cell death induced by virus, although the details of how this occurs remain elusive.109 Apoptosis induced by measles virus was also enhanced by cotreatment with heat shock protein inhibitors in vitro. This was potentially mediated by the effects of HSP inhibitors on rhoA expression, important for measles-induced cell fusion.116 Some studies suggest that certain drug/OV combinations induce autophagic cell death and that intact autophagy pathways are required for the observed combined effect.117,118,119

With respect to using combination therapy to enhance virus spread to and within tumor sites, one group showed that a single dose of angiostatic cRGD peptide treatment before oncolytic virus treatment enhanced the antitumor efficacy of oncolytic HSV.119 These results are somewhat surprising, because restricted blood flow appeared to be beneficial to the virus, contrary to what might be expected. This was found to be associated with decreased tumor production of IFN-γ and decreased infiltration of immune cells within the tumor. It will be interesting to assess whether antiangiogenic drugs can enhance the oncolytic ability of other viruses such as VSV, that induce vascular shutdown120 instead of permeabilization as is observed for HSV. Finally, coadministration of various OVs with proteases such as hyaluronidase has been performed intratumorally in order to increase access of virus to tumors and enhance viral spread.121,122

Immunotherapies. The idea that OVs exert their effects not only directly through lysis of tumor cells but also through induction of an immune response is intriguing and has garnered much attention; both in understanding this immune response on its own and to use other forms of immune modulation to try to enhance it. It is likely that OVs induce some level of antitumoral immunity as a byproduct of oncolysis. As pathogens, viruses elicit toll-like receptor signaling through a variety of TLRs present on a variety of cells including antigen-presenting cells.123-125 In the course of replicating within the tumor, OVs generate pathogen-associated molecular patterns which are ligands for these receptors thus providing two of the major requirements for initiating and enhancing antitumor immune responses: they supply tumor antigens to local dendritic cells through the direct oncolysis of tumor cells, while providing the “danger signals” necessary to promote localized inflammation and dendritic cell activation.

There are numerous examples of OVs initiating antitumor immune responses. This effect has been best demonstrated in studies
using oncolytic HSVs where cured mice generally resist subsequent rechallenge with the same tumor cell line. Several reports from Toda et al. demonstrate the establishment of antitumor immunity in various rodent models using oncolytic HSV. The treatment of immunocompetent mice bearing CT26.x.c. tumors, or orthotopic colon cancer with liver metastases, using the conditionally replicating HSV G207 virus led to regression of the primary injected tumors, and of metastases or contralateral untreated tumors. Importantly, this effect was absent in athymic nude mice, arguing in favor of the induction of antitumoral immunity following viral oncolysis. In an ovarian carcinoma model, Benencia et al. reported the induction of antitumor immune responses postoncolytic viral therapy through the induction of proinflammatory signals and tumor antigen presentation and similar effects were reported following treatment of melanoma using H-1 parvovirus. This enhancement of antitumoral immunity has also been demonstrated in a phase 1 dose-escalation clinical trial using oncolytic measles virus in cutaneous T-cell lymphomas where biopsies displayed heightened IFN-γ mRNA in infiltrating CD4+ and CD8+ T lymphocytes and an overall expansion of the CD8+ T-cell population. Overall, the process by which viral oncolysis leads to induced or enhanced antitumoral immunity is poorly understood and has not really been studied in detail.

Many groups are actively exploring strategies to enhance or directly generate antitumoral immunity with OV. One promising approach is through the use of fusogenic OVs. The formation of multinucleated syncytia following infection is a property of certain viruses, including some being developed as oncolytic such as measles virus. Viruses that are not naturally fusogenic can be engineered to have this property. The fusion of tumor cells within a solid tumor appears to generate two desirable effects, one being the enhanced spread of the virus through the tumor and increased oncolysis along with an increased ability to generate an antitumoral immune response. The syncytial, oncolytic FusOn-H2 HSV-2 induced strong T-cell responses against primary and metastatic 4T1 breast tumors in immune-competent mice while adoptive transfer of splenocytes from the treated mice to naive mice prevented metastasis of 4T1 in the recipients. Additional studies using this oncolytic herpes virus have demonstrated antitumor immune responses following treatment of both neuroblastoma and colon cancer. The induction of antitumor immunity by fusogenic OVs has been suggested to be the result of killing large numbers of tumor cells by nonapoptotic means leading to the production of a mass of inflammatory tumor tissue.

Another approach to enhancing the immunogenicity of OVs has been to engineer them to express various immunostimulatory cytokines. Many candidate cytokines and immunomodulatory factors have been tested in this setting and have shown promise in preclinical testing. As many of the vectors being used are likely to induce some level of proinflammatory immune mediators, it is likely that most of these strategies can only lead to enhanced production of particular mediators. It is also very difficult to predict and control the amount of cytokine produced and there is a theoretical risk of excessive cytokine production if a tumor is heavily infected. The potential immunostimulatory benefits of producing proinflammatory cytokines in the tumor microenvironment justifies this approach.

An emerging strategy for the combination of viral oncolysis with immunotherapy consists of enhancing tumor killing through adoptive cellular therapy. Adoptive transfer of tumor-specific CD8+ T cells into naive recipients showed some efficacy in the B16/OVA melanoma model, and this was heightened by further intratumoral treatment with VSV, providing the necessary local tumor inflammation to recruit and maintain activation of adoptively transferred T cells. One variation on this approach has been to transfer dendritic cells during oncolysis to encourage presentation of the unidentified tumor antigens provided by the viral-mediated destruction of tumor. An alternative approach is to load an oncolytic virus into or onto tumor-specific lymphocytes and use these cells to enhance targeting of the virus to the tumor while potentially providing additional immune-mediated tumor destruction along with viral oncolysis. This is a complex arena as the cells harboring virus may be cytotoxic themselves (i.e., cytokine-induced killer cells) or act to target the virus to tumor or shield it from neutralizing antibodies. This has been recently extensively reviewed by Willmon et al. The partnering of oncolytic viral therapy with adoptive transfer of immune cells holds significant potential, as viral oncolysis may be able to enhance and drive the occasionally efficacious effects of cellular therapies. By feeding transferred antigen-presenting cells and/or enhancing viral delivery while attracting tumor-specific effector cells into the tumor, OVs may partner very well with cellular therapies in the clinic, an area that has not yet been explored to any extent.

Other viruses

It has long been proposed that OVs should be used as combination agents with other viruses. In the setting where an immune response to one virus develops, an appealing idea is to use a second virus to continue the therapy. From a practical point of view, this is far from the clinic as obtaining regulatory approval for two separate experimental modalities will be a challenge. But the challenge may indeed be worth it: a recent article out of the Bell lab (F. Le Beouf, J.-S. Diallo, J.A. McCart, S. Thornton, T. Falls, M. Stanford et al., manuscript submitted.) has shown that the powerful immune evasion genes of VV render cells otherwise resistant to killing by VSV, uniquely sensitive when VSV is used as the second agent. In this case VV is acting as a biologic therapy to suppress the innate antiviral immune system and permit VSV to infect and kill the cells. This is an excellent example of the synergy that can be seen when combining modalities that utilize different mechanisms to kill cells and should guide future design of combination therapies.

CHALLENGES TO COMBINATION THERAPIES

The use of relevant animal tumor models is a concern for all investigators, however it is of particular importance for those studying OVs. Recent studies that have focused on elucidating the role of the immune system in OV therapy found that both the innate and adaptive immune responses contribute significantly to overall efficacy of the OV. Furthermore, antiviral immune responses are both implicated in hindering OV efficacy by inhibiting early infection and required for eventual clearance of the virus. Many of the studies discussed in this review were performed in xenograft models using immunocompromised mice. This is appealing
because it allows testing in human tumors (as opposed to murine tumors) however this comes at a significant cost. Adding to the limitation of these models is the widespread use of tumor cell lines and the scarcity of data using fresh human tumor samples. Although use of human tumors in xenograft models is useful, it is perhaps more clinically relevant to demonstrate OV efficacy in immunocompetent hosts. It will be important to consider these factors when choosing the best combination therapy regimens to develop clinically.

DI SCUSSION AND FUTURE DIRECTIONS

As we have discussed, as OVs move toward clinical use, it is likely that in order to be successful, some type of combination therapy will need to be employed. Rather than simply combining existing modalities, we have attempted to show how certain combinations make the most sense and have a high likelihood of acting in a synergistic manner. Although initially we expect that OVs will be combined with standard validated therapeutic agents (i.e., chemotherapy and radiotherapy), overall we anticipate that novel combinations with other biologics (including other OVs) or immunotherapies will yield better results. It is critical to understand the interplay between the virus and the combination of choice. As we have described earlier, some chemotherapy and radiotherapy modalities may inhibit viral replication, and as such could be used to improve the safety of the virus rather than influencing its efficacy. Studies using OVs are now demonstrating an immune-mediated component to successful therapy in addition to direct oncolysis. Importantly, the induction of an antitumor immune response during oncolysis has the potential to provide prolonged tumor control long after the oncolytic virus has been cleared. Future studies will need to further elucidate the role that the immune system is playing in OV therapy and disease-specific combinations will need to be defined.

The oncotherapy is on to move these novel therapeutics to the clinic. Although combining OVs with the current standards of care may be appealing both from a cost-effective point of view (no need to "develop" the second arm of the therapy) and from a regulatory point of view (getting two novel therapeutics approved for one trial), we hope that this review will challenge the reader to pause and consider whether what he/she is proposing to take to the clinic is rational and will provide the best possible outcome for the patients.

REFERENCES


Combination Oncovirus Therapy

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Appendix 1.2. Mounting a strategic offense: fighting tumour vasculature with oncolytic viruses

Mounting a strategic offense: fighting tumour vasculature with oncolytic viruses

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Blood supply within a tumor drives progression and ultimately allows for metastasis. Many anticancer therapies target tumor vasculature, but their individual effectiveness is limited because they induce indirect cell death. Agents that disrupt nascent and/or established tumor vasculature while simultaneously killing cancer cells would certainly have a greater impact. Oncolytic virotherapy utilizes attenuated viruses that replicate specifically within a tumor. They induce cytotoxicity through a combination of direct cell lysis, antitumor immune stimulation, and recently identified antitumor vascular effects. This review summarizes the novel preclinical and clinical evidence regarding the antitumor vascular effects of oncolytic viruses, which include infection and lysis of tumor endothelial cells, natural or genetically engineered antiangiogenic properties, and combination therapy with clinically approved antiangiogenic agents.

Targeting tumor vasculature

During tumorigenesis, cancer cells initially obtain nutrients and oxygen from neighboring normal tissue [1], but as tumors reach ~1–2 mm in diameter deficits in supply quickly arise. Subsequent clonal evolution selects for cancer cell subpopulations that resist apoptosis [2] and elicit the ‘angiogenic switch’, the pivotal moment when tumors become capable of inducing angiogenesis [1,3] (see Glossary and Box 1). Interestingly, tumors have also developed additional mechanisms, namely, vessel cooption, vasculosuppression, in-tussusception, and vasculogenic mimicry, by which they ensure a blood supply. In essence, any means of establishing blood supply within the tumor stroma will drive its progression and eventually allow metastasis. Tumor vasculature is therapeutically eradicated by two means: (i) antiangiogenesis, a process in which key components necessary for new vessel development are inhibited [4–6]; and (ii) vascular disruption, whereby established blood vessels are specifically targeted and destroyed [4,7,8] (Box 2). A limited number of agents are in clinical use, and they are far from being optimal. Limited effectiveness is multifactorial: (i) cancer cell death occurs through indirect mechanisms of action such as inducing cytostasis and ischemia or by enhancing the delivery of other directly cytotoxic agents [9]; (ii) adaptive and intrinsic mechanisms of drug resistance, which include, but are not limited to, revascularization by upregulating alternative angiogenic signals and stimulation of metastasis and cooption [10]; and (iii) limited penetration and distribution of molecules into tumors [9–13]. Certainly, a therapeutic capable of preventing and eradicating tumor vasculature while simultaneously and directly killing cancer cells would have greater antitumor effects. It would be of additional benefit if this therapeutic could act synergistically when used in combinatorial strategies.

Oncolytic viruses (OVs) demonstrate tumor-specific replication and cytotoxicity. Specificity results from innate or genetically engineered properties that take advantage of the impaired antiviral defense mechanisms within the tumor [14]. In the past two decades, OVs have shown consistent antitumor efficacy in a wide range of preclinical tumor models, leading to their clinical investigation [15–19]. Understanding the mechanisms by which OVs cause cancer cell death has garnered significant interest, and the recently identified antiangiogenic properties of OVs, which inhibit tumor perfusion and progression, have been the topic of multiple preclinical and clinical studies (Figure 1). OVs can infect both developing and established tumor vasculature without harming normal vasculature. The exact mechanisms that govern this tropism are still under investigation; however, it has been hypothesized that this specificity may be partly attributed to the ‘proviral’ state established in endothelial cells (ECs) by vascular endothelial growth factor (VEGF), similar to that observed in Ras-activated cancer cells [20].
Glossary

Adenovirus: non-enveloped, double-stranded DNA virus (36–38 kb) that typically causes mild upper respiratory infections in humans. Oncolytic agents are primarily based on serotype 5 of species C. Pre-existing immunity may limit systemic delivery.

Anogenital: development of a new network of blood vessels within the stroma of a tissue from established vasculature. The degree of blood vessel maturation depends on whether the process is physiological (e.g., fetal development, wound healing, or menstrual cycle) or pathological (e.g., tumor).

See Box 1. Angiostatin: circulating peptide fragments of plasminogen that bind to ATP synthase and abrogates on the surface of endothelial cells. It stimulates endothelial cell apoptosis by inhibiting ATP synthesis and disrupting the functioning of focal adhesion kinase and sodium protein antipporter.

Antivascular therapy: treatment modality that aims to eradicate pathological tissue by targeting its vasculature to impair delivery of oxygen and nutrients. It can be further classified based on the mechanism: (I) Inhibition of new blood vessel development (angiogenesis) or (II) destruction of well-established vasculature (vascular disruption).

Armed virus: oncolytic virus that has been genetically engineered to carry transgenes that enhance its cancer cell death properties beyond selective oncolysis (e.g., disrupting tumor vasculature or activating prodrug).

Avastatin: angiogenic fragment from the a 1 chain of type IV collagen, which inhibits endothelial cell proliferation and migration through apoptosis by activating mitotic pathways.

Bevacizumab: humanized monoclonal antibody with specificity for vascular endothelial growth factor VEGF-A.

Canstatin: a human basement membrane-derived collagen type IV a1 chain that inhibits human endothelial cell migration, murine endothelial cell tube formation, and induces endothelial cell apoptosis.

Chicken embryo chondrocyte matrix (CAM): assay 1: a highly simple, and inexpensive non-mammalian system for studying angiogenesis. A membrane or collagen containing the compound or drug of interest is implanted on the chicken embryo CAM through a hole cut in the eggshell. After ~1–3 days of incubation, angiogenesis is quantified through image analysis or colorimetric detection methods.

Cluster of differentiation 31 (CD31): cell adhesion molecule found on monocytes, platelets, and granulocytes. Because it is also constitutively and highly expressed on the surface of endothelial cells, it is used as an immunohistological marker for the presence of these cells.

Cluster of differentiation 34 (CD34): a type I transmembrane glycoprotein that is expressed on the luminal cell surface and cell-cell junctions of vascular endothelial cells, except in large blood vessels and lymphatic vessels. It is used as an immunohistological marker of endothelial cells.

Co-option: process by which cancer cells form cues that wrap around normal vascular vessels. Eventually these endothelial cells release angiogenesis-2, destabilizing and collapsing the blood vessel itself. In turn hypoxia and nutrient deprivation ensue, thereby stimulating ‘secondary’ angiogenesis.

Cyclooxygenase (COX): enzymes that convert arachidonic acid to prostaglandins and thromboxanes and are present on the cell surface of endothelial cells, platelets, and smooth muscle cells.

EIA protein: protein encoded in the EIA genome of adenoviruses that positively regulates the transcription of early genes. This protein binds and inactivates ribonuclease protein (gnb) to stimulate the cell to progress into S phase in which virus replication can thrive.

Endostatin: a recombinant proteolytic fragment of the C-terminal end of human type XVIII collagen with broad spectrum antiangiogenic properties, such as inducing microvascular endothelial cell apoptosis, inhibiting endothelial cell proliferation and angiogenesis, and interacting with several endothelial cell surface angiogenic receptors (e.g., vascular cell adhesion molecule [VCAM]) by reducing their expression.

Epidermal growth factor (EGF): a family of polypeptide growth factors with a wide variety of biological effects, such as promoting the proliferation and differentiation of many epithelial, and endothelial cells.

Epidermal growth factor receptor (EGFR): a family of cell surface receptors specific for EGF and its related peptides that acts through tyrosine kinase activity.

Erlotinib: a small molecule tyrosine kinase inhibitor that acts on the intracellular domain of EGFR.

Eversin: a homologous envelope of the mammalian extracellular matrix. This small molecule inhibits the mechanistic target of rapamycin (mTOR), previously known as mammalian target of rapamycin. Angiogenesis is inhibited because it decreases the production of VEGF and inhibits vascular endothelial cell response to VEGF.

Fibroblast growth factor receptor (FGFR): a family of small polypeptide growth factors involved in the control of a variety of biological functions including the mitogenesis of various types of cells (e.g., endothelial cells, chemokines, neuronal survival, neurite extension, mesoderm induction, angiogenesis, wound healing, and embryonic development.

Fibroblast growth factor receptor (FGFR): a family of high-affinity cell surface receptors that bind with FGF and its related peptides to internally relay cellular signaling generally through tyrosine kinase activity. Granulocyte macrophage colony-stimulating factor (GM-CSF): a glycoprotein that stimulates proliferation, differentiation, and maturation of granulocytes and monocytes. It inhibits angiogenesis by stimulating transforming growth factor-β to secrete VEGFR-1, which in turn inhibits tumor macrophage VEGF activity.

Heparin: a complex glycosaminoglycan that, when co-infected into cells, triggers a replication-defective virus to copy itself by providing the missing genes and/or enzymes necessary for replication. Heparin simple virus (HSV): enveloped double-stranded DNA virus (120–200 kb) typically known for its neurotropism. Its large genome can carry multiple therapeutic transgenes.

Hypoxia-inducible factor 1 (HIF-1): heterodimeric transcription factor comprised of two subunits (HIF-1α and HIF-1β). Under hypoxia, normal cells express HIF to upregulate angiogenesis through VEGF gene activation.

Interferon gamma (IFN-g): a cytokine produced by mitogenically or antiangiogenically stimulated lymphocytes, in order to regulate the development, of innate and adaptive immunity against pathogens. It acts on endothelial cells, tumor fibroblasts, and macrophages to increase secretion of the angiogenic proteins IP-10 and MIG.

Interferon g-induced protein-10 (IP-10): chemokine secreted by several types of cells (e.g., endothelial cells, fibroblasts, and monocytes) in response to IFN-g. It inhibits endothelial cell migration and induces involution of new blood vessels by triggering endothelial cell anaplasia.

Interleukin (IL): a group of soluble factors that stimulate growth-related activities, cell proliferation and differentiation, and DNA synthesis and secretion of other bioactive molecules in leukocytes and other types of cells. IL-8 is an angiogenic cytokine that enhances endothelial survival and proliferation. IL-8 directly inhibits FGF-induced endothelial cell proliferation in an IFN-γ-independent manner.

Intussusception: process by which tumors stimulate the insertion of intestinal tissue columns into the lumens of pre-existing blood vessels (mother blood vessel). The lumens is portioned to create smaller ‘daughter’ blood vessels. Mean vessel density: surrogate marker that directly reflects the concentration of tumor angiogenesis. The standard method involves manual or software-based quantitation of intratumoral blood vessels.

Measles virus (MV): enveloped, single-stranded negative-sense RNA virus (15–19 kb) that typically produces a potentially serious infection of the respiratory tract in humans. Genetically attenuated strains are used as oncolytic agents.

Monokine induced by interferon-γ (MIG): a chemokine produced by monocytes/macrophages and other cells, including endothelial cells, after stimulation with IFN-γ. It inhibits endothelial cell chemotaxis and differentiation.

Oncolysin: cancer cell destruction through disruption of the cell membrane by the production of a pore (cytolytic hole) and possibly from the accumulation of cytotoxic viral proteins and virions within the host cell.

Oncolysin cytokine (OV): a virus that preferentially infects, replicates within, and destroys cancer cells while producing minimal or no damage to normal tissue. These agents are often attenuated compared with the parental virus.

p53: protein encoded by the TP53 gene, which controls cell proliferation and apoptosis. It also upregulates antiangiogenic molecules (e.g., thrombospondin-1 (TSP-1), brain-specific angiogenesis inhibitor 1 (B1A), ephrin receptor A2 (EPHA2), and proteolytic fragments of antiangiogenic collagens), downregulates angiogenic molecules (e.g., VEGF and FGF), and inhibits hypoxia-sensing systems (e.g., HIF-1).

p300: cellular transcriptional co-activator protein that is important in cell proliferation and differentiation. It also acts as a co-activator of HIF-1α, thereby playing a significant role in stimulating hypoxia-induced genes such as VEGF.

Photodynamic therapy (PDT): a clinically approved and minimally invasive therapy that uses a nontoxic, light-sensitive compound (photosensitizer) that is readily absorbed by abnormal cells. When exposed to a specific wavelength of light, the photosensitizer is activated to produce changes in endothelial cell integrity that ultimately produce vascular disruption.

Plasminogen: precursor of plasmin (fibrinolysin), an active serine protease that digests the fibrin of blood clots. It contains five kringle domains of which the fifth kringle domain induces apoptosis of proliferating endothelial cells and inhibits endothelial cell migration.

Platelet factor 4 (PF4): a chemokine released from the a-granules of platelets. In addition to being involved in platelet aggregation, it inhibits the interaction of FGF and VEGF with their cell surface receptors.

Sorafenib: a small molecule that inhibits cancer cell division and proliferation by blocking the enzyme RAF kinase (a critical component of the RAF/MEK/ERK signaling pathway) and tumor angiogenesis by inhibiting VEGF receptors.

Suramin: a small molecule receptor tyrosine kinase inhibitor that targets several receptors including VEGF.
Thrombospondin (TSP): family of five multifunctional glycoproteins involved in cell adhesion, platelet aggregation, cell proliferation, metastasis, vascular smooth muscle growth, tissue repair, and angiogenesis. It inhibits tumor angiogenesis by binding to the transmembrane receptor CD36 and inducing apoptosis in endothelial cells.

TNF-related apoptosis-inducing ligand (TRAIL): member of the tumor necrosis factor cytokine family capable of inducing cancer cell apoptosis, while causing minimal damage to normal cells.

Trichostatin A (TSA): small molecule histone deacetylase inhibitor that alters VEGF signaling.

Tumor angiogenesis: unregulated development of new, morphologically irregular, and semi-functional blood vessels within the stroma of a tumor. This process is particularly critical for tumors to initiate and sustain growth as well as metastasize.

Unarmed virus: OV that produces cancer cell death without genes overly incorporated to enhance its mechanism of action.

Vaccinia virus (VV): enveloped double-stranded DNA (130-280 kb) pox virus that was used as the vaccine against smallpox.

Vascular endothelial growth factors (VEGFs): family of glycoproteins that stimulates the growth, proliferation, differentiation, and migration of vascular and lymphatic endothelial cells, vascular permeability, and vasodilation via nitric oxide-dependent pathways.

Vascular endothelial growth factor receptors (VEGFRs): a family of target molecules for VEGFs that are highly expressed on the surface of endothelial cells and act via tyrosine kinase.

Vascular permeability: process by which tumors stimulate the participation of circulating stem cell differentiated endothelial precursor cells (angioblasts) deriving from bone marrow or peripheral blood cells to create blood vessels de novo.

Vasculogenic mimicry: process by which cancer cells with multipotent stem-like properties differentiate into endothelial-like cells capable of lining blood vessels.

Vascularization: extracellular fragment of brain specific angiogenesis inhibitor 1 (BAI1) that has potent angiogenic properties by antagonizing the activation of α,β family of integrins.

Vascular stromatitis virus (VSV): enveloped, negative single-stranded RNA virus (13-16 kb) that targets cancer cells with defective IFN response pathways.

It is rarely associated with human disease.

von Willebrand factor (VWF): high-molecular weight plasma glycoprotein produced by endothelial cells and megakaryocytes that mediates platelet adhesion. It also regulates angiogenesis through multiple crosslinking pathways involving α,β median endothelial growth factor receptor 2 (VEGFR-2) signaling, and Ang-2. It is used as an immunohistological marker to demonstrate the presence of endothelial cells.

Antivascular properties have been studied primarily in genetically attenuated OVs, with viral gene deletions aimed at increasing tumor specificity and decreasing toxicity. Some ‘unarmed’ viruses have no specific modification for destroying tumor vasculature and can thus be thought of as ‘innately’ antivascular. Additionally, ‘armed’ OVs have been created that encode antivascular molecules, either to downregulate angiogenic proteins and cytokines or to deliver antiangiogenic factors.

This review summarizes the body of novel evidence that describes the antiangiogenic and vascular disrupting properties of OVs. Both innately antivascular OVs and those that have been ‘armed’ with antivascular agents are discussed. Studies of combination OV therapy with clinically approved antivascular agents are also reviewed. We highlight the emerging clinical evidence on the impact of OVs in patients with highly vascularized tumors, and finally, we discuss several key issues to be considered in order to further elucidate the role of OVs in antivascular therapy.

Innate antivascular properties of oncolytic viruses: Current evidence suggests that there are three inherent mechanisms by which OVs affect tumor vasculature: (i) direct infection of tumor ECs; (ii) induction of virus-mediated immune responses that cause cellular accumulation and decreased tumor perfusion; and (iii) the expression of viral proteins with antiangiogenic properties (Table 1).

Box 1. Tumor angiogenesis

Angiogenesis is a multistep process governed by endogenous stimulators such as VEGF and FGF (II) and inhibitors (e.g., peptides, hormone metabolites, and apoptosis modulators) [90]. Constant feedback, also known as ‘dynamic reciprocity’, between ECs and the extracellular matrix guarantees that proangiogenic and antiangiogenic molecules favor a state of rest. Under both physiological (e.g., wound healing, reproduction, and organogenesis) or pathological (e.g., cancer, ocular diseases, and psoriasis) settings, tissues leave this ‘dormant’ state to meet their growing metabolic needs by inducing angiogenesis [90].

Tumors use the same endogenous angiogenic modulators as normal tissues, but their exponential growth pattern and deregulated balance between stimulators and inhibitors results in an exceptionally rapid proliferation and migration of blood vessel components [90]. Additionally, many cancer cells are programmed to persistently stimulate angiogenesis. The human tumor suppressor p53, which is mutated in approximately 50% of tumors, has significant antiangiogenic properties [91,92]. In the end, tumors give rise to a poorly distributed and dysfunctional vascular network composed of blood vessels with minimal wall stability, irregular branching, abrupt changes in diameter, blind ends, leaky sprouts, and vascular compression [1]. Despite the fact that these abnormal features substantially contribute to the failure of many standard cytotoxic treatment regimens, tumor vasculature per se is an important target for novel anticancer therapies [1].

These mechanisms are not necessarily mutually exclusive given that direct EC infection may lead to the stimulation of a strong immune response, which is an expected reaction to vascular injury [21]. In the following sections, we summarize several viral classes being investigated for their inherent antivascular properties.

Adenovirus: Conditionally replicating adenoviruses are considered truly antiangiogenic because the E1A protein [22,23] interacts with angiogenic cellular proteins such as p300 to downregulate VEGF. An oncolytic adenovirus expressing a mutant E1A, lacking its p300 binding site, is significantly impaired in its ability to inhibit VEGF, expression in pancreatic cancer cells and leads to increased tumor mean vessel density when compared with the same adenovirus expressing wild type E1A [22]. OBP-301, which has intact E1 genes under the control of a human telomerase reverse transcriptase promoter (hTERT), reduced CD31-positive tumor EC staining in subcutaneous colon cancer tumors in an immunocompetent murine model [24] (Figure 1). A replication-deficient adenovirus shows minimal infiltration and no changes in CD31 staining, suggesting that EC loss is virus replication-dependent. Reduced CD31 staining was not observed in the equivalent immunosuppressed tumor model treated with OBP-301. When immunocompetent mice are depleted of their interferon-γ (IFN-γ) response prior to and during virotherapy, the reduction in mean vessel density is partially abrogated relative to virus alone, suggesting the importance of the immune system in the antivascular response [24].

Vaccinia virus (VV): Oncolytic VV specifically infects CD31-positive tumor ECs as early as 24 h after intravenous delivery leading to a loss of vascular density at 48 h in tumors but not normal tissues [25]. Oncolytic VV (double-deleted VV (vvDD)) colocalizes
Box 2. Principles of antivascular therapy

Tumor vasculature can be eradicated by two different strategies – antiangiogenic and vascular disruption – which differ with respect to (i) mechanism of action, (ii) type of cancer cell death induced, (iii) type of susceptible tissue, and (iv) duration of administration required for efficacy (Figure 1).

Antiangiogenesis, the process by which key components of de novo blood vessel formation are inhibited, is achieved by antagonizing the angiogenic signaling pathways between tumor, endothelial, and stromal cells or by impeding EC proliferation, differentiation, and migration [93–98] (Figure 1A). By preventing the delivery of oxygen and nutrients early on, these agents induce cancer cell cytostasis, which not only prevents tumor progression but also limits metastatic potential [6,99–101]. Vasculature function is also normalized because remodeling restores both the interstitial pressure and thickness of the basal membrane; consequently, the intravascular delivery of chemotherapeutic agents can be optimized [102]. Because tumors initially develop vasculature at the periphery, antiangiogenic agents are more likely to succeed if administered in the case of early, small stage, or asymptomatic metastatic disease [103] (Figure 1A). In practice, this type of therapy requires chronic administration as tumors persistently stimulate angiogenesis [4].

Alternatively, vascular disruption selectively destroys well-established tumor vasculature by targeting histological characteristics, such as the presence of certain cytoskeletal and surface membrane proteins, that differentiate it from its normal counterpart [7,104] (Figure 1B). Agents that disrupt vasculature cause extensive cancer cell ischemic necrosis [7,105] by inducing EC apoptosis with subsequent platelet aggregation and vasoconstriction or by directly inhibiting tumor blood flow. Following administration, tumor growth and metastatic potential are hindered, possibly to a greater extent than with antiangiogenic agents [4]. Tumor blood vessels are more established in the core; therefore, vascular disrupting agents appear to be ideal for advanced, large tumors (Figure 1B). Despite treatment, a thin peripheral layer of viable cancer cells remains unharmed as nearby normal tissue and blood vessels supplement oxygen and nutrients [7]. Given that the effects of these agents are immediate, treatment is usually acute [4].

Figure 1. Antivascular therapy of cancer. Examples of antiangiogenesis (A) and vascular disruption (B). Any given agent may possess either property, or a combination of both. (A) In this panel, the global antitumor effects of several compounds are illustrated, including antiangiogenic drugs (e.g., bevacizumab, erlotinib, sorafenib, and TSA) as well as endogenous factors with antiangiogenic properties, such as endostatin, canstatin, angiostatin, TF4, amantadase, TSP-1, IFN, and IL-11. The endogenous factors are of particular interest in CV therapy as they have been used as therapeutic transgenes in many armed viruses. These cytokines mediate their antiangiogenic effect through both IFN-g-dependent (e.g., IL-12, IL-24, IL-18, IFN-10, and MIG) and independent (e.g., GM-CSF, IL-17, and IFN-4) mechanisms. Inhibition of new vessel formation by antiangiogenic agents results in cytostasis at the leading edge of the tumor but leaves a viable tumor core. (B) Examples of vascular disrupting therapy include integrin-binding agents (e.g., CRGDF peptide) and PDT. Vascular disruption results in a large necrotic tumor core with a minimal residual viable rim. Abbreviations: ATP, adenosine triphosphate; CD36, cluster of differentiation 36; CRGDF, cyclic Arg-Gly-Asp peptide; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGF receptor; GM-CSF, granulocyte-monocyte colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon-g-induced protein-10; MIG, monokine induced by interferon-g; mTOR, mechanistic target of rapamycin (previously known as mammalian target of rapamycin); PDT, photodynamic therapy; PF4, platelet factor 4; sVEGFR, soluble vascular endothelial growth factor receptor; TSA, trichostatin A; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
Figure 1. Antivascular and endothelial cell-targeting properties of oncolytic viruses. (A) Indirect inhibition of cancer cell-mediated in vivo angiogenesis by oncolytic adenovirus (AdV-101) [24]. Representative images of human colorectal carcinoma cell line SW620-induced angiogenesis in the absence of treatment (top) and in the presence of supernatant from AdV-101-infected cells show the indirect (interferon-mediated) antiangiogenic effect of the virus. Curved microvessels are shown (white arrows). Adapted with permission from The Journal of Immunology 182(3):1763-1769 [24], copyright 2009, The American Association of Immunologists, Inc. (B) Oncolytic vaccinia virus (vOC2) infects tumor vasculature in C57Bl/6 mice bearing colon carcinoma (K. Ottolino-Perry, unpublished). Mice were treated intraperitoneally with vOC2 and tumors were harvested 6 days later. Representative serial immunohistological sections show CD31 staining of tumor blood vessel endothelium (top) and VV infection of the same structure (bottom). Original magnification: 20×. Bar represents 100 μm. (C) Vesicular stomatitis virus (VSV) infection and tumor perfusion are restricted to the surface of CT-26 colon tumors [28]. BALB/c mice bearing subcutaneous CT-26 tumors were treated intravenously with VSV 24 h prior to perfusion with fluorescent microspheres. Whole (top) and half (bottom) three-dimensional models reveal that the surfaces of both VSV- and phosphate buffered solution (PBS)-treated tumors are perfused. Half models show that the tumor core of the VSV-treated tumor is dramatically less perfused when compared with the PBS-treated tumor. Adapted with permission from Macmillan Publishers Ltd. Molecular Therapy 19(8):886-894 [28], copyright 2011. (D) Oncolytic herpes simplex virus (G207) infects endothelial cells of human alveolar rhabdomysarcoma (HFR) xenografts [30]. Ultrastructure analysis 24 h after treatment revealed enveloped viral particles are frequently enclosed in vacuoles (arrows) in the cytoplasm of endothelial cells (see insets). Abbreviations: EC, endothelial cell; TC, tumor cell; n, nucleus; c, cytoplasm. Adapted with permission from Neoplasia 6(8):725-735 [30], copyright 2004.

with CD31-positive tumor ECs as early as 24 h and up to 6 days after intraperitoneal delivery (K. Ottolino-Perry, unpublished) (Figure 1). A loss of CD31-positive tumor ECs is observed in virus-infected intraperitoneal colorectal cancer (CRC) tumors 6 days post-infection. VV can also lead to ischemic tumor cell death as a result of virus-induced neutrophil accumulation and a subsequent loss of perfusion [26].

Although it has been demonstrated that OVts can infect and kill ECs in vitro, it is still unclear how this process occurs in vivo. Generally, it is assumed that OVts infect ECs from the luminal membrane as they migrate into the tumor. However, there is evidence that VV preferentially infects the basal membrane of polarized ECs [27], suggesting that EC infection may be occurring secondary to tumor infection. It is unclear whether OVts lyse tumor ECs following in vivo infection or whether a persistent infection results. It is also possible that tumor ‘neo-blood vessels’ are differentially susceptible to infection, relative to established tumor blood vessels. These areas are currently under investigation.

Vesicular stomatitis virus (VSV)

Three-dimensional reconstructions of subcutaneous colon cancer tumors created using approximately 1000 serial histological sections have helped visualize the distribution of perfusion, virus, and proliferating tumor cells 24 h after intravenous VSV treatment [28] (Figure 1). VSV directly infected tumor ECs, and despite the fact that the infection was limited primarily to the tumor rim, resulted in a dramatic loss of perfusion and extensive tumor cell death throughout the core of the tumor (Figure 1). Similar to VV, VSV infection of the tumor caused a massive infiltration of neutrophils, leading to a loss of perfusion and an increase in tumor apoptosis secondary to ischemia [26]. Heparin inhibition of VSV-induced clot formation abrogated the loss of perfusion seen with virus treatment alone. Taken together with evidence that neutrophil recruitment is critical to clot formation, these data support a novel mechanism of VSV-induced vascular disruption. It is unclear if other OVts utilize this mechanism.

Herspes simplex virus (HSV)

HSVts have been shown to directly infect tumor endothelium in vivo and significantly decrease the mean vessel density in ovarian, glioma, and rhabdomysarcoma tumors [29-31] (Figure 1). The virus was not detected in normal tissue endothelium by immunohistochemistry (IHC) following intravenous delivery of HSV1716, thus demonstrating virus specificity for tumor endothelium [29]. Ex vivo culturing of ECs harvested from tumor-bearing mice demonstrated increased proliferation rates in ECs derived from tumors, relative to normal heart-derived ECs. The differential proliferation rates may contribute to the increased sensitivity of tumor ECs to viral replication relative to normal endothelium. HSV1716 was also cytotoxic in ex vivo flow-sorted ovarian tumor ECs obtained from patient samples [29]. HSV also specifically inhibits cyclin D1 and VEGF production by activated ECs and tumor cells (the viral protein responsible has not yet been identified) [32].

Oncolytic viruses armed with antiangiogenic factors

OVts have been genetically engineered to carry antiangiogenic genes that (i) deliver antiangiogenic factors [33-49] or (ii) target the expression or function of angiogenic proteins or cytokines [50-57] (Table 2). Arming OVts potentially
Table 1. Unarmed viruses with antiangiogenic and vascular-disrupting properties. *

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus backbone</th>
<th>Cancer type/tumor model (cell lines)</th>
<th>Effect in angiogenesis assays</th>
<th>Delivery route</th>
<th>Effect on vasculature (in vivo)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Ad309</td>
<td>Wt Ad5</td>
<td>Lung/s.c. in nude mice (AS49)</td>
<td>n/a</td>
<td>IT</td>
<td>vWF and virus association (HIC, 60 dpi)</td>
<td>[106]</td>
</tr>
<tr>
<td>Ad-E1A</td>
<td>E1B-55K deleted, AdS E1A inserted</td>
<td>Hepatocellular/s.c. in nude mice (SMMC-7721, HepG2)</td>
<td>n/a</td>
<td>IT</td>
<td># CD34 and VEGF (HIC, 15 dpi)</td>
<td>[23]</td>
</tr>
<tr>
<td>Axe1A/AdB</td>
<td>E1B-55K deleted, Wt E1A</td>
<td>Pancreatic/s.c. in SCID mice (AIPC-1, Panc-1, PK-1, PK-B)</td>
<td>n/a</td>
<td>IT</td>
<td># Capillaries</td>
<td>[22]</td>
</tr>
<tr>
<td>OBP-301</td>
<td>E1A/E1B under hTERT promoter</td>
<td>Color/s.c. in BALB/c or SCID mice (SW620, Colon-20)</td>
<td># Tube formation</td>
<td>IT</td>
<td># Cork-screw vessels (diffusion chamber)</td>
<td>[24]</td>
</tr>
<tr>
<td>VACCINIA VIRUS</td>
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<tr>
<td>vvDD</td>
<td>tk- and vgf- deleted, WR strain</td>
<td>Colon/s.c. in BALB/c mice (CT-26)</td>
<td>n/a</td>
<td>IV</td>
<td># Tumor perfusion and * apoptosis in uninfected cells (HIC, 5 dpi)</td>
<td>[26]</td>
</tr>
<tr>
<td>WR-delb18R</td>
<td>B18R-deleted, WR strain</td>
<td>Lung/s.c. in C57BL/6 mice (CMT-93)</td>
<td>n/a</td>
<td>IV</td>
<td>CD31 and virus colocalization (IF, 1 dpi; HIC, 7 dpi)</td>
<td>[25]</td>
</tr>
<tr>
<td>GLV-1h-68</td>
<td>RLuc-GFP, LacZ, GUS, LVP strain</td>
<td>Prostate/s.c. in nude mice</td>
<td>n/a</td>
<td>IV</td>
<td># CD31 (HIC, 7 dpi)</td>
<td>[84]</td>
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<tr>
<td>VESICULAR STOMATITIS VIRUS</td>
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<tr>
<td>VSV</td>
<td>AVI (unmodified IFN-inducing mutant)</td>
<td>Colons.c. in BALB/c mice (CT26)</td>
<td>n/a</td>
<td>IV</td>
<td>Neutrophil- and coagulation-dependent # in tumor perfusion (1 dpi) Tumor cell death due to ischemia CD31 and virus colocalization (HIC, 24 dpi)</td>
<td>[26,28]</td>
</tr>
<tr>
<td>HERPES SIMPLEX VIRUS</td>
<td></td>
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<tr>
<td>HSV-1716</td>
<td>HSV-I (CP54.5 deleted)</td>
<td>Ovarian/s.c. in C57BL/6 mice (IDB-VEGF, patient samples)</td>
<td># Tube formation (in vitro and plug assay) Cytotoxic in HuVECs</td>
<td>IV</td>
<td>CD31 and virus colocalization in tumor but not normal tissues (HIC, 10 dpi) # MVD (HIC, CD31, 14 dpi) * TF production Cytotoxic in patient tumor endothelial cells (ex vivo)</td>
<td>[29]</td>
</tr>
<tr>
<td>G207</td>
<td>HSV-I (CP54.5 deleted (both copies), CP9 inactivated (encodes RR)</td>
<td>Glioblastoma/s.c. in athymic nude rats (patient samples) Rhabdomyosarcoma/s.c. in athymic mice (KFR)</td>
<td>n/a</td>
<td>IV</td>
<td># MVD and microvessel areas (HIC, 30 dpi)</td>
<td>[31]</td>
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*Abbreviations: dpi, days post-infection; hpl, hours post-infection; hTERT, human telomerase reverse transcriptase promoter; HuVECs, human umbilical vein endothelial cells; IF, intratumoral; IC, intracranial; IFN, interferon; IHC, immunohistochemistry; IV, intravenous; LVP, Lister strain from the Institute for Research on Viral Preparation; MVD, mean vessel density; n/a, not applicable; RR, ribonuclease reductase; s.c., subcutaneous; TF, tissue factor (indicator of endothelial injury); tk, thymidine kinase; VEGF, vascular endothelial growth factor; vgf, vaccinia growth factor; vWF, von Willebrand factor; WR, wild type.

Improves safety compared with standard antiangiogenic therapies because it results in the localized expression of antiangiogenic factors, contingent on the tumor-specificity of the virus.

Targeting angiogenic factors

The angiogenic proteins, VEGF and vascular endothelial growth factor receptor (VEGFR) [50-52,54,55], fibroblast growth factor (FGF) [53], and interleukin (IL)-8 [57], have all been successfully targeted by different OVs in a variety of models. VEGF and VEGFR are the most widely studied targets, and several recent studies have investigated ways to reduce or eliminate VEGF activity. An oncolytic VV encoding a soluble VEGFR-1-Ig fusion protein that binds to human and murine VEGF significantly reduced the mean vessel density and tumor volume in renal cell carcinoma (RCC) following intravenous delivery [51]. Oncolytic VVs (GLV-1h-107, -108, and -109) expressing GLAF-1, an anti-VEGF single chain antibody, significantly decreased the mean vessel density and volume of human lung and pancreatic tumors compared with the parental GLV-1h-68 virus [50]. In another approach, a replication-deficient adenovirus (Ad-Fk1-Fc) expressing soluble VEGFR, coinfected with a replication competent ‘helper’ virus, significantly inhibited human prostate tumor progression in mice and reduced CD31 staining, relative to either virus treatment alone
### Table 2. Armed oncolytic viruses with antiangiogenic factors

<table>
<thead>
<tr>
<th>Targeted pathway/molecule</th>
<th>Virus (species)</th>
<th>Arming protein/molecule</th>
<th>Cancer type or tumor model</th>
<th>Effect on assays of angiogenesis</th>
<th>Delivery route</th>
<th>Effect on vasculature (in vivo)</th>
<th>Efficacy (v/-)</th>
<th>Refs</th>
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<tr>
<td><strong>Targeting angiogenic pathways</strong></td>
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<tr>
<td>VEGF/VEGFR</td>
<td>GLV-1h107, R9 (VV)</td>
<td>Anti-VEGF Ab</td>
<td>Pancreatic and lung/s.c. in nude mice</td>
<td>n/a</td>
<td>IV</td>
<td># MVD (IHC, CD31, 21 dpi)</td>
<td>+</td>
<td>[50]</td>
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<tr>
<td></td>
<td>GLV-1h109 (VV)</td>
<td>Anti-VEGF Ab</td>
<td>Soft tissue sarcoma and prostate/s.c. in nude mice</td>
<td>n/a</td>
<td>IV</td>
<td># MVD (IHC, CD31, 7 dpi)</td>
<td>+</td>
<td>[107]</td>
</tr>
<tr>
<td></td>
<td>vDO-VEGFR-1-lg (VV)</td>
<td>Soluble VEGFR</td>
<td>Renal/s.c. in nude and BALB/C mice</td>
<td>Cytotoxic in HuVECs</td>
<td>IT or IV</td>
<td># MVD (IF, vWF study end)</td>
<td>+</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>Ad-FLK-1-Fc + dT52/947 (Adv) ‘helper virus’ (Adv)</td>
<td>Soluble VEGFR</td>
<td>Prostate/s.c. in CD1 nude or SCID mice</td>
<td>n/a</td>
<td>IT or IV</td>
<td># CD31 (IHC, CD31, 21 dpi)</td>
<td>+</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Ad-DB7-KOX (Adv)</td>
<td>VEGF transcriptional repressor</td>
<td>Glioma/s.c. in nude mice</td>
<td># VEGF</td>
<td>IT</td>
<td># MVD (IHC, CD31, 10 dpi)</td>
<td>+</td>
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<td></td>
<td>Ad-DB7-shVEG (Adv)</td>
<td>Anti-VEGF short hairpin RNA</td>
<td>Glioma/s.c. in nude mice</td>
<td># VEGF</td>
<td>IT</td>
<td># MVD (IHC, CD31)</td>
<td>+</td>
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<td>EGF/EGFR</td>
<td>ZD55-878-1 (Adv)</td>
<td>Soluble EGFR</td>
<td>Colon/s.c. in BALB/c nude mice</td>
<td># HuVEC proliferation (CM)</td>
<td>IT</td>
<td># MVD (IHC, CD31, 30 dpi)</td>
<td>+</td>
<td>[56]</td>
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<td>FGF/FGFR</td>
<td>bG47 D-dnFGFR (HSV)</td>
<td>Dominant-negative FGFR</td>
<td>Glioma and malignant peripheral nerve sheath tumors/s.c. nude mice</td>
<td>n/a</td>
<td>IT</td>
<td>n/a</td>
<td>+</td>
<td>[53]</td>
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<td><strong>Targeting angiogenic cytokines</strong></td>
<td></td>
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<td>IL-8</td>
<td>Ad-DB7. U6shIL8 (Adv)</td>
<td>Anti-IL-8 short hairpin RNA</td>
<td>Breast, HCC, and lung/s.c. in nude mice</td>
<td># Tube formation and migration (CM)</td>
<td>IT or IV</td>
<td># MVD (IHC, CD31, 7 and 28 dpi)</td>
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<td>[57]</td>
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<td><strong>Delivering antiangiogenic factors</strong></td>
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<td>Vasculostatin</td>
<td>RAMBO (HSV)</td>
<td>Vistat (extracellular fragment of BAII)</td>
<td>Glioma/s.c. and i.c. in nude mice</td>
<td># Tube formation and migration</td>
<td>IT</td>
<td># Hb (angiogenesis assay)</td>
<td>+</td>
<td>[35]</td>
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<td>3A.5ENVE (HSV)</td>
<td>Vistat (ICP34.5 under nestin promoter)</td>
<td>Glioma/s.c. and i.c. in nude mice</td>
<td># Tube formation and migration (CM)</td>
<td>IT</td>
<td># MVD (IHC, CD31)</td>
<td>+</td>
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<td>Endostatin</td>
<td>HSV-Endo (HSV)</td>
<td>Murine endostatin</td>
<td>Colon/s.c. in BALB/c mice</td>
<td># Vessel formation (CAM)</td>
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<td># CD31 (IHC, 22 dpi)</td>
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<td>Lung/s.c. and metastatic in BALB/c mice</td>
<td># HuVEC proliferation (CM)</td>
<td>IT</td>
<td># MVD (IHC, CD31, 3 dpi)</td>
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<td>A6618 (HSV)</td>
<td>Endostatin–angio inhibitor fusion</td>
<td>Non-small cell lung/s.c. in SCID mice</td>
<td>Cytotoxic in HuVECs</td>
<td>n/a</td>
<td>IT</td>
<td># MVD (IHC, vWF, 2 dpi)</td>
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<td>CNHK200-mE (Adv)</td>
<td>Murine endostatin</td>
<td>HCC/s.c. in nude mice</td>
<td>n/a</td>
<td>IT</td>
<td># MVD and occlusive morphology (IHC, CD31, 21 dpi)</td>
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<td>CHK500-mE (Adv)</td>
<td>Murine endostatin</td>
<td>Nasopharyngeal/s.c. in BALB/c nude mice</td>
<td>n/a</td>
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<td># MVD (IHC, CD34, 35 dpi)</td>
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<td>Type IV collagen</td>
<td>CRA-Ad-Cam (Adv)</td>
<td>Canstatin</td>
<td>Pancreatic/s.c. in BALB/c nude mice</td>
<td>n/a</td>
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<td># MVD (IHC, CD34, 35 dpi)</td>
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Table 2 (Continued)

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<th>Targeted pathway/ molecule</th>
<th>Virus (species)</th>
<th>Aiming protein/molecule</th>
<th>Cancer type/tumor model</th>
<th>Effect on assays of angiogenesis</th>
<th>Delivery route</th>
<th>Effect on vascularule formation</th>
<th>Efficacy b (±)</th>
<th>Refs</th>
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<tr>
<td>VEGF</td>
<td>ZD6554-VEGF-251 (Adv)</td>
<td>VEGF-251 (secreted isoform)</td>
<td>Cervical and colon/s.c. in BALB/c nude mice</td>
<td>Cytotoxic in HuVECs # Tube formation # Vessel formation (CAM)</td>
<td>IT # MVD (IHC, CD31, 7 dpi)</td>
<td>+</td>
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<td>Plasminogen</td>
<td>ZD5555/5555SmK5 (Adv)</td>
<td>Kringe domain 5 (human wt or mutant)</td>
<td>Colon/s.c. in BALB/c nude mice</td>
<td>Cytotoxic in HuVECs # Tube formation # Vessel branches (CAM)</td>
<td>IT # MVD (IHC, CD31)</td>
<td>+</td>
<td>[34]</td>
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<td>Arresten</td>
<td>CRA45/11-DA24/ TRAIL/ arresten (Adv)</td>
<td>TRAIL (apoptotic effects only) and arresten</td>
<td>Glioblastoma/s.c. in BALB/c nude mice n/a</td>
<td>IT # MVD (IHC, CD31, 25 dpi)</td>
<td>+</td>
<td>[63]</td>
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<td>Thrombospondin</td>
<td>T-TSP-1 (HSV)</td>
<td>Thrombospondin-1</td>
<td>Gastric cancer/s.c. in nude mice n/a</td>
<td>IT # MVD (IHC, CD31, 7 dpi)</td>
<td>+</td>
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<tr>
<td>Angiostatin</td>
<td>G47D-mAngio (HSV)</td>
<td>Angiostatin</td>
<td>Glioblastoma/c. in nude mice n/a</td>
<td>IT # MVD (IHC, CD31, 3 dpi)</td>
<td>+</td>
<td>[60]</td>
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</tbody>
</table>

Delivering antiangiogenic cytokines

| IL-12                     | NV1042 (HSV) | IL-12 | Squamous cell/s.c. in C3H/HeJ mice # Tube formation | IT # MVD and Hb (plug assay, 12 dpi) | + | [44] |
| IL-18                     | ZD5555-IL-18 (Adv) | IL-18 | Prostate/s.c. in C57BL/6 and C3(1)/T-Ag mice n/a | IT # CD31 (injected but not contralateral tumor) | + | [43] |
| IL-24                     | Ab.DD3-1E1A-IL-24 (Adv) | IL-24 | Prostate/s.c. in nude mice n/a | IT # CD34 and VEGF (IHC, 49 dpi) | + | [49] |
| PF4                       | bG47D-D-PF4 (HSV) | PF4 | Glioma and peripheral nerve sheath cancer/s.c. in nude mice | Cytotoxic in HuVECs # Migration | IT # MVD (IHC, vWF, 8 dpi) | + | [38] |
| GM-CSF                    | NV1034 (HSV) | GM-CSF | Prostate/s.c. in C57BL/6 and C3(1)/T-Ag mice n/a | IT n/a | - | [43] |
| JX-594                    | GM-CSF | JX-594 | HCC/patients n/a | IT # VEGF (serum) # Perfusion (CT, 6 dpi) | + | [39] |

aAbbreviations: Ab, antibody; Adv, adenovirus; ARA, aortic ring assay; BAI1, brain-specific angiogenesis inhibitor 1; CAM, chick chorioallantoic membrane; CD31, cluster of differentiation 31; CM, conditioned media; CT, computed tomography; dpi, days post-infection; EGF, epidermal growth factor; EGFRI, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FRET, fluorescence resonance energy transfer; FMT, fluorescence molecular tomography; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hemoglobin; HCC, hepatocellular carcinoma; HSV, herpes simplex virus; HuVECs, human umbilical vein endothelial cells; Ic, intracranial; IF, immunofluorescence; IHC, immunohistochemistry; IL, interleukin; IT, intratumoral; IV, intravenous; MVD, microvessel density; n/a, not applicable; PF, platelet factor; sc., subcutaneous; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VEGI, vascular endothelial cell growth inhibitor; Votat, vasotocin; vWF, von Willebrand factor; wt, wild type.

bEfficacy improved over unamed parental virus.

[54]. Antiangiogenesis has also been documented in a murine xenograft colon cancer model following treatment with an adenovirus expressing soluble human VEGFR (hVEGFR) [56]. Two adenoviruses, Ad-DE1-KOX and Ad-DB7-KOX, have been constructed to express the VEGF-A transcriptional repressor F435-KOX [52] or a VEGF-targeted short hairpin RNA [55], which target VEGF at the level of transcription or translation, respectively. After infection with either virus, in vivo VEGF expression and tumor CD31 staining were significantly impaired relative to parental virus. Viral delivery of VEGF-signaling inhibitors may overcome some of the delivery issues that have been encountered with traditional anti-VEGF immunotherapies. Although less well studied, other targets have proven successful in preclinical studies and warrant further investigation. The oncolytic HSV bG47D-DnFGFR expresses a dominant negative fibroblast growth factor receptor (FGFR) that dimerizes with endogenous FGFR without stimulating downstream signaling [53]. This virus is cytotoxic to human ECs in vitro and significantly reduced tumor growth relative to bG47 D in murine models of malignant peripheral nerve sheath tumor and human glioblastoma.
An oncolytic adenovirus expressing a short hairpin specific to mRNA encoding the cytokine IL-8 reduced tumor cell IL-8 production in vitro [57]. Conditioned media, from infected tumor cells, inhibited EC tube formation and migration, and in glioma, lung, and metastatic breast tumor xenograft models reduced tumor growth as well as expression of IL-8, CD31, and VEGF from the tumor.

Delivering antiangiogenic factors
Endogenous antiangiogenic agents, such as endostatin, canstatin, plasminogen kringle 5, vascuostatin, arresten, thrombospondin (TSP), angiostatin, and ILs (see Figure 1 in Box 2), have also been exploited as therapeutic transgenes in a variety of OVs. Infection with HSV expressing endostatin significantly reduced tumor volume [40,46,58] and CD31 staining [40,58] relative to parental viruses, and similar improvements were observed following treatment with adenoviruses expressing endostatin [37,42]. Arming HSV to express TSP-1 increased its efficacy over the parental virus as shown by decreased tumor burden secondary to decreased mean vessel density [59]. Angiostatin-armed HSV administered simultaneously with bevacizumab increased virus distribution and the angiostatin-mediated inhibition of VEGF expression, which increased the survival of the animals by inhibiting angiogenesis and decreasing tumor invasion [60].

Adenovirus delivery of genes encoding other potent inhibitors of angiogenesis known to induce EC apoptosis [61,62], such as canstatin [36] and plasminogen kringle 5 [34], also effectively inhibit in vivo tumor angiogenesis and improve tumor responses relative to parental viruses. Significant cancer cell apoptosis and a reduction of mean vessel density was observed when a conditionally replicating adenoviral vector AD5/11-D24 was double armed with TRAIL and arresten [63], suggesting the benefit of arming viruses with multiple transgenes that target different pathways.

An adenovirus armed with vascular EC growth inhibitor (VEGFI-251) revealed secondary benefits [45] when it was found to induce levels of tumor cell apoptosis in vitro that exceeded those induced by the parental virus. By modulating caspase-8 and inducing the mitochondrial apoptotic pathway, secreted VEGF-251 enhanced tumor cell apoptosis in addition to limiting angiogenesis. RAMBO, an oncolytic HSV encoding vascuostatin, efficiently reduced in vivo tumor mean vessel density and perfusion in glioma models [35]. The second generation of this virus (34.5ENVE), encoding the virulence factor ICP34.5 under control of a glioma-specific nestin promoter, showed superior therapeutic efficacy compared with RAMBO [47].

NV1042, an IL-12-expressing HSV, reduced in vivo prostate tumor growth with greater efficacy than its counterpart NV1034 that expresses granulocyte macrophage colony-stimulating factor (GM-CSF) [43]. NV1042 stimulated significantly more CD4+ and CD8+ T cell and macrophage infiltration and reduced the growth of non-injected contralateral tumors. IL-12 stimulation of two antiangiogenesis mediators, monokine induced by interferon-g (MIG) and interferon-g-induced protein-10 (IP-10), significantly decreased tumor vasculature [43,44]. In models of human melanoma [48] and RCC [49], the adenovirus ZD55-IL-18 not only reduced tumor volume but also decreased CD34 and VEGF expression, compared with a GFP-expressing control. Expression of platelet factor 4 (PF4) by oncolytic HSV significantly increased in vitro cytotoxicity in human ECs inhibiting their migration compared with the parental virus [38]. Additionally, virus delivery of PF4 reduced the mean vessel density and improved survival in two glioma xenograft models [38]. Similarly, administration of IL-24-expressing oncolytic adenovirus completely regressed subcutaneous prostate tumors in mice and significantly reduced the mean vessel density relative to the parental virus [33].

OVs targeted to tumor vasculature endothelium Superior targeting specificity for tumor ECs can be obtained using OVs genetically engineered to interact with the overexpressed integrin subunit combinations found on the surface of these cells. For example, the measles virus (MV) displaying a mutated form of the integrin binding protein echistatin on its envelope (MV-ERV) selectively infected established blood vessels in the choroidal anecious membrane (CAM) assay [64] and reduced tumor burden compared with the parental MV following intratumoral injection in a subcutaneous human multiple myeloma xenograft model. A second study confirmed that MV expressing wild type echistatin infected neovascularure in two in vivo models of angiogenesis [65].

Combining OVs with antivascular therapeutics
Combining OVs with clinically approved antivascular agents (Box 2) has emerged as an interesting strategy for dramatically reducing tumor growth. One of the challenges in evaluating the effectiveness of combination therapies is the wide variation in study design, particularly with respect to treatment schedules and drug selection.

Initially, simultaneous administration of bevacizumab, a humanized monoclonal antibody, and adenoenures (Ad5/3-D24, Ad5-D24RbG, and Ad5pK7-D24) did not improve survival in mice [66]. Nevertheless, a later study using a different adenovirus (Ad922-947) reported that conditionning mice with bevacizumab before virotherapy significantly improved viral distribution within tumors and reduced tumor growth relative to monotherapy [67]. In contrast to the results with adenovirus, bevacizumab preconditioning with HS did not improve the tumor distribution of the virus [68], but administering bevacizumab 13 days post-GLV-1h-68 (Lister strain V) infection significantly inhibited tumor growth compared with monotherapy [50]. Similarly, when HSV infection was followed by bevacizumab treatment it improved survival rates of mice bearing human Ewing’s sarcoma, a finding that was likely due to continued antiangiogenic effects once the virus was eliminated by the immune system [68].

Combining the epidermal growth factor receptor (EGFR) small molecule inhibitor erlotinib with oncolytic HSVs (G207 and hrR3) did not lead to synergistic effects, despite the fact that each agent has antiangiogenic effects in the same models and erlotinib does not affect in vitro viral replication [69]. Simultaneous administration of sorafenib with JX-594 does inhibit viral replication in vitro; however, sequential therapy (OV followed by sorafenib) was superior to monotherapy in murine models [17].
Pretreatment with a single dose of cyclic Arg-Gly-Asp (cRGD) peptide before HSV-1 hr3 administration reduces tumor vascular permeability, leukocyte infiltration, and IFN-γ levels, while increasing viral titers within tumor tissue, consequently prolonging median survival relative to monotherapy [70]. Given the role of IFN-γ in the antiviral immune response it is likely that the cRGD-induced reduction of IFN-γ was significant in improving OV delivery and efficacy.

The mechanistic target of rapamycin (mTOR) inhibitor RAD001 (everolimus) has been shown to prolong the survival of tumor-bearing mice when given in combination with oncolytic adenovirus [71]. RAD001 was withheld the day before and the day of virus administration to create a VEGF burst at the time of injection, and an immunodeficient model was used to control for the potential contributions of immunosuppressive effects known to be a result of everolimus therapy. Unlike in mice treated with monotherapy, in mice treated with combination therapy histological analysis revealed tumor vasculature collapse with extensive central necrosis.

The concurrent administration of oncolytic HSV (G47 D) and trichostatin A (TSA) also enhanced antiangiogenesis and antitumor efficacy [32]. Synergy depended upon viral replication, but did not depend on the dosing sequence, viral genetic alterations, infectivity, or replication kinetics. Both treatments individually reduced cyclin D1 via different mechanisms, consequently inhibiting cell proliferation and VEGF secretion. The observed synergy is thought to be cyclin D-dependent because cells with low levels of cyclin D or those treated with anti-cyclin D antibody were less susceptible to combination therapy.

Copper is an important cofactor for many angiogenic proteins (e.g., VEGF). The combination of an antiangiogenic copper chelator (ATN-224) with another oncolytic HSV (rQnsetin34.5) decreased the mean vessel density and improved virus propagation in intracranial glioma tumors [72]. Interestingly, ATN-224 was also found to increase the serum stability of the virus.

Combining oncolytic VV with photodynamic therapy (PDT) improved survival in nude mice bearing neuroblastoma or head and neck squamous cell carcinoma xenografts versus mice treated with virus alone [73]. Decreased tumor size and increased viral titers in tumor were observed in mice treated with combination therapy compared with either monotherapy. These results were attributed to the ability of PDT to cause endothelial barrier dysfunction, which increases permeability and virus delivery.

One innovative approach to combination therapy utilized VEGF stimulation to activate tumor endothelium with the aim of supporting viral replication. Preconditioning tumor endothelium with transient exposure to VEGF supported oncolytic reovirus or VSV replication [20]. Preconditioning was generated either by administrating VEGF to tumors lacking VEGF expression or by inducing a VEGF burst through the administration and withdrawal of bevacizumab to VEGF-expressing tumors. It was hypothesized that VEGF–VEGFR2 intracellular signaling established a proviral proliferative state similar to that of Ras-activated tumor cells [20]. It was hypothesized that this activated state allowed OVs to replicate in poorly susceptible EC lines in vitro. VEGF stimulation in immunocompetent mice triggered an innate immune-mediated vascular collapse, substantial tumor regression, and eventually cured established tumors in immunocompetent mice [20]. This proviral state has also been induced by exposing endothelium to paclitaxel [74].

Prolonged exposure or high doses can compromise the delivery of drugs to tumors and increase areas of poor perfusion, low pH, and high interstitial pressure, all of which make tumors particularly resistant to standard cytotoxic therapies [9]. Because many OVs replicate in hypoxic and acidic environments without losing efficacy (e.g., VV and HSV) [75–78], these viral candidates may be ideal for combining with antiangiogenic agents, leading to sustained synergy after the normalization period.

Antiangiogenic drugs can also have immunomodulatory effects that could synergistically improve combination therapy with OVs. Proangiogenic factors such as VEGF and FGF contribute to the ‘immunosilent’ state typical of tumors by downregulating adhesion molecules on ECs and consequently restricting leukocyte extravasation [79]. Other antiangiogenic agents have been shown to improve leukocyte extravasation [79] and the recruitment of leukocytes and other immune mediators into tumors. In the environment of local inflammation created by OVs, these leukocytes may become stimulated to induce antitumor immune responses.

Proangiogenic viruses

Some viruses have evolved to induce a proangiogenic state by coding or upregulating expression of angiogenic factors, such as the parapoxvirus orth that encodes a homolog of VEGF [80], or by inhibiting the expression of the antiangiogenic proteins TSP-1 and TSP-2, as in the case of certain HSV recombinants [81]. This increases tumor vascularity and can lead to tumor growth, which is a distinct disadvantage when using these viruses as oncolytic. Consequently, when these viruses were engineered as oncolytic agents, deletions were made to counteract this feature [72,81–83].

In some cases, the timing of analysis may lead to the finding of angiogenic stimulation. For example, GLV-1h68 (VV) was shown to decrease the mean vessel density in prostate tumor xenografts early after infection (7 days post-infection) [84], yet have no effect on mean vessel density in breast tumor xenografts late after infection (42 days post-infection) [85]. Similarly, G207 (HSV) was shown to increase vascularization in a glioma model late after infection (27 days post-infection) [81], but had anti-vascular effects in vitro and early after treatment in a rhabdomyosarcoma model (8 days post-infection) [30]. In a clinical study, increased vascularization (CD31) was observed late (14 days post-infection) with HF10 (HSV)-treated breast cancer nodules compared with saline-injected nodules in the same patient [86].

Clinical data

To date, only JX-594, an oncolytic VV expressing GM-CSF (see Figure 1 in a Box 2), has been reported to have
antivascular properties in humans. In a Phase II dose-escalation clinical trial, JX-594 was administrated intratumorally at 3-week intervals to patients with primary hepatocellular carcinoma (HCC) or with liver metastases [41]. Of 14 patients treated, 3 with HCC, a hypervascular and VEGF-rich tumor, were subsequently studied as a subgroup and discussed in another study [39]. For the first time in humans, an OV was confirmed to cause objective vascular shutdown as shown by a 43% decrease in tumor perfusion relative to baseline 6 days post-treatment on perfusion computer tomography. Significantly reduced levels of circulating VEGF (28–45% up to 192 h post-treatment) and the induction of antivascular cytokines, specifically TNF-α and IFN-γ [39], were also observed.

Intratumoral delivery of JX-594 followed by sorafenib in three HCC patients was associated with a significant decrease in tumor perfusion with subsequent necrosis (rate: 50–100%), starting at 2.5 weeks post-sorafenib treatment [17] (Figure 2). The authors also reported a case of a patient with metastatic RCC who had a complete tumor response after combination treatment with JX-594 and sunitinib [17]. Four years after treatment the patient was reported to be alive.

IHC performed on biopsies from patients with CRC, ovarian carcinoma, leiomyosarcoma, and non-small cell lung cancer enrolled in a Phase I trial [87] documented dose-dependent EC infection 7 days after a single intravenous infusion of JX-594. Serial dynamic magnetic resonance of 16 patients from a Phase II trial for HCC and CRC demonstrated that JX-594 induced progressive, durable vascular disruption with loss of perfusion starting 5 days post-treatment and lasting up to 8 weeks [87]. This phenomenon was observed not only in tumors injected intravenously or intratumorally but also in non-injected tumors, suggesting an immunological bystander effect. Wound-healing sites were unaffected by treatment.

Concluding remarks and future perspectives
A growing number of studies demonstrate that the antivascular properties of many OVs significantly contribute to their overall antitumor efficacy (Figure 3). Targeting angiogenesis alone will not overcome the multifaceted nature of tumor vasculature: therefore, exploiting OVs with vascular-disrupting properties and using OVs in combination with antivascular agents represent promising strategies. To advance OVs as antivascular therapeutics, future research should focus on understanding the specific mechanisms of action of individual OVs and how these influence the optimal treatment schedule and potential drug combinations.

Critical to establishing the role of OVs as antivascular agents is carrying out appropriately designed studies. Two aspects of study design must be considered: (i) the appropriate time point(s) to measure the effects of OV infection on vasculature, and (ii) informative measures of infection and vascularization. The time point after treatment in which infection is quantified could affect whether or not the antivascular effects of OVs are observed. Studies have shown immediate antivascular effects following OV administration.
Figure 3. Antivascular properties of oncolytic viruses and antivascular therapies. Several oncolytic viruses (OVs) have inherent antivascular properties that can be categorized as antiangiogenic or vascular disrupting. Direct infection of endothelial cells (ECs) by vesicular stomatitis virus (VSV) leads to a cascade of inflammatory responses, cell death, loss of perfusion, and subsequent ischemic tumor cell death [26]. Vaccinia virus (VV) also infects tumor ECs and results in a loss of tumor perfusion (the exact mechanism is unknown) [26]. Adenovirus exerts a direct antiangiogenic effect through its E1A protein (see inset) [22,23]. E1A interacts with cellular p300, a vascular endothelial growth factor (VEGF) transcription cofactor, to inhibit VEGF transcription resulting in decreased EC migration, proliferation, and survival. OVs can be effectively combined with traditional antiangiogenic therapies by taking advantage of their vascular normalization properties. During oncolytic virotherapy, leaky vessels allow circulating immune cells to easily infiltrate into virus-infected tumor beds and potentially halt the progression of infection. Binding of cyclic Arg-Gly-Asp (cRGD) or anti-VEGF therapies (e.g., bevacizumab) to EC targets can result in vascular normalization leading to decreased immune cell infiltration and antiviral cytokine production [70], as well as increased virus titers in the tumors [60,68], as has been done with adenovirus and herpes simplex virus (HSV).

[26,28], and clinical data have shown rapid antivascular effects, such as a decrease in VEGF levels, as early as 1 h post-OV treatment [39]. In cases where angiogenesis is measured at a later time point relative to the timing of maximum antitumor response, an absence of an antiangiogenic response or an apparent increase in angiogenesis may lead investigators to overlook the antivascular potential of their virus. Secondly, IHC for endothelial markers provides a two-dimensional ‘snap shot’ of the structure of the tumor vasculature without providing functional information. Based on the published methods for detecting angiogenesis in solid tumors, CD31 or CD34 staining may be more informative when combined with other stains, such as anti-Ki-67 or a smooth muscle actin [88]. For more functional information, measuring perfusion within a whole tumor could be employed, as was done by Breitbach et al. [28]. Another option is to utilize first-pass perfusion computed tomography, a novel non-invasive approach for quantifying tissue perfusion that monitors changes in perfusion over the course of treatment [89].

In addition to the timing and methods of evaluating OV effects on tumor vasculature, inherent differences in the models, such as their dependence on vascularization, could also affect whether a given virus is observed as having antivascular properties. Importantly, within viral species, differences in the viral backbone can affect the antivascular properties of an OV. As investigators endeavor to improve the antivascular effects of OVs, either through genetic aming or combination therapy, it would be prudent to work with viruses that are inherently antivascular (e.g., adenoviruses with E1A expressing backbones as opposed to E1A mutants).

The optimal sequence and timing of combination therapy and basic aspects of establishing which regimens are synergistic has yet to be determined. Ideally, dosing schedules should be designed with an understanding of the mechanisms of action for each individual therapy. Unfortunately, these actions are not always fully understood, especially for OVs, which typically have multiple, complex antitumor mechanisms.
Clinical studies comparing multiple combinations and sequences will be necessary. Nevertheless, it seems likely that drugs that improve delivery and enhance virus uptake by tumors will be more effective when delivered prior to oncolytic virotherapy, whereas the ideal timing of agents with immunomodulatory properties will depend on the effect of the specific agent on the antiviral response and virus-induced antitumor immune response.

Intensive research on the role of OVs as antivascular agents has led to several important conclusions. Overall, OVs have an array of distinctive advantages over other antivascular agents: (i) specificity for multiple tumor components (tumor and ECs); (ii) direct tumor lysis; (iii) VEGF-independent mechanisms of action, which are particularly useful because traditional antiangiogenic agents (e.g., bevacizumab) often fare poorly in heterogeneous tumor cell populations with fluctuating patterns of angiogenic gene expression; (iv) capacity to carry and specifically deliver antiangiogenic molecules; and (v) induction of an antitumor immune response. As we develop a more comprehensive understanding of the interaction of OVs with tumor vasculature, we will be able to strategically combine them with antivascular agents to optimize their synergistic, antiangiogenic, and vascular disruptive properties.

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References

30. Cinati, J., Jr et al. (2004) Multimutated herpes simplex virus g207 is a potent inhibitor of angiogenesis. Neoplasia 6, 725–735
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Appendix 1.3. Antiproliferative Effects of $^{111}$In- or $^{177}$Lu-DOTATOC on Cells Exposed to Low Multiplicity-of-Infection Double-Deleted Vaccinia Virus Encoding Somatostatin Subtype-2 Receptor

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Antiproliferative Effects of $^{111}$In- or $^{177}$Lu-DOTATOC on Cells Exposed to Low Multiplicity-of-Infection Double-Deleted Vaccinia Virus Encoding Somatostatin Subtype-2 Receptor

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Abstract

Oncolytic viruses may be limited in their ability to infect and lyse tumor cells because of penetration barriers and viral elimination by the immune system. Combining virotherapy with targeted radiotherapy that uses $^{111}$In- or $^{177}$Lu-DOTATOC may address such issues by spatially enhancing antitumor effects through bystander and/or cross-fire phenomena. In this study, a double-deleted vaccinia virus (vvDD) encoding the gene for somatostatin subtype-2 receptor (sstR-2) infected MC-38 murine colon adenocarcinoma cells and increased their sstrR-2 expression by 2-fold. A low multiplicity-of-infection (MOI = 0.1) of vvDD and short exposure time (48 hours) preserved MC-38 viability (>80%–90%) for up to 3 days, permitting targeting of sstrR-2 by $^{111}$In- or $^{177}$Lu-DOTATOC. $^{111}$In-DOTATOC, alone or in combination with vvDD, was less effective than $^{177}$Lu-DOTATOC at decreasing the growth of sstrR-2-gene-transfected human embryonic kidney (HEK)-293 cells or MC-38 cells in monolayer. However, $^{111}$In- or $^{177}$Lu-DOTATOC combined with vvDD provided equivalent growth inhibition of HEK-293 or MC-38 cells as spheroids, suggesting a bystander effect from $^{111}$In-DOTATOC. Growth of the cells was reduced 4-fold (from 20% to <5%) at 8 days in this case. Further evaluation of low-MOI vvDD in combination with $^{111}$In- or $^{177}$Lu-DOTATOC for the treatment of MC-38 tumors in mice is planned.

Key words: antiproliferative effects, $^{111}$In-DOTATOC, $^{177}$Lu-DOTATOC, multiplicity-of-infection (MOI), vaccinia virus

Introduction

The somatostatin subtype-2 receptor (sstR-2) class is an important target for peptide-directed radiotherapy that uses $\beta$-emitting octreotide analogs (e.g., $^{90}$Y-DOTATOC or $^{177}$Lu-DOTATATE) for the treatment of neuroendocrine malignancies.\(^1\) Impressive tumor responses leading to improved survival and quality of life of patients have been observed with this therapy.\(^1,2\) Clinical responses and partial tumor responses have also been achieved in some patients with sstrR-2-positive malignancies treated with higher doses of $^{111}$In-pentetreoctide, an octreotide analog that emits subcellular-range Auger electrons in addition to $\gamma$-rays that are used in tumor imaging.\(^3,4\) Many different tumor types express sstrR-2, but these receptors are most highly and homogeneously expressed in neuroendocrine malignancies.\(^5,6\)

The introduction of exogenous sstrR-2 into tumors that have relatively low levels of these receptors using a recombinant double-deleted vaccinia virus (vvDD) engineered to include the sstrR-2 gene in its genome has been studied.\(^7\) vvDD has deletions of the viral thymidine kinase (TK) and vaccinia growth factor (VGFI) genes.\(^8\) These deletions render the virus reliant on rapidly dividing host cells for replication (e.g., cancer cells), as it requires the high levels of thymidine

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phosphate present in dividing cells. VGF deletion also attenuates the ability of the virus to promote the proliferation of surrounding cells, further decreasing its efficiency to replicate in normal cells. VvDD, owing to its large size (200 nm in diameter), selectively accumulates in tumors because the vascular “leakiness” often present facilitates its extravasation. The encoded sstr-2 are expressed in infected host cells late in the viral replicative cycle but prior to tumor cell lysis. It was previously shown that tumor infection following intraperitoneally (i.p.) administered vvDD encoding the sstr-2 gene permitted enhanced imaging of subcutaneous MC-38 murine colon cancer xenografts in athymic mice at 72 hours after intravenous injection of 111In-pentetreotide, thus demonstrating that these virally encoded sstr-2 were correctly displayed on the surface of tumor cells and were capable of binding radiolabeled octreotide analogs. In another study, treatment of athymic mice bearing subcutaneous MC-38 tumors with 107 plaque-forming units (pfu) of i.p.-administered vvDD caused tumor regression at 12 days after virus injection, owing to the potent oncolytic properties of vvDD. Brader et al. recently reported that a recombinant oncolytic vaccinia virus harboring the human nonepinephrine transporter (hNET) gene allowed SPECT and PET imaging of orthotopic mesothelioma with 123I-labeled dobenzylyguanidine (MIBG) and 124I-MIBG, respectively.

The antitumor properties of vvDD that acts as a gene delivery vector capable of introducing the sstr-2 gene into tumor cells may be combined with targeted radiotherapeutics (177Lu or 186Y-DOTATOC or 111In-pentetreotide) directed against these receptors. This approach may be advantageous by relying solely on the oncolytic properties of the virus to eradicate tumor cells. One benefit is the spatial increase in antitumor effects on more distant and noninfected cells, provided by the “cross-fire” effect of the moderate-energy and millimeter-range β-particles emitted by 177Lu- or 186Y-DOTATOC targeted at virally infected cells. This could be especially important in situations where the virus is unable to penetrate homogeneously and infect all cells in larger tumor deposits. Another advantage is that the “bystander” effects as a consequence of cell death from the low-energy and micrometer-range Auger electrons emitted by 111In-pentetreotide (as well as the β-particles from 177Lu- or 186Y-DOTATOC) could kill more proximal but noninfected cells. Such effects would continue to inhibit tumor growth in the event of virus elimination by the host immune system. To realize the augmentation in antitumor effects provided by sstr-2-targeted radiotherapeutics, low-MOI vvDD must be used to achieve viral sstr-2 expression before infection leads to widespread tumor cell lysis. In this study, the effects of low-infectivity amounts of vvDD (MOI = 0.1) on the growth of MC-38 murine colon adenocarcinoma cells were evaluated. This cell line was chosen because it is tumorigenic in C57BL mice, which would allow an evaluation of vvDD alone or in combination with sstr-2-targeted radiotherapeutics in an immunocompetent mouse tumor model in future studies. MC-38 cells grown as spheroids were exposed to vvDD alone or in combination with 111In- or 177Lu-DOTATOC. The antiproliferative potentials of 111In- and 177Lu-DOTATOC on human embryonic kidney (HEK)-293 cells stably transfected with the sstr-2 gene were compared. The growth-inhibitory effects of vvDD combined with these radiotherapeutics on HEK-293 cells exposed as spheroids were also studied.

Materials and Methods

HEK-293 and MC-38 cells and spheroids

HEK-293 cells stably transfected with the sstr-2 gene were donated by Dr. Ujendra Kumar, University of British Columbia, Vancouver, BC, Canada. MC-38 murine colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium for HEK-293 cells was supplemented with 72 µg/L of gentamicin (G418) (Sigma-Aldrich, Oakville, ON, Canada). Spheroids were prepared using a modification of the method reported by Ivascu and Kubbes. Briefly, 96-well plates were precoated with 25 µL of well of 1% polyHema (Sigma-Aldrich) and air dried for 1 hour. HEK-293 cells in T175 flasks were recovered using Accutase (Sigma-Aldrich) and seeded into the precoated plates at a density of 104 cells per 200 µL of medium. The plates were centrifuged at 1500 x g for 10 minutes and then cultured at 37°C and 5% CO2 for 3 days to form the spheroids.

Radiotherapeutics

1,4,7,10-tetraazacyclododecane-N2,N2',N4,N4'-tetracetic acid (DOTA)-conjugated D-Phel-Tyr octreotide (DOTATOC) was generously donated by Dr. Helmut Macosko, University of Basel, Switzerland. DOTTATOC (1 µg in 50 µL of 250 mM ammonium acetate buffer, pH 5.5) was radiolabeled by heating with 25-40 MBq of 111InCl3 (MDS-Nordion, Kanata, ON, Canada) or 177LuCl3 (PerkinElmer, Waltham, MA) at 95°C for 30 minutes as previously reported. 111In- or 177Lu-DOTATOC were purified on a reversed-phase C18 SepPak cartridge (Waters, Milford, MA) eluted first with 3 mL of 400 mM sodium acetate buffer (pH 5.0) and then with 3 mL of methanol. The methanol fractions were collected, evaporated to dryness under N2, and reconstituted in 1.0 mL of 150 mM NaCl. The final radiochemical purity was >95% as determined by instant thin layer silica gel chromatography using 100 mM sodium citrate (pH 5.0) or by reversed-phase high performance liquid chromatography (HPLC) as described by Storch et al. HPLC analysis was performed on a Jupiter 5u 300A C-18 column (250 x 4.6 mm ID; Phenomenex, Torrance, CA) fitted to a PerkinElmer HPLC system composed of a Series 200 pump, a diode array detector monitoring at 280 nm, and a Flow Scintillation Analyzer in-line radioactivity detector (PerkinElmer). Gradient elution was performed using 95% of 0.1% trifluoroacetic acid and 5% CH3CN increasing to 100% CH3CN over 37 minutes. The flow rate was 0.75 mL/minute.

Double-deleted vaccinia virus

The vvDD virus is a genetically modified version of the Western Reserve strain that has deletions of the TK gene and VGF genes produced in Dr. J. Andrea McClintock’s laboratory at the University Health Network. VvDD was further engineered to incorporate the sstr-2 gene into its genome under the control of a vaccinia late promoter. The virus was constructed and propagated as previously reported.

Levels of sstr-2 expression on cell lines

The radioligand binding affinity and levels of sstr-2 on HEK-293 cells stably transfected with the sstr-2 gene were
measured in direct saturation radioligand binding assays using \(^{111}\text{In-}\) and \(^{177}\text{Lu-DOTATOC}.\) Briefly, increasing concentrations (0-119 \text{nmol/L}) of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) in serum-free medium were incubated in triplicate at 4°C for 2 hours with 1\times 10^6 cells cultured overnight in 6-well plates. Unbound radioactivity was removed, and the adherent cells were rinsed twice with 150 mM NaCl. The cells were then dissolved in 100 mM NaOH and recovered. The cell-bound and unbound fractions were measured in a \(\gamma\)-counter (PerkinElmer Model 1480). Nonspecific binding was evaluated by repeating the assay in the presence of 400 nmol/L of unlabeled somatostatin-14 (Sigma-Aldrich). The specific cell-bound radioactivity was plotted against the unbound radioactivity, and the curve fitted to a 1-site receptor-binding model by nonlinear regression analysis using Prism software version 4.0 (GraphPad Inc., San Diego, CA). The dissociation constant \((K_d)\) and the maximum binding sites per cell \((B_{max})\) were estimated.

Flow cytometry was used to estimate the relative levels of sst2-2 on MC-38 cells infected or not infected with vVDD for 48 hours, by comparing the mean fluorescence intensity (MFI) values with those of HEK-293 cells, for which the receptor density was measured in the direct radioligand binding assays described above. For flow cytometry, 5\times 10^6 cells were incubated with 1\mu g of fluorescein isothiocyanate-labeled anti-sst2 polyclonal antibodies (Novus Biologicals, Littleton, CO) in 100 \mu L of 150 mM NaCl at room temperature for 30 minutes in 1.5 mL Eppendorf tubes. The cells were recovered by centrifuging at 450\times g, rinsed twice with 150 mM NaCl, and then fixed with 500 \mu L of 3.7\% formaldehyde at room temperature for 20 minutes. The cells were recovered, rinsed twice with 150 mM NaCl, and then sorted on a FACScan flow cytometer (BD Biosciences, Bedford, MA). Approximately 10,000 events were recorded, and the data sets were analyzed using CELLQuest software version 3.3 (BD Biosciences).

Subcellular distribution and radiation dosimetry of \(^{111}\text{In-}\) and \(^{177}\text{Lu-DOTATOC}\)

The kinetics of internalization and nuclear importation of \(^{111}\text{In-DOTATOC}\) over a 24-hour period were measured in HEK-293 cells by subcellular fractionation. Briefly, 1\times 10^6 cells seeded overnight in 6-well plates were incubated in triplicate with 25 nmol/L of \(^{111}\text{In-DOTATOC}\) in serum-free medium at 37°C. Unbound radioactivity was removed, and the cells were rinsed twice with 150 mM NaCl. Cell surface-bound radioactivity was then displaced by incubating for 10 minutes with 1 mL of 200 \mu M sodium acetate/500 mmol/L NaCl buffer, pH 2.5. Following removal of this fraction, the cells were lysed on ice by exposure to 1 mL of lytic buffer from a nuclei isolation kit for 22 minutes (Nuclei EZ Prep Kit; Sigma-Aldrich). The lysed cells were centrifuged at 3000\times g for 5 minutes, and the supernatant containing the cytoplasmic fraction was separated from the pellet containing the cell nuclei. Radioactivity on the cell surface, or in the cytoplasmic or nuclear fractions was measured in a \(\gamma\)-counter. Subcellular localization of \(^{177}\text{Lu-DOTATOC}\) was determined only after 4 hours of incubation with HEK-293 cells. The cumulative radioactivity in each of the subcellular compartments (\(A (\text{Bq}\cdot\text{sec})\)) was estimated from the kinetic data, assuming receptor saturation with \(^{111}\text{In-}\) and \(^{177}\text{Lu-DOTATOC}\) (specific activity 9 MBq/\mu g). The mean radiation-absorbed dose to the nucleus \([D (\text{Gy/Bq}\cdot\text{sec})]\) was calculated and compared using the cellular dosimetry model of Goddu et al.16,17

Relative antiproliferative potency of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\)

The antiproliferative potency of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) for HEK-293 cells was measured using the WST-1 cell proliferation assay (Roche Diagnostics, Laval, QC). This assay measures the conversion of the WST-1 tetrazolium salt to a colored formazan complex by mitochondrial dehydrogenase in viable cells. Increasing amounts (0.3-20 ng) of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) (specific activity 6-9 MBq/\mu g) were incubated for 4 hours at 37°C with 5\times 10^5 cells contained in 500 \mu L of 150 mM NaCl in 1.5 mL Eppendorf tubes. Controls consisted of HEK-293 cells exposed to unlabeled DOTATOC (20 ng) or \(^{111}\text{In-}\) or \(^{177}\text{Lu-ace}\) (using an amount of radioactivity equivalent to that complexed to 20 ng of DOTATOC) or cells exposed to 150 mM NaCl vehicle. Following exposure, the cells were recovered, counted, and seeded into wells in a 96-well plate at a density of 8\times 10^3 cells/well. Growth medium was added (200 \mu L/well) and the plates incubated at 37°C and 5% CO\(_2\) for 4 days. In a separate experiment, HEK-293 cells were exposed to 60 ng of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) and their growth was measured over 8 days. Finally, 10 \mu L of WST-1 reagent was added to each well, and the plates were incubated for 3.5 hours at 37°C. An additional 100 \mu L of 150 mM NaCl was then dispersed into each well, and the absorbance was measured at 400 nm in a plate reader (BioTek Synergy 2, Winosook, VT) with subtraction of the absorbance at 600 nm (reference wavelength).

Antiproliferative effects of low-MOI vVDD

The antiproliferative effects of low-MOI (0.1) vVDD on HEK-293 or MC-38 cells in monolayer were evaluated by adding 5\times 10^5 pfu of vVDD in 3.0 mL of growth medium containing 2.5% FBS to 5\times 10^5 cells in 175 flasks. After 2 hours, 8 mL of growth medium was added and the cells cultured for 48 hours at 37°C. In preliminary experiments, 48 hours was found to be a suitable time period for vVDD exposure at these MOI values. Longer exposure times greatly reduced cell viability (results not shown). The vVDD-treated cells were recovered, counted, and seeded into wells at a density of 8\times 10^3 cells/well in a 96-well plate and cultured for up to 8 days at 37°C and 5% CO\(_2\). HEK-293 or MC-38 spheroids were similarly infected with vVDD at MOI = 0.1 for 48 hours, and then incubated for 24 hours with the radiopharmaceuticals. The spheroids were then dissociated by trypsinization and plated at 8\times 10^3 cells/well into 96-well plates and cultured for up to 8 days at 37°C and 5% CO\(_2\). The growth of cells exposed to vVDD in monolayer or as spheroids was measured by the WST-1 assay as described earlier.

Combined antiproliferative effects of \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) and vVDD

The effects of combining low-\*infectivity (MOI = 0.1) amounts of vVDD with \(^{111}\text{In-}\) or \(^{177}\text{Lu-DOTATOC}\) (60 ng) on the growth of HEK-293 cells exposed in monolayer or HEK-293 or MC-38 cells grown as spheroids were studied. Infected cells were exposed to the radiopharmaceuticals for 24 hours. Monolayers and spheroids were dissociated by
trypsinization and cultured for up to 8 days at 37°C and 5% CO₂. Cell growth was measured by the WST-1 assay.

**Statistical analysis**

Statistical comparisons were made using Student’s t-test ($p < 0.05$).

**Results**

**Levels of sstr-2 expression on cell lines**

$^{111}$In- and $^{177}$Lu-DOTATOC bound with high affinity to HEK-293 cells stably transfected with the sstr-2 gene, with $K_d$ values of $8.4 \pm 2.7$ and $11.4 \pm 1.2$ nmol/L, respectively (not significantly different; $p > 0.05$, $n = 4$). The $B_{max}$ values were $(1.4 \pm 0.1) \times 10^6$ and $(1.1 \pm 0.1) \times 10^5$ receptors/cell for $^{111}$In- and $^{177}$Lu-DOTATOC, respectively (not significantly different; $p > 0.05$, $n = 4$). Based on the flow cytometry analysis, there was a 2-fold greater expression of sstr-2 on MC-38 cells infected with vVD for 48 hours than on noninfected MC-38 cells (MFI = 10.0 and 6.0, respectively; Fig. 1). By comparison with the flow cytometry results for HEK-293 cells (MFI = 6.0), there were $\approx 2 \times 10^3$ sstr-2/cell on vVD-infected MC-38 cells.

**Subcellular distribution and radiation dosimetry of $^{111}$In- and $^{177}$Lu-DOTATOC**

On incubating HEK-293 cells with $^{111}$In-DOTATOC at 37°C, approximately 75%-80% of radioactivity remained on the cell membrane, 20%-25% was internalized, and 2%-5% was imported into the nucleus from 4 to 24 hours (Fig. 2). There were no significant changes in these fractions of radioactivity over this time period. A similar distribution of radioactivity was observed for $^{177}$Lu-DOTATOC at 4 hours (74% on the cell membrane, 22% in the cytoplasm, and 3.6% in the nucleus). For radiation dosimetry calculations, $^{111}$In- or $^{177}$Lu-DOTATOC distribution was assumed to be 75% membrane bound, 20% in the cytoplasm, and 5% in the nucleus, with very rapid uptake (based on the results shown in Fig. 2) and elimination from subcellular compartments only by radioactive decay. Based on these assumptions, the radiation-absorbed dose deposited in the nucleus of HEK-293 cells was estimated to be 3.5 times higher for $^{177}$Lu-DOTATOC than for $^{111}$In-DOTATOC (3.46 vs. 1.05 Gy; Table 1).

**FIG. 2.** Percent of cell-bound radioactivity localized to the cell membrane, internalized into the cytoplasm, or present in the nucleus of human embryonic kidney-293 cells incubated for selected times at 37°C with $^{111}$In-DOTATOC.
$^{111}$In- AND $^{177}$Lu-DOTATOC AND VACCINIA VIRUS

<p>| Table 1. Radiation-Absorbed Doses Deposited in the Nucleus of Human Embryonic Kidney-293 Cells from the Auger Electrons Emitted by $^{111}$In-DOTATOC or the $\beta$-Particles Emitted by $^{177}$Lu-DOTATOC |</p>
<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>$\tilde{A}^a$ (Bq/sec)</th>
<th>$S^b$ (Gy/Bq/sec/10^{-9})</th>
<th>$D^c$ (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-DOTATOC</td>
<td>Membrane 6290</td>
<td>0.77</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm 1680</td>
<td>1.18</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Nucleus 419</td>
<td>9.09</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1.05</td>
<td>3.46</td>
</tr>
<tr>
<td>$^{177}$Lu-DOTATOC</td>
<td>Membrane 15,000</td>
<td>1.32</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm 4000</td>
<td>2.03</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Nucleus 1000</td>
<td>6.71</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3.67</td>
<td>3.46</td>
</tr>
</tbody>
</table>

$^a$ It was assumed that a single human embryonic kidney-293 cell with $1 \times 10^3$ somatostatin subtype-2 receptor/cell would be targeted to receptor saturation using $^{111}$In- or $^{177}$Lu-DOTATOC (specific activity 9 MBq/mg) and that there would be rapid binding and distribution to the subcellular compartments with elimination only by radioactive decay $[A = A_0 e^{-\lambda t}]$, where $\lambda = 2.9 \times 10^{-6}$/sec$^{-1}$ for $^{111}$In and $1.2 \times 10^{-6}$/sec$^{-1}$ for $^{177}$Lu. It was assumed that 75% of the radioactivity would be present on the cell membrane, 20% in the cytoplasm, and 5% in the nucleus (Fig. 2). $^b$-Values were obtained from References 16,17 using a measured cell radius of $8 \mu m$ and nucleus radius of $6 \mu m$. $^c$-Dose to the nucleus from radioactivity in the indicated subcellular compartment.

**Relative antiproliferative potency of $^{111}$In- or $^{177}$Lu-DOTATOC**

There were no significant differences in the ability of $^{111}$In- or $^{177}$Lu-DOTATOC (specific activity 6-9 MBq/\mu g) to decrease the growth of HEK-293 cells exposed to increasing amounts (0.3-20 ng) of these radiopharmaceuticals in monolayer and cultured for 4 days (Fig. 3A). The amounts of $^{111}$In- or $^{177}$Lu-DOTATOC incubated with $5 \times 10^5$ cells corresponded to a 2- to 150-fold excess, based on the measured str-2 density ($1 \times 10^7$ receptors/cell). The mean (±standard error of mean [SEM]) growth of HEK-293 cells exposed to 20 ng of $^{111}$In-DOTATOC was 63.4% ± 5.1%, compared to $53.6\% \pm 0.4\%$ for those exposed to $^{177}$Lu-DOTATOC ($p = 0.10$). The growth of HEK-293 cells exposed to unlabeled DOTATOC (20 ng) or an amount of $^{111}$In- or $^{177}$Lu-acetate equivalent to the radioactivity bound to DOTATOC was $87.3\% \pm 7.0\%$, $91.9\% \pm 14.3\%$, and $71.9\% \pm 6.0\%$, respectively. In a separate experiment using 60 ng of the radiopharmaceuticals, $^{177}$Lu-DOTATOC was not significantly more effective at inhibiting the growth of HEK-293 cells than $^{111}$In-DOTATOC for up to 4 days (37.6% ± 1.6% vs. 29.8% ± 9.5%, respectively; $p = 0.30$, Fig. 3B). However, regrowth of HEK-293 cells exposed to $^{111}$In-DOTATOC at 7 days was significantly greater than for cells exposed to $^{177}$Lu-DOTATOC (66.3% ± 11.3% vs. 16.9% ± 2.0%, respectively; $p = 0.05$).

**Antiproliferative effects of low-MOI vDd**

The effects of low-infectivity (MOI = 0.1) amounts of vDd on the growth of HEK-293 cells exposed in monolayer or as spheroids, or MC-38 cells exposed as spheroids are shown in

![Image](image-url)

**FIG. 3.** (A) Effect of increasing amounts of $^{111}$In- or $^{177}$Lu-DOTATOC (specific activity 6-9 MBq/\mu g) on the growth of HEK-293 cells cultured for 4 days. (B) Effect of 60 ng of $^{111}$In- or $^{177}$Lu-DOTATOC on the growth of HEK-293 cells cultured for up to 8 days. HEK, human embryonic kidney.
FIG. 4. Effect of low multiplicity-of-infection (=0.1) double-deleted vaccinia virus (vDD) exposure on the growth of HEK-293 or MC-38 cells in monolayer or spheroid culture for up to 8 days. HEK, human embryonic kidney.

Figure 4. Following 48 hours of exposure to vDD and subsequent culture for up to 3 days, there was a modest (10%–20%) decrease in the growth of vDD-infected cells compared to noninfected cells. However, after 8 days in culture, the growth of vDD-infected cells reduced to <20%. There was no significant difference in the antiproliferative effects of vDD when HEK-293 cells were exposed as monolayer or as spheroids, or between HEK-293 cells and MC-38 cells grown as spheroids.

Combined antiproliferative effects of \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC and vDD

The effect of \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC combined with vDD on the growth of HEK-293 cells exposed in monolayer and then cultured for up to 7 days is shown in Figure 5A. The mean growth (±SEM) of HEK-293 cells was reduced to 27.8%±2.3% by \(^{111}\text{In}\)-DOTATOC + vDD and to 6.6%±1.3% by \(^{177}\text{Lu}\)-DOTATOC + vDD at 7 days (p = 0.015). The growth of HEK-293 cells cultured for 8 days following exposure as spheroids was decreased to 15.1%±4.1% by \(^{111}\text{In}\)-DOTATOC + vDD and to virtually zero by \(^{177}\text{Lu}\)-DOTATOC + vDD (Fig. 5B). Similarly, the growth of MC-38 cells cultured for 8 days after exposure as spheroids to \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC combined with vDD was reduced to 4.9%±0.01% and 5.0%±0.3%, respectively (p = 0.77; Fig. 5C).

A comparison of the antiproliferative effects of \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC or vDD or these agents in combination on MC-38 cells cultured following exposure as spheroids is shown in Figure 6. At 2 days, growth of MC-38 cells exposed to \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC single treatments decreased significantly compared to cells exposed to vDD alone (84.0%±0.8% or 80.2%±3.3%, respectively, vs. 98.1%±2.9%; p = 0.023 and p = 0.022). Growth of MC-38 cells exposed to \(^{111}\text{In}\)-DOTATOC combined with vDD was also significantly less compared to cells exposed only to \(^{111}\text{In}\)-DOTATOC (75.7%±0.6% vs. 84.0%±0.8%; p = 0.014). However, there was no significant difference in the growth of cells exposed to \(^{177}\text{Lu}\)-DOTATOC combined with vDD compared to cells exposed to \(^{177}\text{Lu}\)-DOTATOC alone at this time point (79.8%±5.2% vs. 80.2%±3.3%; p = 0.95). At 8 days, combining vDD with \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC significantly decreased the growth of MC-38 cells from 18.0%±1.6% to 4.9%±0.1% or 5.0%±0.4%, respectively (p = 0.008 or p = 0.008). At this time point, there was no significant difference in the growth of MC-38 cells exposed to vDD combined with \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC or single treatments with these radiopharmaceuticals (4.9%±0.1% or 3.7%±1.2%; p = 0.85 or p = 0.41).

Discussion

Infection with vDD (MOI = 0.1) augmented sstr-2 expression on MC-38 cells by 2-fold as revealed by flow cytometry (Fig. 1). This increase in sstr-2 density corresponded to \(\sim 2 \times 10^5\) receptors/cell and was comparable to that previously reported (2.7-fold increase) for MC-38 cells.

FIG. 5. Effect of low multiplicity-of-infection (=0.1) double-deleted vaccinia virus (vDD) exposure combined with 60 ng of \(^{111}\text{In}\)- or \(^{177}\text{Lu}\)-DOTATOC (specific activity 6–9 MBq/μg) on the growth of (A) HEK-293 cells in monolayer culture for 7 days, (B) HEK-293 cells in spheroid culture, and (C) MC-38 cells in spheroid culture. HEK, human embryonic kidney.
exposed \emph{in vitro} to vvDD at a higher MOI (=1) measured by radioligand binding assays using $^{111}$In-pentetretidine.\textsuperscript{7} A balance must be achieved between host cell infection and viral ssr-2 gene expression and the powerful oncolytic action of vvDD.\textsuperscript{8} Extensive cell lysis by vvDD may prevent targeting of endogenous as well as virally encoded ssr-2 by $^{111}$In- or $^{177}$Lu-DOTATOC. Radiopharmaceutical targeting is required to take advantage of the potential bystander and cross-fire effects of these agents \emph{in vivo} on noninfected tumor cells, which could spatially enhance the antitumor effects of oncolytic viral therapy. Brader et al.,\textsuperscript{9} employing an analogous engineered GLV-1h99 vaccinia virus encoding the hNET gene, concluded that there was a 24–48 hour “window” in infected MSTO-211H mesothelioma host cells, during which sufficient hNET transporters were displayed for targeting with $^{131}$I- or $^{125}$I-MIBG but prior to cell lysis caused by the virus. In the present study, viability of MC-38 cells was relatively preserved for up to 3 days following a short, 48-hour exposure to vvDD (MOI = 0.1), with <10%–20% decreased cell growth compared to noninfected cells (Fig. 4). After 8 days, vvDD diminished the viability of MC-38 cells to <20%. Brader et al.\textsuperscript{9} similarly found no significant decrease in the viability of MSTO-211H cells for up to 2 days after infection with GLV-1h99 vaccinia virus (MOI = 0.1), but 98% decreased cell survival at 7 days.

VvDD encoding the ssr-2 gene could be combined with $^{111}$In- or $^{177}$Lu-DOTATOC to exploit the Auger electron or $\beta$-particle emissions, respectively, but the relative potency of these two analogs to kill cells expressing ssr-2 has not been reported. Clinically, $^{177}$Lu-DOTATATE is more effective in producing tumor responses than $^{111}$In-pentetretide in patients with ssr-2-positive malignancies.\textsuperscript{10} Nonetheless, Auger electron emitters can cause extensive and lethal DNA damage in cells in which they are internalized, especially if these radionuclides are imported into the cell nucleus.\textsuperscript{11–14} Auger electron emitters have also been associated with a bystander effect on proximal nontargeted cells.\textsuperscript{15,16,21,22} $\beta$-emitters would similarly be expected to have a bystander effect, could also kill distant nontargeted cells through a cross-fire effect.\textsuperscript{11,22} In this study there were no significant differences in the growth inhibition of HEK-293 cells by $^{111}$In- or $^{177}$Lu-DOTATOC (60 ng) up to 4 days in culture (Fig. 3A). Radiation this time period $^{111}$In-DOTATOC-treated cells exhibited regrowth, reaching >66% of that of untreated HEK cells at 7 days (Fig. 3B). In contrast, $^{177}$Lu-DOTATOC-treated cells did not proliferate up to 7 days. Based on the subcellular distribution of $^{111}$In-DOTATOC (Fig. 2), HEK-293 cells exposed to $^{111}$In-DOTATOC were estimated to receive a 3.5-fold lower radiation dose to the nucleus than cells exposed to $^{177}$Lu-DOTATOC (Table I). The distribution of $^{177}$Lu-DOTATOC was measured only at 4 hours, but since this was similar to that of $^{111}$In-DOTATOC at this time point, it was assumed for radiation dose estimation that there would be no differences in the subcellular distribution of these two radionuclides over their lifetimes. However, such differences if present would affect comparison of radiation-absorbed doses for $^{111}$In- and $^{177}$Lu-DOTATOC. The lower calculated average radiation-absorbed doses deposited by $^{111}$In-DOTATOC were mainly due to the small percentage of radioactivity localized in the nucleus (<5%; Fig. 2), but these doses may have been sublethal, allowing cell regrowth. Use of DOTATOC analogs modified with nuclear-localization sequence peptides as reported by Gini et al.\textsuperscript{23} could greatly increase the radiation absorbed doses and enhance the lethality of $^{111}$In-DOTATOC.

The greater antiproliferative potency of $^{177}$Lu-DOTATOC compared to $^{111}$In-DOTATOC on HEK-293 cells exposed in monolayer up to 7 days was also evident when combined with vvDD (Fig. 5A). In contrast, equivalent growth inhibition was observed for HEK-293 or MC-38 cells exposed as spheroids to $^{111}$In- or $^{177}$Lu-DOTATOC combined with vvDD (Fig. 5B, C). The increased effectiveness of $^{111}$In-DOTATOC-vvDD combination therapy toward HEK-293 or MC-38 spheroids compared to monolayer exposure may be attributed to a bystander effect more prominent in these three-dimensional cultures.\textsuperscript{24} Bystander effects have been described for cells expressing hNET exposed as monolayers or spheroids to $^{125}$I-MIBG, $^{131}$I-MIBG, or $^{211}$At-MABG, which emit Auger electron, $\beta$-particle, or $x$-radiation, respectively.\textsuperscript{11,25} The cross-fire effect from $^{177}$Lu-DOTATOC on MC-38 or HEK-293 spheroids could potentially enhance cell killing compared to $^{111}$In-DOTATOC, which does not have such an effect. However, the contribution of this effect may have been minimized by the short incubation time (24 hours) before cell dissociation and subsequent culturing, or by the size of the spheroids (400 µm in maximum diameter), which would cause some $\beta$-radiation (mean path length 2 mm) to be deposited outside the spheroid volume. One limitation of exposing MC-38 spheroids to low-MOI (=0.1) vvDD was that almost 20% of these cells remained viable after 8 days (Fig. 6). However, combining vvDD with $^{111}$In- or $^{177}$Lu-DOTATOC reduced this proportion to <5%. This suggests that this combined treatment might assure a low percentage survival in vvDD-infected and radiopharmaceutical-targeted cells in tumors \emph{in vivo}, while allowing the destruction of distant noninfected or nontargeted cells through bystander and/or cross-fire effects.
Although vVDD effectively killed MC-38 cells exposed \textit{in vitro} in monolayer or as spheroids, requiring a reduction in MOI to minimize the oncolytic effects and to allow expression of \textit{str} gene for targeting with \textsuperscript{111}In- or \textsuperscript{177}Lu-DOTATOC, this may not be the case when this virus is used \textit{in vivo} to treat tumors. Infection of all tumor cells in a lesion may not be easily achieved due to limitations in penetration owing to the relatively large size of the virus (20-30 nm in diameter). Moreover, stimulation of the immune system may arrest viral spread within a lesion, further contributing to heterogeneities in the oncolytic effects of the virus, although recruitment of the immune system may aid in tumor destruction. Nonetheless, combining vVDD encoding the \textit{str} gene with \textsuperscript{111}In- or \textsuperscript{177}Lu-DOTATOC may help address tumor infectivity barriers by permitting killing of distant noninfected cells through bystander and/or cross-fire effects as discussed. These issues will be examined in future studies in which vVDD will be combined with \textsuperscript{111}In- or \textsuperscript{177}Lu-DOTATOC for the treatment of MC-38 tumors \textit{in vivo} in immunocompetent C57BL mice. Ultimately, the aim is to use combined oncolytic virotherapy and targeted radiotherapy in the treatment of tumors in humans. It is encouraging that a Phase I trial of another oncolytic vaccinia virus has recently commenced.\textsuperscript{27}

Conclusions

To conclude, \textit{in vitro} exposure of MC-38 cells to low-MOI vVDD encoding the \textit{str} gene for 48 hours enhanced the expression of these receptors while minimizing the oncolytic effects of the virus for 3 days in culture. Although \textsuperscript{111}In-DOTATOC was less effective than \textsuperscript{177}Lu-DOTATOC in inhibiting cell growth in monolayer, these agents exhibited equivalent cytotoxicity alone or in combination with vVDD on HEK-293 or MC-38 cells grown as spheroids. The greater effectiveness of \textsuperscript{111}In-DOTATOC in spheroid cell cultures may be because of a potent bystander effect. These results warrant further testing \textit{in vivo} of low-infectivity amounts of vVDD combined with these radiotherapeutic agents for treatment of MC-38 tumors in immunocompetent C57BL6 mice. Ultimately, the aim is to translate combined oncolytic vVDD virotherapy and targeted radiotherapy of tumors to phase I trial in humans.

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Disclosure Statement

No competing financial interests exist.

References


2. Kweekeboom DJ, De Herder WW, Kam BL, et al. Treatment with the radionuclide somatostatin analog [\textsuperscript{177}Lu-DOTA\textsubscript{2}Tyr\textsuperscript{3}] octreotate: Toxicity, efficacy, and survival. J Clin Oncol 2008;26:2124.


12. Storch D, Béche M, Walker MA, et al. Evaluation of \textsuperscript{99m}Tc/EDDA/HYNIC\textsuperscript{1}octreotide derivatives compared with \textsuperscript{111}In-DOTATOC,Tyr\textsuperscript{3},Ther\textsuperscript{5}octreotide and \textsuperscript{111}In-\textsuperscript{DTA}\textsuperscript{-5}octreotide: Does tumor or pancreas uptake correlate with the rate of internalization? J Nucl Med 2005;46:1361.


Appendix 2.1 Schematic of vvDD-RFP genome

Sites of left (5’TK) and right (3’TK) segments of vaccinia thymidine kinase gene, synthetic late promoter (pSynL), synthetic early/late promoter (Pse/l), 7.5 promoter (p7.5), human somatostatin receptor 2 (hSSTR2) gene and xanthine-guanine phosphoribosyl transferase (gpt) gene are indicated. The vaccinia growth factor (VGF) sites within the inverted terminal repeats are interrupted by the insertion of the lacZ gene.
Appendix 2.2. Method for calculating the vessel distribution index

A) Total tumor area was divided into two regions, A_i (inner area) and A_o (outer area) with between 49.01-50.99% of the total area included in A_i. Multiple tumors per mouse (range 2 – 15 tumors) were analyzed individually (using the same settings). The total number of positive pixels in each region (P_i and P_o) was determined using ImageScope software.  

B) The vessel distribution index (VDI) was used to measure the relative distribution of CD31 positive staining pixels for each individual tumor.  

C) To ensure our data reflected the 3-dimensional distribution of VV, positive staining in A_i was determined at three levels 100 to 250 µm apart. The positive staining in A_i for each tumor was calculated as the average staining in A_i over all three levels. Multiple tumors per mouse were analyzed and the overall average of VV staining in A_i was compared between mice treated on day 8 or day 12.

\[
\text{VDI} = \frac{P_o / A_o}{P_i / A_i}
\]

- VDI = 1, even distribution
- VDI > 1, more staining in outer area
- VDI < 1, more staining in inner area

\[A_i = \text{Inner area}\]
\[A_o = \text{Outer area}\]
\[P_i = \text{Positive pixels in inner area}\]
\[P_o = \text{Positive pixels in outer area}\]
Appendix 2.3 RT-PCR for VV in the blood

Viral genome in the plasma was quantified by RT-PCR at the indicated time points (n = 2 at 1 - 6 hr and n = 1 at 24h). Bars represent the mean ± SD.
Appendix 2.4 Supplementary Methods

Viral DNA Extraction. Blood samples were collected by cardiac puncture immediately prior to sacrifice. Samples were centrifuged, red blood cell pellets were discarded and supernatant (plasma) was stored at -80°C until further analysis. Lysis buffer was added to thawed samples to a total volume of 600 µl. Proteinase K (0.2 mg/ml; Fermentas International INC., Burlington, Ontario, Canada) was added and tubes were incubated at 55°C overnight. DNA was extracted using a standard phenol/chloroform protocol. DNA was precipitated from the aqueous phase in 2-propanol with glycogen (0.05 µg/ml) following incubation on a rotating rack for 20 minutes at room temperature. Samples were centrifuged at 10 000 xg for 10 minutes and the supernatant was discarded. DNA pellet was washed with 70% ethanol and air-dried. Pellets were rehydrated in 0.1x TE buffer. DNA concentration was determined by spectrophotometry.

Real-time Polymerase Chain Reaction. Real-time (RT) PCR was performed using LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics, Laval, Quebec Canada) as per the manufacturers protocol. The following primers, directed to vaccinia virus a34 gene, were used: TGGCATAGGAACATTTCTGCAT (forward) TGTGTATCGTATTGTATCCATCCATTG (reverse). All PCRs contained 5 µg of sample DNA, 15ul of PCR Reaction Mixture (4 µl Light Cycler® Master Mix + 10 µl PCR-grade water + 0.5 µl of each primer). Samples were run on a LightCycler® 1.5 (Roche Diagnostics). Runs included one positive (viral DNA at known concentration) and two negative (water) controls. Total viral particles in each sample were calculated based on comparison of sample CP values to a previously generated standard curve. Viral particle values were converted to pfu based on the previously established ratio of $1.2 \times 10^{10}$ virus particles is equivalent to $5 \times 10^8$ pfu.
Appendix 3.1. Effect of OX on vvDD viral gene expression and replication

Viral RFP expression was evaluated in the presence and absence of OX. Cells were infected at MOI 0.1 and treated with increasing concentrations of drug. Fluorescence microscopy was performed at 24 – 72h post treatment (a) and virus replication was determined (b-d). Data represents the mean plaque forming units (pfu) of triplicate values ± SD. *p < 0.05.
Appendix 3.2. Cytotoxicity and synergy in DLD1 cells treated with vvDD and SN-38 at a 1:1 dose ratio

Dose response (A) and drug-drug interactions (B) were evaluated in DLD1 cells treated with vvDD and SN-38 at a 1:1 (MOI:µM) dose ratio. (B) The combination index (CI) was less than 1 at all doses tested (0.001, 0.01, 0.1, 0.5, 1) indicating synergistic cell killing. Data represent mean fraction viability relative to DMSO treated controls ± SEM (A) or CI at the doses tested.
Appendix 3.3. Effect of vvDD and SN-38 on cell cycle in HT29 cells

HT29 cells were treated with vvDD (MOI = 0.1) and/or SN-38 (0.02 μM) and analysed by flow cytometry following DAPI staining at 48 hpi. The experiment was performed in triplicate and bars represent the mean ± SD. * p < 0.05, one-way ANOVA with bonferroni’s multiple comparison test.
Appendix 3.4. Combination vvDD and CPT-11 therapy in a syngenic model of PC

C57BL/6 mice bearing IP MC38 tumors were treated with IP VV (day 12; $10^9$ pfu) and/or IP CPT-11 (day 10, 14, 18, 22; 30 mg/kg) or vehicle alone. Survival was significantly improved in all treatment groups relative to the control group, however combination therapy was not better than either monotherapy. Median survival was: 21 d (HBSS), 26 d (CPT-11, $p = 0.0003$), 24 d (vvDD, $p = 0.023$) and 26.6 d (vvDD + CPT-11, $p = 0.0003$).
Appendix 4.1. SSTR-2 expression in a syngeneic PC model treated with vvDD-SSTR2

C57BL/6 mice bearing IP MC38 tumours were treated with vvDD-SSTR2 (10^9 pfu) on day 12 and tumours were harvest at 4, 6 and 8 dpi. Tumours were fixed in formalin and stained with anti-VV (left panel) or anti-SSTR2 (right panel) antibodies.