COMBINATION $^{111}$In AND $^{177}$Lu –DOTATOC AND VACCINIA VIRUS ONCOLYTIC THERAPY FOR SSTR2-POSITIVE TUMOURS

By

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A THESIS SUBMITTED IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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Combination of $^{111}$In and $^{177}$Lu-DOTATOC and Vaccinia Virus Oncolytic Therapy for sstr2-Positive Tumours

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Abstract

Radiolabeled somatostatin analogues based on octreotide have proven useful in the management of somatostatin receptor subtype 2 (sstr2)-positive tumours in clinical trials. The aim was to compare the potency and evaluate the combination of $^{111}$In- and $^{177}$Lu-DOTATOC with a double-deleted version of vaccinia virus (ddVV), an oncolytic virus for inhibiting the growth of sstr2-expressing human embryonic kidney (HEK-293) cells or MC-38 murine colon cancer cells grown as monolayers or as spheroids. Cytotoxicity studies were carried out using ddVV, $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC, individually or in combination on MC-38 spheroids, HEK-293 cells and spheroids. HEK-293 cell growth in spheroids was reduced to 17.2 ± 4.9% and 26.5 ± 6.3 % with $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC alone and 13.1 ± 7.1% and 0% in combination, respectively. MC-38 spheroids showed greater toxicity in combination treatment. Combination of ddVV with $^{111}$In- or $^{177}$Lu-DOTATOC is only advantageous in monolayer culture. No advantage was observed in spheroid models.
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List of Abbreviations

CAR – Coxsackie virus – adenovirus receptor
CEV – Cell-associated enveloped virus
ddVV – double-deleted vaccinia virus
DOTA – 1, 4, 7, 10-tetraazacyclododecane-N, N’, N”, N”’-tetraacetic acid
DOTATOC – DOTA conjugated to Tyr³ Octreotide
DTPA – diethylenetriaminepentaacetic acid
EEV – Extracellular enveloped virus
EnGFR – Endothelial growth factor receptor
GBq – gigabecquerel (unit of radioactivity)
GEP – Gastroenteropancreatic tumours
GM-CSF – Granulocyte-macrophage-colony-stimulating factor
GPCR – G-protein coupled receptor
HEK-SSTR2 – Human embryonic kidney cells expressing somatostatin receptor subtype 2
HSV-1 – Herpes simplex virus 1
IC – Internal conversion
ICAM1 – Intercellular adhesion molecule
IEV – Intracellular enveloped virus
IFN – Interferons
IMV – Intracellular mature virus
IV – Immature virions
mCi – millicurie (unit of radioactivity)
NLS- Nuclear localisizing sequence peptide
NDV – Newcastle disease virus
NEMT – Neuroendocrine Malignancies
OV – Oncolytic virus
PFU – plaque forming unit
RT-PCR – Reverse transcription – polymerase chain reaction

SFV – Semliki forest virus

SRIF – Somatotropin release inhibiting factor

SSTR2 – Somatostatin receptor subtype 2

TK – Thymidine kinase

VEGF – Vascular endothelial growth factor

VSV – Vesicular stomatitis virus
CHAPTER 1

INTRODUCTION
1.1 Somatostatin and Somatostatin Receptors

1.1.1 Somatostatin receptors

Somatostatin receptors (SSTRS) are members of the G-protein coupled receptor family (GPCR). Based on structure, there are 3 different classifications within this receptor family which include the glucagon-receptor-like family, the GABA-receptor-like family and the rhodopsin-like family of which somatostatin receptors are members. The GPCRs contain 7 transmembrane alpha helices connected by six loops that have an extracellular domain for binding of their ligands and an intracellular domain for signal transduction. Activation of these receptors by the ligands results in the transmission of signals to their intracellular domain, which subsequently gives rise to the inhibition of adenyl cyclase activity. This inhibition thereby causes a decrease in intracellular cyclic AMP levels, a reduction in the activity of calcium channels and, consequently, a reduction in intracellular calcium levels, cell proliferation, activation of tyrosine phosphatases and, finally, an upregulation of antimitotic activities. Other induced signal transduction pathways include the Na⁺ -H⁺ exchangers, Src, Erk 1 and 2, MAP kinase, p38 mitogen activation, and protein kinase. Changes in the regulation of these pathways due to activation of sstr2 lead to responses such as the inhibition of cell proliferation, inhibition of endocrine and exocrine secretion, modulation of neurotransmission, inhibition of motor and cognitive functions, decreased intestinal motility, absorption of nutrients and ions, and vascular contractility.

1.1.2 Somatostatin receptor subtypes

To date, a total of 5 somatostatin receptor subtypes have been cloned using immunohistochemistry methods, in vivo mRNA purifications, RT-PCR analyses, and receptor binding assays. These receptors are named in the chronological order of their discovery, i.e. numbers 1 through 5. All five subtypes are known to have different origins due to their presence on different chromosomes in various tissues. They are structurally related as GPCRs, but functionally different. The functional difference is depicted by the variations in their amino and carboxyl terminal sequences which result in different binding affinities and specificities to ligands, and differences in intracellular signals transmitted upon activation. The most commonly expressed subtype is sstr2 which is found in the pancreatic islets, specific regions of the brain, the adrenal glands and the kidneys as well as in the peripheral nervous system and the immune system.
System \(^{9-11}\). Sstr3 and sstr5 are found mainly in lymphocytes whereas sstr4 is found in human placenta and fetal and adult lung tissue.

**Fig 1.1: Schematic of Signal transduction of somatostatin receptors**

1.1.3 Mechanism of action

Several hypotheses have been proposed regarding the fate of the somatostatin receptor before and after activation by the ligand. A study conducted by Grant et al.\(^{12}\) showed that GPCRs assemble on the cell membrane as monomeric, homodimeric, and heterodimeric units, depending on the family of GPCRs to which they belong. Upon activation they tend to dimerize, remain unaffected, or dissociate into monomeric units. It was discovered that receptor dimerization is
expressed differently within the GPCR family and is a function of ligand binding, signaling, receptor desensitization, and receptor trafficking. Somatostatin receptors dissociate into monomers upon extracellular ligand binding in a concentration-dependent manner, and then transmit signals in the intracellular region. This leads to rapid phosphorylation in the intracellular region by the GPCR kinases, followed by the recruitment and binding of β-arrestin1 and β-arrestin2 to the receptor. Binding of the arrestins culminates in endocytosis of the receptor-ligand complex by clathrin-coated vesicle formation. The ligand dissociates from the receptor due to the acidified lysosomes. Receptors are desensitized and recycled back to the membrane; the ligands are left in the intracellular compartment of the cell.

1.1.4 Ligand-dependent internalization of the receptors.

As mentioned above, receptor internalization and desensitization are a major part of the mechanism of action of this family of receptors; however, the kinetics of internalization vary among the different subtypes. It has been argued that this difference in kinetics is due to the nature and concentration of GPCR kinases present in different cell types, the varying concentrations of arrestins among different cell types, and the strength of the arrestin-receptor-ligand complex bond.

Receptor internalization is an important factor in molecularly-targeted radiotherapy of sstr-positive cells especially when radioisotopes with short energy range, for example $^{111}$In, are used. Froidevaux and Reubi further suggested that downregulation of SSTR could be induced by receptor internalization. In a related study, Luttrell and colleagues argued that although the binding of β-arrestin and receptor internalization are both processes that result in receptor downregulation, these are the same processes that are required for the initiation of mitogenic signals from GPCRs.

1.1.5 Somatostatin receptors in tumours.

The attraction of SSTRs in oncological research is the abundance of certain subtypes in tumours, thereby creating a platform that could and has been used to target these malignancies. Such targeted therapeutic uses rely on the anti-angiogenic and antiproliferative properties of the receptors previously discovered in normal cells expressing the receptors. An example of drug that utilizes these properties are sandostatin and lanreotide (somatostatin analogues used for acromegaly and gastroenteropancreatic neuroendocrine malignancy GEP NEM therapy).
More recently, the therapeutic uses of these receptors have shifted towards their ability to be used as routes to shuttle toxic materials into malignant cells, potentially causing more damage to the tumour. Examples of these toxic materials include radionuclides discussed in detail later in this chapter.

Among the tumours that express SSTRs are the neuroendocrine malignancies (NEM): islet cell tumours, carcinoids, paragangliomas, pheochromocytomas, medullary thyroid carcinomas, and gastric tumours. Other tumours that express SSTRs include pituitary adenocarcinomas, small cell lung cancer, breast carcinomas, lymphomas, renal cell cancers, brain tumours, prostate cancer, ovarian cancer, hepatocellular cancer, and nasopharyngeal carcinoma, most of which express subtype 2. An advantage of targeting SSTRs in malignant cells compared with normal cells is the overexpression of these receptors and also the multiple expression of more than one subtype of the receptor. This characteristic, indeed, allows for increased targeting of the ligands by using more than one somatostatin analogue which can also target the other subtypes of the receptor on these tumour cells for therapeutic purposes.

1.1.6 Somatostatin and somatostatin analogues

Somatostatin, also known as somatotropin-release-inhibiting factor (SRIF), refers to a group of regulatory peptide hormones which are either 14 or 28 amino acids long. Both peptide subtypes are derived from one gene. Prosomatostatin is enzymatically cleaved to produce the ss-14 or ss-28 peptide. The discovery of somatostatin dates as far back as the 1970s when ss-14 was first discovered. It was known mainly as a neurotransmitter found in the central nervous system as well as in the cerebral cortex, the hypothalamus and the brain stem. Later studies showed the presence of somatostatin in the gastrointestinal tract and the pancreas. The ss-28 peptide is believed to be the circulating form of the hormone. Other cells producing somatostatin include those of the thyroid gland, adrenal gland, submandibular gland, the kidneys, prostate gland, and placenta. Both peptide subtypes are responsible for inhibiting the release of growth hormone, insulin, glucagon, and gastrin – all inhibition properties exhibited by SSTRs.

Since the discovery of the presence of SSTRs in malignant tumours, the potential use of somatostatin as a growth-inhibitory ligand was prevented by its short in vivo half-life of only 3 minutes. This short half-life is due to the presence of naturally occurring L-isomer amino acid residues that are susceptible to endogenous peptidase and protease cleavage once administered into the systemic circulation. Subsequently, several analogues have been synthesized and evaluated for their binding properties to all subtypes of the receptor (Fig. 1.1). These analogues are modified versions of ss-14 which, in all cases, have amino residues between positions 7 to 10.
of the original ss-14 preserved to retain their functions in receptor binding, yet have the enzyme cleavage sites present in ss-14 removed and replaced with D- isomers of the amino acid residues (tryptophan and phenylalanine at positions 1 and 4 in octreotide). These modifications, which include the cyclization of some residues in the molecule, improve receptor binding and promote higher retention in systemic circulation as a result of decreased susceptibility to enzymatic degradation. They include octreotide, lanreotide, vapreotide, MK 678, MK363-301, and demotate, depreotide. The most widely used analogue is octreotide, which consists of 8 amino acids.

1.2 Imaging and Targeted Radiotherapy Exploiting Somatostatin Receptors

1.2.1 Nuclear medicine imaging

As mentioned previously, several malignancies often express a high density of somatostatin receptors. This advantageous characteristic promoted their use in the clinical imaging of
neuroendocrine tumours. The first radiolabeled peptide used for this purpose was \([^{123}\text{I}-\text{Tyr}^3-\text{octreotide}]\) in studies carried out by Krenning et al. The results of these studies indicated that it was not the optimal radiolabeled peptide because of its high lipophilicity. As a result, it was prone to high gastrointestinal excretion from the liver and extensive bowel radioactivity was often observed. Consequently, a high background is often observed which makes assessment of intra-abdominal tumours difficult after injection with \([^{123}\text{I}-\text{Tyr}^3-\text{octreotide}]\). Another shortcoming in the use of \([^{123}\text{I}-\text{Tyr}^3-\text{octreotide}]\) is the deiodination of the radiolabeled peptide resulting in the uptake of released radioiodine in normal organs which has the tendency to further increase the background in these organs. Other radiolabeled peptides were, therefore, evaluated to achieve more rapid elimination after systemic administration.

\(^{111}\text{In}-\text{DTPA-octreotide} (\text{\textsuperscript{111}In-pentreotide, Mallinckrodt Medical, St Louis, MO, USA})\) was approved by the U.S. FDA in 1994 for imaging of neuroendocrine tumours. Its use resulted in better detection and diagnosis of tumours because of rapid renal elimination and the longer physical half-life of \(^{111}\text{In}.\) This is particularly advantageous because a longer half-life of \(^{111}\text{In}-\text{octreotide}\) allows for later imaging time points where tumour accumulation of the radiopharmaceutical is greater and background radioactivity is lower. In other words, the tumour-to-background ratio of the radiopharmaceutical is improved and a diagnostic image of the tumour is obtainable. More recently, \(^{68}\text{Ga-DOTA-Tyr}^3\text{octreotide} (\text{Fig 1.2}),\) which is comprised of a positron emitter \((^{68}\text{Ga})\) has been conjugated to a more stable chelator (DOTA) and octreotide. This allows for imaging to be performed by positron emission tomography (PET), and has been found to be much more sensitive than \(^{111}\text{In-DOTATOC}.\) As a result it has been used instead in somatostatin receptor scintigraphy. This higher sensitivity is due to the higher spatial resolution and volume sensitivity of PET, the imaging modality used compared to single photon emission computed tomography (SPECT) which is employed for \(^{111}\text{In-DOTATOC}\) imaging. In addition, the lower kidney accumulation of \(^{68}\text{Ga-DOTA-Tyr}^3\text{octreotide}\) and shorter imaging time (total imaging procedure takes about 2 hours) compared with \(^{111}\text{In-DOTATOC}\) made its use attractive. Other radiopharmaceuticals employed for sstr2-positive tumours include \([^{99m}\text{Te-EDDA/HYNIC}]\text{octreotate},\) \(^{64}\text{Cu-DOTATOC},\) \(^{64}\text{Cu-TETA-OC},\) \(^{11}\text{C-labeled octreotide},\) and \(^{18}\text{F-labeled octreotide} (\text{Fig 1.2}).\) As a result of the success of targeting sstr2-positive cells with radiolabeled octreotide described above, the use of cytotoxic doses and analogues of these radiolabeled peptides was considered promising for therapy of neuroendocrine tumours, gastroenteropancreatic tumours, some colon tumours, prostate tumours and some ovarian tumours, to mention a few.
Table 1.1:
Physical properties of radionuclides commonly used in targeted radiotherapy of somatostatin receptor-positive tumours.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Physical Half-life (days)</th>
<th>Radiation Type</th>
<th>Maximum Energy (keV)</th>
<th>Maximum Range in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In$^a$</td>
<td>2.8</td>
<td>Auger electron IC</td>
<td>25 245</td>
<td>1 to 10 µm 550 µm</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>2.7</td>
<td>Beta</td>
<td>2284</td>
<td>12 mm</td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>6.7</td>
<td>Beta</td>
<td>497</td>
<td>2 mm</td>
</tr>
</tbody>
</table>

$^a$ γ emissions of $^{111}$In are omitted in this table because only therapeutic emissions are considered.

1.2.2 Auger electrons

Radiation damage due to molecularly-targeted radiation is caused by the emitted particles from these radioisotopes. The most commonly used radioisotopes in this regard emit Auger electrons or beta radiation. Auger electrons were first discovered by a French scientist named Pierre Auger in 1925 $^{63}$, but have been investigated more recently for radiotherapy. These electrons are ejected from unstable atoms that decay by electron capture. In this decay process, a proton in the nucleus of the atom captures an electron in the inner shell orbital. A vacancy is created in the shell which is filled by an electron from a higher shell. Transitions of electrons from higher to lower orbitals emit energy which is transferred to another electron, resulting in the ejection of the electron known as “Auger electron”. Although these electrons are highly energetic (25 keV), they only travel a range of a few hundred nanometers in tissue. As a result, they are only useful when targeted to cells and brought into close proximity to the nucleus of these cells to produce the required detrimental effect. Reilly et al have shown in several studies $^{64,65}$, the toxicity of $^{111}$In-trastuzumab in HER2/neu positive tumours and particularly the enhanced toxicity brought about by increasing the nuclear localization of the $^{111}$In-labeled antibody using a nuclear localizing peptide sequence (NLS). This enhanced toxicity as a function of increased nuclear localization was recorded to be a 6-fold increase compared to when $^{111}$In-labeled trastuzumab without the NLS was used $^{64}$. 
Fig 1.3: Schematic diagram of DOTA and DTPA

* Peptide is either octreotide or octreotate

Fig 1.4: Schematic diagram of different radiolabeled somatostatin analogues
1.2.3 Targeted radiotherapy

In addition to the use of radiolabeled octreotide for tumour imaging, a number of radionuclides have been employed for conjugation to octreotide or its analogues which is in turn used for therapeutic purposes based on the type of radiation emitted. The most common radionuclides employed in this manner are $^{111}$In, $^{177}$Lu and $^{90}$Y. These radionuclides differ in the type of radiation emitted, energy of the radiation emitted, and distance traveled by the emitted radiation in tissue (Table 1.1). The best response clinically has been obtained with beta-emitting nuclides such as $^{90}$Y and $^{177}$Lu. The first radiolabeled somatostatin analogue to be used in targeted radiotherapy was $^{111}$In-DTPA-octreotide. Prior to clinical use, studies in rats and mice using $^{111}$In-DTPA-octreotide (Fig 1.2) showed promising results in the regression of small-sized (<1 cm) subcutaneous CA20948 pancreatic tumour deposits reported by Capello et al $^{66}$.

Breeman and colleagues reported a study where 50 patients with end-stage neuroendocrine tumours expressing sstr2 were treated with a cumulative dose up to 160 GBq of $^{111}$In-pentrreotide. At the stage of disease of these patients, no large reductions in tumour size or significant remission were expected as most of the patients had end-stage disease and other treatment modalities were ineffective. Therapy with unlabeled octreotide or other somatostatin analogues which included lanreotide produced an objective response of only 2% and disease stabilization of 45% in this population $^{33}$. Despite this, a 17.5% overall response (complete and partial response) was observed post-radiolabeled octreotide therapy in the 40 eligible patients. However, if end-stage patients were removed, the therapeutic efficacy was 64% in the remaining 28 patients. Based on these factors, these responses were considered good. Toxicities observed were to the bone marrow. Of the evaluable 40 patients, 3 patients at doses above 100 GBq developed myelodysplastic syndrome $^{33}$. The reason for such toxicity was not discussed by the authors of the paper. The same group reported a non-randomized Phase II study comprising 27 patients with gastroenteropancreatic tumours that, after treatment with a cumulative dose of 13.32 GBq over a 2-month period, only resulted in tumour regression in 27% of patients, but with clinical benefits in 62% and a median survival of 18 months $^{67}$. Patients in this study were not at the end-stage as in the previous study, hence a low dose of radiolabeled octreotide was enough to induce a higher response rate than observed in the earlier Phase 1 study in which cumulative doses up to 160 GBq were administered. A similar study conducted by Schipper et al $^{68}$ showed a 23% response post-therapy in 19 patients with advanced neuroendocrine tumours. Most of these beneficial results
were associated with small tumours that responded to the short-range Auger electrons produced by $^{111}$In. The Auger electrons are likely responsible for the antitumour effects of these $^{111}$In-labeled octreotide analogues, but this occurs only when internalization of the radionuclide brings the Auger electrons in close proximity to the cell nucleus where their energy can be imparted to DNA causing strand breaks. Also indirect damage to the cells is caused by free radicals produced from the collision of these energetic electrons with water molecules.

In larger tumours greater than 1 cm in diameter, $^{111}$In-labeled-octreotide treatment was not as effective $^{33}$. Consequently, $^{90}$Y-DOTATOC also known as Octreother ($^{90}$Y-SMT487; Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) $^{69}$ and $^{90}$Y-DOTA-lanreotide were introduced based on the outcome of several animal studies $^{70}$. The properties of $^{90}$Y such as the longer range (10 mm) and higher beta energy (2.2 MeV), are more conducive to treating larger tumours (Table 1). Using this $^{90}$Y agent, an increased response to therapy has been observed in patients with tumours expressing sstr2 receptors. $^{90}$Y-DOTA-octreotide (Fig 1.2) was shown to have 10% to 33% response to therapy in patients with large tumours whereas $^{111}$In-pentreotide (Fig 1.2) had been ineffective $^{45,54,71,72}$. In a Phase 1 study carried out by Bodei et al in which an injected cumulative dose of 3.7 to 7.4 GBq/m$^2$ was used, a relatively high tumour regression rate (29%) was observed with $^{90}$Y-DOTATOC $^{73}$. Valkema and colleagues only observed a 20% tumour response rate in patients with neuroendocrine gastroenteropancreatic tumours $^{54,73,74}$. Otte et al $^{75}$ reported 25% response in 28 patients treated with 1.1 GBq of $^{90}$Y-DOTA-octreotide while Paganelli et al $^{76}$ achieved 23% overall response (complete and partial) in the 30 patients they treated in a similar manner. Both Chinol et al and Waldeher and colleagues reported a 24% overall response in patients with neuroendocrine tumours $^{31,32,77}$. In a similar study with $^{90}$Y labeled to lanreotide, Britton and colleagues $^{78}$ treated patients with sstr2-positive tumours using a cumulative dose of 8.58 GBq. This analogue was mildly efficacious showing a 14% tumour regression and 41% disease stabilization, with low toxicity in patients as evidenced by no occurrence of either renal or liver impairment $^{78}$. One major concern regarding the use of $^{90}$Y labeled somatostatin analogues, however, lies in the pronounced dose-dependent toxicities in patients during and after treatment, in some instances resulting in renal failure. The high-energy radiation produced by the radionuclide $^{90}$Y tends to result in prolonged irradiation of the kidney and bone marrow resulting in severe side effects. In fact, the median absorbed radiation dose from $^{90}$Y has been shown to be twice the dose from $^{111}$In to the kidneys $^{79}$. Consistent with this fact was a report by Cybulla et al describing a 78-year-old patient who developed deteriorated renal function 15 months after radiotherapy. The patient was previously diagnosed with a small
intestine carcinoid and metastases in multiple locations, and then treated with $^{90}\text{Y}$-DOTATOC. In subsequent related studies with $^{90}\text{Y}$-DOTATOC, toxicity to the kidney was reduced by the co-administration of D-lysine and argininine amino acids which block the accumulation of $^{90}\text{Y}$-DOTATOC in the kidney.

Due to these complications, $^{177}\text{Lu}$, which emits a beta particle with a shorter range and less energy compared to $^{90}\text{Y}$, has been evaluated in animal studies for possible use in management of sstr2-positive tumours. In a study executed by Mearadji et al, $^{177}\text{Lu}$-DOTATATE (Fig 1.2) caused significant tumour regression in a Lewis rat model with liver metastasis after treatment with 370 MBq. The same results were observed in two other independent studies with CA20948 tumour models implanted into the flanks of rats receiving up to 555 MBq of radioactivity and in nude mice with a cumulative dose up to 120 MBq. These promising results gave rise to clinical studies carried out by De Jong and colleagues in 2001. One of the first studies by this group using $^{177}\text{Lu}$ involved 35 patients with neuroendocrine gastroenteropancreatic malignancies (NEM GEP). The maximum dose administered to the patients per cycle was 200 mCi (7.4 GBq). The final cumulative dose ranged between 600 mCi and 800 mCi (22.2 and 80.0 GBq). Overall therapeutic response, which included complete, partial and minor response in these patients, was 38%, of whom 41% had stable disease and 21% had progressive disease. Later a 47% overall response was observed with this same therapeutic agent in a separate study with a similar group of 131 NEM GEP patients who were administered a similar dose. Prior to these studies, they showed $^{177}\text{Lu}$ to have 3 to 4 times lower radiation absorbed dose to kidneys and 2 to 3 times lower dose to the liver compared to $^{90}\text{Y}$-DOTATOC. Although only a couple of studies have been reported with $^{177}\text{Lu}$-DOTATOC/DOTATATE, therapy with this agent showed better potential for use in future clinical studies.
1.3 Gene Therapy and Oncolytic Viruses

Gene therapy is generally proposed for use in oncology for the introduction of genes into physiologically defective cells with the purpose of destroying such cells, i.e. through the use of suicide genes to enhance an immune response against them or by sensitizing the cells to other therapies \(^{85,86}\). This option, known as virotherapy, has been successfully employed in tumour management where it has been associated with the use of certain viruses. To an extent, gene therapy aims to study the interaction between viruses, immune responses to these viruses, and the tumour environment \(^{87}\). As reported in a few review papers, the onset of virotherapy goes as far back as 1912 when a patient previously diagnosed with cervical carcinoma experienced tumour regression after being bitten by a dog infected with rabies \(^{86-89}\). This discovery was later tested in 1920 with Newcastle disease virus (NDV) and influenza virus where their antitumour properties were confirmed \(^{89}\). The study of these viruses in patients proceeded into the late 1940s and early 1950s with wild type adenovirus and a different strain (adenoidal-pharyngeal-conjunctival) virus which was used in patients with cervical carcinomas \(^{89,90}\). Within the same period, the measles virus was found to cause tumour regression in patients with Burkitt’s lymphoma and Hodgkin’s disease. Regressions were also observed in lymphoblastic leukemic patients who were coincidentally injected with the measles virus and rubella \(^{90}\). These viruses used in tumour treatment are termed oncolytic viruses (OV).

Initially, gene therapy was concentrated on replication-incompetent viruses because of the fear of uncontrolled viral spread if replication-competent viruses were used. It was soon discovered that these viruses had low therapeutic efficacy after systemic administration due to poor distribution to the site of action, poor penetration into the tumour, and inefficient virus replication in the tumour leading to insufficient levels of gene transfer and expression \(^{88,90}\). The use of replication-competent viruses such as strains of adenoviruses, herpes simplex virus 1, Newcastle disease, and vaccinia virus eliminate these shortcomings.

1.3.1 Specificity and selectivity of oncolytic viruses

One of the major characteristics of oncolytic viruses is their ability to differentiate between normal and malignant cells, thereby causing the infection of only cancerous cells. They have been
shown to have a wide range of methods for recognizing and infecting tumours. All of these methods are solely based on the exploitation of abnormal cellular pathways in tumour cells that differentiate them from normal cells.

1.3.2 Virus gene defects and deletions

Over the years, several viruses have been engineered to possess qualities allowing them to “seek” tumours. Some of these qualities involve the deletion of genes that are necessary for viral replication. This property is exhibited in an engineered strain G207 of HSV-1 where the ICP6-γ34.5 gene is deleted, therefore, making the virus selective for rapidly proliferating cells, yet diminishing its neurovirulence.

This method to improve specificity is also used to engineer two different adenovirus strains. The first is Onyx-015 where deletion of the E1B-55K-/E3B gene leads to its selective distribution into cells that have lost the p53 pathway and have late mRNA transportation. The second is Delta-24 with a deletion of E1A-CR2 gene. This deletion enhances Delta-24’s transportation into cells that have lost the G1-S checkpoint control and pRB functions, both of which are characteristics of tumour cells. Vaccinia virus western strain JX-594 was engineered to have the thymidine kinase (TK) gene deleted in order to allow its passage into rapidly proliferating cells and replaced with granulocyte-macrophage colony-stimulating factor. Alternatively, Western reserve strain have been engineered to have both the TK gene and vaccinia growth factor (VGF) gene deleted to increase its specificity for rapidly proliferating cells.

1.3.3 Tumour-specific receptors and promoters.

These viruses are engineered to seek certain moieties expressed on the surface of tumour cells. Adenovirus is such an example whereby the coxsackie virus-adenovirus receptor (CAR)/integrin-binding gene has been replaced with a tumour-targeting ligand gene and another adenovirus with a Delta-24RGD gene deletion. These two deletions lead to selective binding in tumour cells.

Some viral genes have been subjected to replication under the instruction of tumour-specific promoters as a mechanism of selective replication in these cells compared to normal cells. In the adenovirus strain CG7870, the E1A gene and the E1B gene have been placed under
the control of the rat probasin promoter and PSA promoter/enhancer, both found in prostate tumours. Another example is the BM24-TE strain of HSV-1. It has been engineered to function only under the influence of Wnt/β-catenin-promoter/enhancer and in the presence of Wnt/β-catenin-overexpressing tumours such as certain colorectal carcinomas and hepatoblastoma.

1.3.4 Tumour-specific abnormalities

The most prominent of these subtypes are viruses that seek cells with defective interferon (IFN) production. IFNs are essential for initiating the immune response against abnormal cells. Tumour cells are known to be deficient in the secretion of IFNs, resulting in the inability of the immune system to recognize them as defective. This phenomenon has the same effect in viruses: for example, vesicular stomatitis virus (VSV) was reported to have low pathogenicity due to its sensitivity to IFNs. Other viruses that are selective based on the IFN defect are as follows: i) Newcastle disease virus, an enveloped negative-sense and single-stranded RNA virus, ii) measles virus, a negative-stranded and enveloped RNA virus, and iii) Semliki forest virus (SFV), a positive-stranded enveloped RNA virus. In addition to IFNs, the measles virus selects cells based on their expression of the CD46 gene which tumour cells are known to overexpress. Another selectivity factor is based on the ras signaling pathway. In some viruses such as NDV, VSV and Reo virus (a non-enveloped double-stranded RNA virus), increased activation of the Ras/Raf pathway promotes the selective partitioning and replication of these viruses. Herpes virus in contrast replicates preferentially in tumours having a defective ras pathway.

Other selectivity mechanisms include those based on defective pathways such as translational control and cell growth regulation as seen in VSVs, uncontrolled proliferation due to abnormal mitochondrial apoptotic pathways, activation of the AKT signaling pathway, and overproduction of anti-apoptotic proteins.

1.3.5 Mechanism of cell killing of viruses

Oncolytic viruses exhibit toxicity mainly by the direct lysis of infected cells and subsequent release of virion progeny into the surrounding cells. Over-productivity and higher-than-normal demand of cellular processes as a result of the amplification of virus particles through replication can also cause cell destruction. Some oncolytic viruses have genes that allow them to produce cytotoxic proteins during their replication. Examples of these are adenoviruses that...
express E3, 11.6kDa death proteins, and E4ORF4 proteins late in the replication cycle \(^8^9\). Alternatively, other oncolytic viruses activate an antitumour immune response on infected cells, either specifically or non-specifically. Tumour cells are known to be slightly immunogenic because of the low expression of major histocompatibility complex (MHC) antigens and stimulatory cytokines that tend to activate some immune responses \(^8^9\). An example of the specificity of this mechanism is exhibited in adenoviruses that express E1A proteins which, upon production, enhance the ability of tumour necrosis factor (TNF)-mediated killing upon cell infection \(^8^9\). Non-specific antitumoural immunity is observed in HSV-1 with cytotoxic T lymphocyte (CTL) responses \(^8^9\).

Other sets of oncolytic viruses include those that have been genetically engineered to incorporate genes which, upon virus infection, are expressed and result in sensitization of cells to chemotherapy and or radiotherapy. An example of such virus is the ONYX strain of adenovirus that produces E1A protein which induces a high level of p53 and, consequently, renders the cells susceptible to DNA damage by chemotherapy and radiation \(^8^9\). A study by Kirn et al showed a different cytotoxicity mechanism exhibited by vaccinia virus post-infection of cells. They observed that vaccinia virus specifically infected and destroyed tumour endothelial cells, resulting in the loss of tumour vasculature density \(^9^3\). Also, Breitbach et al in a different study reported that administration of this virus causes activation of inflammatory chemo-attractants CXCL1 and CXCL5 and neutrophils that contribute to the reduction in tumour vasculature \(^9^4\).

1.3.6 Successes and limitation in oncolysis

Several advances in virotherapy over the years have improved the efficacy of treatment. Some limitations which have been overcome include the immune response of the host cells, the attenuation of the virus permitted by genetic engineering of the virus, and improvements in the understanding of the tumour microenvironment \(^8^7\). The first attempt to circumvent the immune response limitations was undertaken in the 1970s where an immunomodulatory agent cyclophosphamide was used in combination with oncolytic viruses \(^9^5-9^7\). The outcome was an increase in virus spread, transgene expression and anti-tumoural efficacy \(^9^5\). Another study was undertaken whereby cytokine-induced killer cells (CIK) whose primary purpose is to seek out, bind and destroy tumour cells, were isolated from patients. CIKs were injected with the virus and re-injected into systemic circulation of the patients. These cells were used as “homing devices” to
direct viruses to the site of action, thereby bypassing the immune system. This study reported a synergistic effect for both the CIKs and the virus 98.

An attempt to improve virus infection in tumours may be co-administration or the engineering of the viruses with matrix modifying agents. Reports of this approach include adenoviruses engineered to encode relaxin, a matrix-degrading protein, and co-administration of bacterial collagenase MMP-1,8 with HSV1. Both were shown to enhance the spread of the viruses and ultimately increase the efficacy of the virus treatment 99,100.

1.4. Vaccinia Virus

1.4.1 Origin and history

Vaccinia was first proposed as a vaccine for smallpox in 1950 101. It belongs to the orthopox genus along with the variola virus, cowpox, monkey pox, and camel pox. Smallpox is a deadly disease caused by variola virus. It started in the 3rd century and has a 30% mortality rate in a vaccinated population 101,102. Its high mortality rate is due to the ease of transmission of the disease from person to person by inhalation of respiratory droplets, contact with, and/or deposit of infected material on nasal, oral or pharyngeal mucous membranes 90,103,104. The earliest smallpox prevention vaccination was performed in the 10th century in China. In 1796, Dr Edward Jennar of Gloucestershire, England found cowpox to be a better vaccine. These developments led to the discovery and worldwide use of vaccinia virus for the eradication of the disease proclaimed in 1979 reviewed by Shah et al. 90. The pox virus family is known to be highly similar in DNA composition and retains great gene conservation 105. They are double-stranded DNA viruses with large, ovoid or brick-shaped virions 88. To date, several strains of vaccinia virus exist, some of which include Western Reserve, Ankara, Lister, NYCBH and Wyeth strains.

1.4.2 Properties, characteristics and types of vaccinia

Vaccinia virus is a DNA virus with a genome about 200 nm in length that encodes up to 200 genes. It encodes enzymes required for its replication and transcription upon entry into host cells. Upon infection and entry of the virus, two different viral particles are produced, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). These two particles
differ in the viral proteins they encode making them structurally, antigenically and functionally different 106.

The IMVs are the first set of virus particles to be produced. They contain immature virions with double-stranded DNA, structural proteins, transcriptional enzymes required to initiate replication, and core proteins which are later cleaved by proteolytic enzymes to form more IMVs 106. These IMVs remain in the cell until lysis. A subset of IMVs is further coated with a layer of membrane derived from the trans-Golgi network and early tubular endosomes. They are delivered to the host cell surface at which point they become cell-associated enveloped virus (CEV) or, if they bud out of the host cell, they become extracellular enveloped virus (EEV) 105. In a review by Vanderplassehen, he argued the importance of the second membrane in EEVs. He stated that although this outer membrane is susceptible to physical damage which could be induced by physical stress, it is protective against immune aggression caused by neutralizing antibodies in circulation and it mediates the binding of the virus onto the cell surface 107. In addition, the different membranes covering these two distinct virus particles allow for different mechanisms of entry into cells as discussed below.

1.4.3 Mechanism of infection

Two different virus particles are produced in host cells, but are removed from the cells at different times. Both EEV and IMV bind to the cell, shed their membranes, and expose naked virions covered by proteins required for transport into the cytoplasm of the target cell. The core virions are then transported further into the cell on microtubules via the interaction between the surface viral proteins and the microtubules, thus creating a sac-like structure in the cytoplasm named the pox factory 105,106. In this factory, early mRNA transcription results in the uncoating of the core proteins that have been imbedded into the virus package, leading eventually to viral DNA replication. After early viral replication, immature virions (IVs) are assembled and processed to form IMVs. As explained previously, some of these IMVs are then wrapped by early endosomes and the trans-Golgi network to form a double membrane and Intracellular enveloped virus (IEV). IEVs are transported to cell surfaces on microtubules to become CEVs which, in turn, are exposed at the cell surface. Polymerization of an actin tail located under the CEV often occurs in order to drive the virus into a neighboring cell or to disconnect the CEV from the exterior of the host cell to form EEV 105,106,108.
The mechanism of virus particle binding and entry has been debated over the years and complicated by the presence of two similar, but distinct particles. It has been deduced that both viral particles enter the cell by endocytosis, particularly macropinocytosis, in a pH-dependent and independent manner\(^{109}\). The IMVs have been shown to enter cells by fusion with the cell in a pH-independent way with the help of viral proteins on the surface after which the virions are exposed directly to the cytoplasm. EEVs, however, are controlled with a more complicated pH-dependent mechanism involving the activation of signaling pathways that trigger actin-mediated membrane ruffling and blebbing\(^{109}\). Internalization of the virus into a large vacuole or macropinosomes formed at the plasma membrane ensues. This vacuole creates an acidic environment which dissolves the outer layer of the EEV converting it to an IMV. Conformational changes are induced in the virus proteins in order to facilitate the fusion of the naked EEV or IMV with the endosomal membrane\(^{105,107,109}\). After fusion, viral protein uncoating begins and the virus starts a new cycle as explained earlier.

1.4.4  Vaccinia as an oncolytic virus

Over the years, the oncological properties of the vaccinia virus have been characterized. In 1982, vaccinia virus was introduced as a vector for the expression of genes in mammalian cells\(^{110}\). It has the advantage of carrying its own replication machinery which, upon infection, completes its whole replication cycle in the cytoplasm without the use of the host DNA machinery and without incorporating itself into the genome of the host. Another advantage of vaccinia is its ability to infect a variety of hosts due to the presence of some immunoglobulin proteins and some complement control proteins on the surface of the EEV particle that mediate interactions between cells or between soluble molecules and cells\(^{107}\). Other advantages include the following: i) its ability to hold up to 25 kb of foreign DNA in its genome, ii) its wide host range, iii) high rate of protein synthesis which can be beneficial in terms of expressing proteins that are incorporated into its genome, iv) availability of transport, secretion and processing enzymes required for post-translational modifications which makes it independent of the host enzymes, v) ability to produce biologically active proteins in tissue culture, vi) its use in the analysis of mutant forms of proteins, and vii) use for studying changes in cell cycle, protein synthesis and immune response in tissue cultures upon virus infection\(^{102-104,111}\).
For therapeutic purposes in oncology, a replication-competent version of vaccinia virus has mostly been employed. Its main advantage is due to the increased expression of incorporated genes upon replication of the virus, thus improving its efficacy. Vaccinia virus is used in oncology because of the toxicity it exerts on host cells. The major reason for this toxicity is the direct lysis of the host cells after replication. Upon infection, vaccinia virus induces several cytopathic effects including the rearrangement of the cellular cytoskeleton and the inhibition of host gene expression, for example Rb and p53 genes that are responsible for the regulation of POL111 transcription and, ultimately, for promoting the replication of the virus in the host cells.\(^\text{112}\)

1.4.5 Specificity and selectivity of vaccinia virus

The specificity of wild type vaccinia virus is still unknown; however, studies have shown it to replicate particularly in rapidly proliferating cells. Another hypothesis argues that due to the size-limited movement of vaccinia in the systemic circulation, the virus partitions only into areas with high vascular endothelial growth factor (VEGF) production with associated neovascularure and with leaky vasculature. This leaky vasculature is often observed in tumours, as is the increased rate of vaccinia virus partitioning. It is also worth mentioning that rapidly replicating ovarian follicles mimic this phenomenon. Studies carried out to evaluate the toxicity of vaccinia virus showed extensive damage to the ovaries with wild type virus, nevertheless no damage was experienced with a recombinant version of the virus explained below.\(^\text{113}\)

1.4.6 Double-deleted vaccinia virus

Several studies have shown the effectiveness of vaccinia virus after genetic engineering.\(^91,114-116\). One modification is the deletion of the thymidine kinase (TK) gene in the virus genome. Due to the amplification of this gene in tumours caused by their ever-replicating nature, in some cases through abnormal EGFR signaling, an increase in the E2F-mediated TK expression is observed hence, a proportional increase in thymidine triphosphate concentration. Thymidine triphosphate is a nucleotide that is phosphorylated by the thymidine kinase gene and also an important nucleotide required for DNA replication. The increased population of this nucleotide in rapidly dividing cells promotes the increased concentration of the virus in tumour cells.\(^91,114\).
Another modification is the deletion of the vaccinia growth factor (VGF) in the virus. This factor is a protein expressed and secreted early in the replication of the virus. VGF binds epidermal growth factor receptors (EGFR) on surrounding resting cells and stimulates proliferation in these cells. Increased proliferation attracts the mature virions to the cells and leads to their infection. The absence of this factor, on the other hand, restricts virus replication to cells that are naturally replicating rapidly without the need for VGF, i.e. mostly tumour cells. The deletion of both the TK and VGF genes in the western strain of the virus has shown tremendous increase in the partitioning of the virus to tumour cells as compared to normal cells.

Lastly, a newly discovered modification is the deletion of two viral genes that encode anti-apoptotic proteins and serine protease inhibitor SpI-1 and SpI-2 in vaccinia virus resulting in the vSp strain. This deletion is also found to increase tumour targeting upon systemic administration of the virus. Further research in this area is ongoing.

1.4.7 Vaccinia in clinical use

The ongoing use of vaccinia virus in the smallpox eradication program has provided a proven safety profile for its use in humans. As a result, several clinical investigations from phases 1 to 3 have evaluated the outcome of vaccinia virus treatment in different diseases and malignancies. Most of these studies have incorporated foreign genes that influence the cytotoxic effects on infected cells. A study by Hawkins et al modified the Ankara strain of the virus. The modified virus was studied in clinical trials for the treatment of colorectal cancer. Treatments were well tolerated. Results showed an improved disease-free survival in high risk patients.

Another strain of vaccinia virus, JX-594 (a western strain in which the TK gene is replaced with the granulocyte-macrophage-colony-stimulating factor GM-CSF), was evaluated in a Phase 1 study of patients with liver malignancy. Upon intratumoural administration of this virus, it was shown to accumulate exclusively in the tumours while being well tolerated by patients. Clinical benefits were, however, not recorded for this study. Researchers have deduced that this modified strain causes cell destruction which relies on cell cycle and EGFR-ras pathway-activation abnormalities. As with all strains of vaccinia virus, it replicates and expresses GM-
CSF, a transgene responsible for stimulating antitumoural immunity. This eventually leads to cell destruction by necrosis\textsuperscript{119,120}.

In another study carried out in South Korea in 2008 by Park and colleagues\textsuperscript{120}, patients with hepatocellular malignancy, colorectal cancer, melanoma, and lung cancer were enrolled in a Phase 1 trial. Patients were treated with JX-594, a vaccinia strain that has the thymidine kinase gene deleted and replaced with the GM-CSF transgene. Of the 10 patients treated, 30\% experienced a partial response, 60\% had stable disease and 10\% exhibited progressive disease. In the patient group with hepatocellular malignancy, toxicity encountered was associated with direct hyperbilirubinaemia as a result of tumour swelling and bile duct obstruction\textsuperscript{120}. In a related study, Lui and colleagues recorded 100\% response in 3 hepatocellular cancer patients also treated with JX-594\textsuperscript{119}.

Subsequently, the successful clinical outcome of the use of vaccinia virus in the treatment of melanoma patients in Stages I through III of the disease encouraged more clinical trials reviewed by Nemunaitis and Edelman\textsuperscript{121}. One study showed 71\% overall response post-treatment in 7 melanoma patients\textsuperscript{121}. In other studies, the antitumour effects of vaccinia virus were augmented by the addition of the p53 gene into the virus, a gene responsible for promoting apoptosis. Upon replication of the virus, apoptosis is induced by a different mechanism than that which causes virus-only cell lysis. The addition of the p53 gene produced promising results as a potent inducer of apoptosis and tumour killing agent in glioma cells\textsuperscript{90}.

In other malignancies, vaccinia virus was engineered to incorporate the prostate specific antigen (PSA) gene into its genome in an attempt to improve selectivity as discussed above. Its cytotoxicity was assessed in patients with prostate cancer with elevated PSA. Intradermal inoculations of this agent lead to the stabilization of disease in 27\% of patients\textsuperscript{122}.

Other gene insertions that have been tested are those of co-stimulatory molecules ICAM 1 and CD70\textsuperscript{115,122}, endothelial monocyte activating factor EMAP-11, cytokines such as interleukin 2, viral antigens such as HPV 16 viral proteins, HIV-1 envelope protein, tumour-associated antigens such as MART 1, tyrosinase and CEA\textsuperscript{123}, PSA and Melan A peptide genes, markers such as Lac Z pr luciferase, prodrug-converting enzyme cytosine deaminase, etc\textsuperscript{122}.
Due to the highly immunogenic nature of vaccinia virus, ways of improving its efficacy in the treatment of malignancies have shifted to strategies to reduce the immune response mounted by the host upon infection. Such strategies include the co-administration of immunomodulators like cyclophosphamide with the virus. Cyclophosphamide is well known to reduce and, in some cases, inhibit the recruitment of natural killer (CD68+), microglia/macrophage (CD163+) cells, IFN-γ, and neutralizing antibody induction and regulatory T cells (Tregs) in infected cells\textsuperscript{108,124}. Inhibition of immune response and improvement in the efficacy of treatment are the ultimate goals.

As already discussed, the toxicity of oncolytic viruses is dependent on multiple pathways such as direct lysis of the cell, disruption of cell cycle processes, induction of apoptosis, and production of necrosis. As a result, in combination with other cytotoxic agents, no cross-reactivity with the other agent is expected nor should resistance be a problem due to the multiple pathways of the viruses which are independent of the other toxic agents. Also, because one of the major limitations for the use of oncolytic viruses in cancer therapy is due to their immunogenicity, combination with other therapeutic agents should result in overall increased efficacy of virus treatment in infected cells. Such a therapeutic agent is cyclophosphamide, an immunomodulant that would inhibit neutralizing antibodies released as a result of immune response against the virus. This effect promotes the circulation of the virus to target areas and, hence, increases efficacy of the virus therapy. This advantage has been promoted in several clinical studies over the years.

On numerous occasions, the combination of direct radiation treatments with different replication-competent strains of herpes simplex virus 1 such as NV1066, R3616, R7020, R3616/G207, and G207, have been found to be more efficacious and add synergistic effects to virus-alone treatment in hepatoma, colorectal and cervical tumour models in mice as well as malignant pleural mesothelioma cells\textsuperscript{125-130}. In a study by Jorgensen et al, however, radiation therapy added no benefit to HSV1 virotherapy\textsuperscript{131}. On the other hand, no study has been reported to date about using vaccinia virus in combination with radiation treatment for the management of any tumour.
1.5 Combinations of Vaccinia Virus and Targeted Radiotherapy

Individually, vaccinia virus and $^{177}$Lu-DOTATOC or $^{111}$In-pentetreotide have shown some potential in the management of certain malignancies. In vaccinia virus-alone therapy, the main limitation is due to the high immunogenic nature of the virus in vivo resulting in a short residence time in the body and, hence, low efficacy after systemic administration. On the other hand, the use of $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC is limited by the amount of somatostatin receptor expressed on tumours which, in turn, determines the extent of binding and internalization of the radiolabeled octreotide into the tumour to effect tumouricidal damage. An additional limitation is the maximum tolerated dose of either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC that can be injected in patients in an attempt to avoid toxicity of the conjugated radonuclide to the kidneys and other vital organs.

To overcome these limitations, it was proposed that a combination therapy approach using both virotherapy (vaccinia virus) and radiation therapy ($^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC) could be employed. In this approach, it is expected that both therapeutic agents impact both complementary and additive advantages to either of the agents alone in the inhibition of cell growth. It was discussed earlier that the vaccinia virus strain used has been engineered to contain the sstr2 which, upon infection of the cells and tumours, express these receptors. $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC added to these tumours targets the receptors expressed through the virus as well as any endogenous receptors in the tumour. Hence, the virus reduces cell growth directly due to its oncolytic nature while increasing targeting of the radiolabeled octreotide. In addition, the expression of the receptors through the virus addresses in part the limitation posed by the somatostatin receptor subtype heterogeneity present on tumours expressing the receptors by incorporating a known exogenous receptor (sstr2). This could further improve the efficacy of ddVV-sstr2 treatment combined with either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC.

Treatment of cells with $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC causes toxicity based on the energy of the radiations emitted by these two different radionuclides. As a result of the energy of these radiations and their path length in tissue, toxicity to these cells could be direct or indirect. A direct effect is caused by DNA strand breakage by the high linear energy transfer (LET) Auger electrons emitted from $^{111}$In and by the low LET beta particles emitted from $^{177}$Lu. Indirect toxicity is a result of the generation of DNA-damaging free radicals by the radiation or by
bystander effects produced from cells that have been lethally damaged by the emitted particles. Indirect toxicity is also caused by crossfire effects from the long-range beta particles from $^{177}$Lu which damages cells not targeted with the radiopharmaceutical but those cells within the 2 mm range of the radiation emitted by $^{177}$Lu-DOTATOC residing on a targeted cell. These factors would improve the cytotoxicity imposed by vaccinia virus alone as well as increasing the rate of cell kill due to the extensive damage done to tumour cells before the onset of the immune response in vivo.

McCart et al. have experience with the double-deleted version of the Western strain of vaccinia virus $^{91}$ and, subsequently, have created a genetically modified version of this virus which incorporates the $sstr2$ gene in its genome (ddVV-$sstr2$ or simply ddVV) $^{116}$. In their published reports, they have shown significant expression of these receptors on the cell membrane following virus infection of cells with low basal levels of the receptor $^{116}$. In the same study, in vivo targeting of the $sstr2$ receptors populated in subcutaneous MC-38 (murine colon cancer) xenografts by the virus was achieved using $^{111}$In-pentreotide ($^{111}$In-DTPA-octreotide) $^{116}$. This was accomplished by injection of $10^9$ pfu of vaccinia virus into the mice bearing the xenografts followed by the injection of 5.5 MBq of $^{111}$In-pentreotide six days later. The uptake of the radiopharmaceutical by the cancer cells which correlated with the targeting of the receptors expressed by the virus was verified by imaging of the xenografts using the gamma radiation from the $^{111}$In-pentreotide.

An increase in receptor population has been discussed already as a strategy to increase the efficacy of radiolabeled octreotide in the management of sstr2-positive cells. The use of this genetically modified vaccinia strain (ddVV) would, in effect, increase receptor population on infected cells which would be available for targeting with radiolabeled octreotide, creating an added advantage over the use of double-deleted vaccinia virus (ddVV) only. Also, another advantage of this combination is the low dose of virus required for injection into patients in order to produce the desired growth inhibitory effect in tumour cells. In previous clinical trials with vaccinia virus, up to four doses of $3 \times 10^9$ pfu often needs to be administered for any clinical benefit to occur $^{119,120}$. In this proposed combined virus and targeted radiotherapy approach, a lower dose compared to this may be required to effect as much toxicity as observed in previous trials with the virus alone and may further improve therapeutic outcome.
1.6 Hypothesis and Specific Aims

1.6.1 Hypothesis

It was hypothesized that the combination of targeted radionuclide therapy using $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC with double-deleted vaccinia virus (ddVV) encoding the somatostatin receptor subtype 2 (sstr2) in cells overexpressing somatostatin receptors or those with low levels of receptor expression would produce superior cytotoxicity compared to using either vaccinia virus, $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC alone in cells grown as monolayers or in spheroid culture.

1.6.2 Specific aims

The specific aims to test this hypothesis were as follows:

1. Characterization of the cell binding properties and cellular internalization properties of $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC in human embryonic kidney cells (HEK-293) transfected with somatostatin receptor subtype 2 gene.

2. Characterization of the receptor expression using flow cytometry in HEK-293 cells; transfected and non-transfected with the somatostatin receptor subtype 2 and murine colon cancer (MC-38) cells infected and non-infected with ddVV.

3. Evaluation of the cytotoxicity of $^{111}$In-DOTATOC in comparison to $^{177}$Lu-DOTATOC as single agents in HEK-sstr2 cells grown as monolayer, and as single agents in both HEK-293 and MC-38 spheroids.

4. Evaluation of cytotoxicity of vaccinia virus (ddVV) alone or in combination with either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC in HEK-293 cells, HEK-293 spheroids, and MC-38 spheroids.

Comment

It should be noted that this concept was tested for the first time in this study using in vitro cell culture systems. The full effect of combining $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC with vaccinia virus encoding the somatostatin receptor subtype 2 (sstr2) would, however, require further in vivo studies in tumour xenograft mouse models.
CHAPTER 2

Growth Inhibitory Effects of $^{111}$In- or $^{177}$Lu-DOTATOC Alone or in Combination with Cytolytic Vaccinia Virus on Somatostatin Receptor-Positive Cells

All of the studies described in this chapter were performed by Olayinka Akinlolu except for the initial virus infection of HEK-293 cells and MC-38 cells for the flow cytometry experiment which was done by Kathryn Ottolino-Perry
Abstract

Introduction: Molecularly targeted radiotherapy using radiolabeled somatostatin analogues has proven effective for treating tumours expressing somatostatin subtype 2 receptors (sstr2). Additionally, double-deleted vaccinia virus (ddVV) is a promising new cancer treatment capable of preferentially infecting and lysing tumour cells. The recombinant virus can also introduce sstr2 into tumour cells for targeting with radiolabeled somatostatin analogues. Our objectives in this study were to compare the cytotoxic potency in vitro of ddVV treatment to that of $^{111}$In- or $^{177}$Lu-DOTATOC as well as evaluate the effects of the virus in combination with these sstr2-targeted radiotherapeutics.

Methods: Human embryonic kidney (HEK-293) cells transfected with the sstr2 gene to overexpress sstr2 or MC-38 murine colon cancer cells endogenously expressing low levels of sstr2 were treated in monolayer or spheroid culture with ddVV or $^{111}$In- or $^{177}$Lu-DOTATOC alone (0.3 to 20 ng) or in combination. Treated monolayer cells were re-plated at a density of $8 \times 10^3$ cells per well in a 96-well plate. Spheroids were treated for 48 h with ddVV or 24hr with $^{111}$In- or $^{177}$Lu-DOTATOC, then dissociated and replated as for monolayer cells. In combination experiments, HEK-293 cells in monolayer or HEK-293 or MC-38 spheroids were first infected with ddVV at a multiplicity of infection (MOI) of 0.1 for 48 h. Cells or spheroids were then treated with 20 ng of either $^{111}$In- or $^{177}$Lu-DOTATOC for 4 h. Both HEK-293 and MC-38 spheroids were cultured for 24 h, then dissociated into cells and plated. Cell growth was measured using the WST1 assay after 5 d for $^{111}$In- or $^{177}$Lu-DOTATOC alone and for 8 d for combination treatments.

Results: $^{111}$In or $^{177}$Lu-DOTATOC (20 ng) alone reduced HEK-293 cell growth compared to untreated cells to $63.4 \pm 8.8\%$ and $53.6 \pm 0.9\%$, respectively (p=0.03). An increase in the amount of the radiopharmaceuticals to 60 ng resulted in reduction of cell growth to $44.5 \pm 9.7\%$ for $^{111}$In-DOTATOC and $29.9 \pm 12.3\%$ for $^{177}$Lu-DOTATOC. Combining ddVV with $^{111}$In- or $^{177}$Lu-DOTATOC decreased growth to $22.9 \pm 8\%$ and $6.6 \pm 1.8\%$, respectively. Treatment with ddVV alone decreased growth to $37.6 \pm 20.2\%$. In spheroids, treatment with ddVV virus alone, or in combination with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC decreased cell growth to $18 \pm 2.7\%$, $4.9 \pm 0.02$ and $5.0 \pm 0.6\%$ after 8 d, respectively compared to $^{111}$In-DOTATOC ($17.3 \pm 4.8\%$) or $^{177}$Lu-DOTATOC ($26.6 \pm 6.3\%$) alone. In MC-38 spheroids, cell growth after 8 d was reduced to...
18.5 ± 9.9% in spheroids treated with ddVV alone, 13.1 ± 7.1% in ddVV and $^{111}$In-DOTATOC treated spheroids and 0% in spheroids treated with ddVV and $^{177}$Lu-DOTATOC.

**Conclusion:** Combining ddVV with $^{111}$In- or $^{177}$Lu-DOTATOC is more effective at reducing the growth of sstr2-expressing cells in monolayer compared to treatment with either $^{111}$In- or $^{177}$Lu-DOTATOC alone or ddVV treatment. In spheroids however, the toxicity of either $^{111}$In- or $^{177}$Lu-DOTATOC as single agents was the same as observed when combined with ddVV. Hence, combination therapy in spheroids is not more advantageous than single treatment of either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC.
2.0 Introduction

Targeted radiotherapy of neuroendocrine malignancies expressing somatostatin receptors, particularly of the subtype 2 class (sstr2) using $^{177}\text{Lu}$ or $^{90}\text{Y}$-DOTATOC has proven successful, achieving tumour remissions and prolonging patient survival in clinical trials $^{27,29,35,45,132,133}$. These agents emit moderate or high energy $\beta$-particles, respectively, of a relatively long range (2-10 mm). $^{111}\text{In}$-pentetreotide is a related analogue which emits very low energy Auger electrons (in addition to its $\gamma$-emissions) that are damaging to DNA and lethal to cells, but only if the decay occurs in close proximity to the nucleus due to the ultrashort submicrometer range of these electrons. $^{111}\text{In}$-pentetreotide has also shown promise for the treatment of sstr2-expressing malignancies yielding clinical improvement in patients as well as decreases in the levels of circulating tumour-associated hormones, although tumour regression has been infrequent $^{33,46,134}$.

It is generally accepted that combination therapies are more effective than single anticancer agents in eradicating tumours. One new class of cancer therapeutics which would be attractive to combine with targeted radiotherapeutics is cytolytic virus therapy. These viruses have the capability of infecting tumours in which they express their genes, replicate, and ultimately lyse and destroy cancer cells $^{89-91,135,136}$. Our laboratory has constructed a double-deleted recombinant cytolytic vaccinia virus (ddVV) which preferentially infects and replicates in tumours. Due to its large size, it relies on the vascular leakiness often found in tumours to extravasate. Because of the engineered deletion of the viral thymidine kinase ($\text{TK}$) gene, our ddVV also relies on high levels of the host TK for replication $^{91,111,116}$. In addition, this recombinant engineered ddVV encodes the human $\text{sstr2}$ gene which allows tumour-selective viral delivery and expression of exogenous sstr2 proteins in cancer cells. This property could permit augmentation of the existing levels of sstr2 in tumours, render sstr2 expression more homogenous due to the infectious spread of the virus within the tumour, or introduce sstr2 into malignant cells not expressing these receptors. This, in turn, may provide an opportunity to target more effectively sstr2 with radiotherapeutics such as $^{177}\text{Lu}$- or $^{90}\text{Y}$-DOTATOC or $^{111}\text{In}$-pentetreotide. Indeed, in previous work, we showed that ddVV efficiently delivered exogenous sstr2 to subcutaneous MC-38 murine colon carcinoma xenografts in mice enhancing tumour imaging with $^{111}\text{In}$-pentetreotide $^{116}$. Thus, combinations of ddVV and sstr2 targeted radiotherapeutics such as $^{177}\text{Lu}$- or $^{90}\text{Y}$-DOTATOC or $^{111}\text{In}$-pentetreotide would be worthwhile exploring. To our knowledge, there have been no direct comparisons of the relative cytotoxic potency of ddVV and targeted radiotherapeutics. Moreover, despite the potential for enhanced cell killing by combining
ddVV encoding the sstr2 gene with sstr2-targeted radiotherapeutics, there have been no studies that have yet examined this approach. In this report, we describe the cytotoxicity of 177Lu- or 111In-DOTATOC alone or in combination with ddVV on human embryonic kidney (HEK-293) cells transfected with the sstr2 gene to overexpress sstr2 as well as in MC38 tumour cells with low sstr2 levels. Both monolayer and spheroid cultures were used to examine the effects of the short range Auger electron emissions from 111In or the longer range β-particles from 177Lu, as well as to study the delivery issues for 3-dimensional vs. 2-dimensional cultures. These studies are expected to inform on subsequent in vivo targeted radiotherapy experiments using ddVV and 177Lu/111In-DOTATOC in athymic mice bearing human tumour xenografts.

2.1 Materials and methods

2.1.1 Monolayer and spheroid cell culture

Human embryonic kidney (HEK-293) cells stably expressing sstr2 were generously provided by Dr. Ujendra Kumar, University of British Columbia, Vancouver, BC, Canada. MC-38 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium for HEK-293 cells was supplemented with 724 µg/L of geneticin (G418) (Sigma-Aldrich, Oakville, ON, Canada). Spheroids were prepared using a modification of the method reported by Ivascu et al. Briefly, 96-well plates were pre-coated with 25 µL per well of 1% polyHema, then air dried for 1 h. HEK-293 cells in T175 flasks were recovered using Accutase (Sigma-Aldrich) and seeded into the pre-coated plates at a density of 10^4 cells per 200 µL of medium. The plates were centrifuged at 1,500 x g for 10 mins and then incubated at 37 ºC/5% CO₂ for 3 d.

2.1.2 Radiopharmaceuticals

1, 4, 7, 10-tetraazacyclododecane-N, N’, N”, N”'-tetraacetic acid conjugated to Tyr³-Octreotide (DOTATOC) was generously provided by Dr. Helmut Maecke, University of Basel, Switzerland. DOTATOC (1 µg in 50 µL of 250 mM ammonium acetate buffer, pH 5.5) was radiolabeled by heating with 25-40 MBq of 111In (MDS Nordion, Kanata, ON, Canada) or 177Lu-
chloride (PerkinElmer, Waltham, MA, USA) at 95°C for 30 mins. $^{111}$In- or $^{177}$Lu-DOTATOC were purified on a reversed-phase C$_{18}$ Sep-Pak cartridge (Waters, Milford, MA, USA) eluted first with 3 mL of 400 mM sodium acetate buffer, pH 5 to elute the unbound radionuclides, then with 3 mL of methanol to elute the pure radiopharmaceuticals. The methanol fractions were collected, evaporated to dryness using nitrogen and reconstituted in 1 mL of normal saline. The final radiochemical purity (RCP) was determined by instant thin layer-silica gel chromatography (ITLC-SG) eluted with 100 mM sodium citrate, pH 5.0 and by reversed-phase HPLC. HPLC was performed using a Jupiter 5u 300A C-18, 250 × 4.6 mm column fitted to a PerkinElmer HPLC system composed of a Series 200 pump, diode array detector monitoring at 280 nm and flow scintillation analyzer in-line radioactivity detector. A gradient elution system of 0.1% trifluoroacetic acid (TFA) and acetonitrile as solvents A and B, respectively, were used. The gradient system included: 95% A (5% B) at 0 mins, 55% A (45% B) at 30 mins, 0% A (100% B) at 32 mins, 0% A (100% B) at 34 mins and 95% A (5% B) at 37 minutes. The flow rate was 0.75 mL/min.The sstr2 binding affinity of $^{111}$In- and $^{177}$Lu-DOTATOC was determined in direct saturation radioligand binding assays using HEK-293 cells transfected with the sstr2 gene. Briefly, increasing concentrations (0-119 nmols/L) of $^{111}$In- or $^{177}$Lu-DOTATOC in serum free medium were incubated in triplicate with 1 × 10$^6$ cells cultured overnight in 6-well plates at 4°C for 2 h. Unbound radiopharmaceuticals were removed and the adherent cells rinsed twice with normal saline and then recovered by dissolving in 100 mM sodium hydroxide. The cell-bound and unbound fractions were measured in a $\gamma$-counter (PerkinElmer Model 1480). Non-specific binding was evaluated by repeating the assay in the presence of 400 nmols/L of unlabeled somatostatin-14. The specifically cell-bound radioactivity was plotted vs. the unbound radioactivity and the curve fitted to a nonlinear regression curve model using GraphPad Prism® software Version 4, to estimate the dissociation constant ($K_d$) and the maximum binding sites per cell ($B_{max}$).

2.1.3 Double deleted Vaccinia virus (ddVV)

Double-deleted vaccinia virus (ddVV) is a genetically modified version of the Western Reserve strain VV that has the thymidine kinase (TK) gene and vaccinia growth factor gene deletions. It was constructed as described by McCart et al. $^{111,139}$ The ddVV was further engineered to incorporate the somatostatin receptor subtype 2 (sstr2) gene in its genome $^{116}$.

2.1.4 Evaluation of sstr2 expression on cells
The expression of sstr2 on HEK-293 cells (transfected/non transfected) or MC-38 cells with/without ddVV infection was examined by flow cytometry. Briefly, $5 \times 10^6$ cells were incubated with 1 µg of fluorescein isothiocyanate (FITC)-labeled anti-sstr2 polyclonal antibodies (Novus Biologicals, Littleton, CO, USA) in 100 µL of normal saline at room temperature (RT) for 30 mins in 1.5 mL Eppendorf tubes. The cells were recovered by centrifuging at 450 × g, rinsed twice with normal saline and then fixed with 500 µL of 3.7% formaldehyde at RT for 20 mins. The cells were recovered, rinsed twice again with normal saline and then sorted on a FACScan flow cytometer (BD Biosciences, Bedford, MA). Approximately 10,000 events were recorded and datasets were analyzed using CELLQuest software Ver. 3.3 (BD Biosciences, San Jose, CA, USA). Results were reported as the mean fluorescence intensity (MFI). Mean fluorescence intensity shift was calculated by subtracting the fluorescence intensity of the non-sstr2-expressing cells from that of cells expressing the sstr2 either by infection with virus or gene transfection.

2.1.5 Measurement of internalization and nuclear importation

The internalization and nuclear importation of $^{111}$In- or $^{177}$Lu-DOTATOC 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 nmols/L of $^{111}$In- or $^{177}$Lu-DOTATOC in serum-free growth medium for 4 h at 37°C. Unbound radiopharmaceuticals were removed and the cells rinsed twice with normal saline. Cell-surface bound radioactivity was then displaced by incubation for 10 mins with 1 mL of 200 mmols/L sodium acetate/500 mmols/L NaCl buffer, pH 2.5. Following recovery of this fraction, the cells were lysed on ice by treatment for 22 mins with 1 mL of lysis buffer from a nuclei isolation kit (Nuclei EZ Prep Kit product no NUC101-1KT, Sigma-Aldrich). The lysed cells were centrifuged at 3,000 × g for 5 mins and the supernatant containing the cytoplasmic fraction separated from the pellet containing the nuclei. Radioactivity in the cell-surface, cytoplasmic and nuclear fractions was measured in a γ-counter.
2.1.6 Treatment of HEK-293 cells in monolayer culture

The effect of exposure to $^{111}$In- or $^{177}$Lu-DOTATOC alone or in combination with ddVV on the growth of HEK-293 cells expressing sstr2 was determined. Increasing amounts (0.3-20 ng) of $^{111}$In- or $^{177}$Lu-DOTATOC (specific activity 6-9 MBq/µg) were incubated for 4 h at 37°C with $5 \times 10^5$ cells contained in 500 µL of normal saline in 1.5 mL Eppendorf tubes. Controls consisted of HEK-293 cells treated with normal saline, unlabeled DOTATOC or $^{111}$In- or $^{177}$Lu acetate. 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 µL) to each well and the plates incubated at 37°C/5% CO$_2$ for 4 d.

For vaccinia monotherapy or combination experiments, HEK-293 cells were first infected with ddVV for 48 h at a MOI of 0.1. In preliminary experiments, 48 h was found to be the optimal time point for the viral expression of sstr2; later time points reduced cell viability due to virus-induced cell lysis (not shown). Infection was achieved by adding $5 \times 10^5$ pfu of virus in 3 mL of growth medium containing 2.5 % fetal calf serum to $5 \times 10^6$ cells in T75 flasks. At 2 h after the addition of the virus, 8 mL of growth medium was added to the cells and cultured for 48 h at 37°C. $^{111}$In- or $^{177}$Lu-DOTATOC (60 ng) was then incubated with $5 \times 10^5$ ddVV-infected cells for 4 h at 37°C (a greater amount of DOTATOC was used for combination experiments to assure receptor saturation of the larger number of cells used). The treated cells were recovered, counted and seeded into wells ($8 \times 10^3$ cells/well) in a 96-well plate as described above and cultured for 8 d at 37°C/5% CO$_2$. Cell growth was measured each day to evaluate the kinetics of toxicity of the agents. Controls in these studies consisted of HEK-293 cells infected with ddVV alone. Cell growth was measured colorimetrically using the WST-1 assay which relies on the conversion of the WST-1 (Roche Diagnostics Canada, Laval, QC, Canada) tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to a coloured formazan complex by mitochondrial dehydrogenase in viable cells. To each well was added 10 µL of WST-1 reagent, and the plates incubated for 3.5 h at 37°C. An additional 100 µL of normal saline was dispensed into each well and the absorbance measured at 440 nm and 600 nm in an Ultrospec 2100pro UV/Visible plate reader. Absorbance at 600 nm (reference wavelength) was subtracted from that at 440 nm (the wavelength of absorbance of the formazan complex). The effect of different amounts of ddVV (MOI = 0.05 or 0.1) and time of infection (15 h or 48 h) on the growth of HEK-293 cells were also investigated.
2.1.7 Treatment of HEK-293 and MC-38 cells in spheroid culture

The effect of $^{111}$In- or $^{177}$Lu-DOTATOC on the growth of HEK-293 or MC-38 spheroids (400 µm in diameter) in culture was determined by incubating 6 spheroids in 500 µL of normal saline for 4 h at 37°C with 0.3-20 ng of the radiopharmaceuticals (specific activity 6-9 MBq/µg). Controls consisted of spheroids treated with normal saline, unlabeled DOTATOC or $^{111}$In- or $^{177}$Lu acetate alone. The spheroids were transferred into growth medium and cultured for 24 h before trypsinizing for 30 mins at 37°C. Cells from the dissociated spheroids were counted and seeded into 96-well plates at a density of $8 \times 10^3$ cells/well and cultured for an additional 5 d at 37°C/5% CO$_2$. For combination studies, HEK-293 or MC38 spheroids were first infected with ddVV for 48 h at a MOI of 0.1. Virus-infected spheroids (6-10 spheroids per well) were then incubated with 20 ng of $^{111}$In- or $^{177}$Lu-DOTATOC in 200 µL of normal saline for 4 h at 37°C and cultured for 24 h. The spheroids were dissociated by trypsinization, then seeded into 96-well plates and cultured for up to 8 d at 37°C/5% CO$_2$. The growth of the cells was determined colorimetrically using the WST-1 assay as described previously. The effect of different amounts of ddVV (MOI = 0.05 or 0.1) and time of infection (24 or 48 h) on the growth of HEK-293 or MC-38 cells treated as spheroids were also investigated.

2.1.8 Statistical analysis

All results are expressed as mean ± standard deviation. Student’s t-test was used for statistical comparisons. P values ≤ 0.05 were considered statistically significant.

2.2 Results

2.2.1 Radiopharmaceuticals

The radiochemical purity of $^{111}$In- and $^{177}$Lu-DOTATOC was greater than 90% measured by ITLC-SG in 100 mM sodium citrate, pH 5.0 ($R_f$ values = 0-0.1 for ($^{111}$In- and $^{177}$Lu-DOTATOC), 1 for ($^{111}$In- and $^{177}$Lu). The retention times ($t_R$) of $^{111}$In- and $^{177}$Lu-DOTATOC when analyzed by reversed phase HPLC were 22.0 mins and 22.1 mins, respectively, which were identical to that of unlabeled DOTATOC ($t_R$=22.1 mins), demonstrating no change in polarity with incorporation of these radionuclides. Both $^{111}$In- and $^{177}$Lu-DOTATOC bound with high
affinity to sstr2 on HEK-293 cells (Table 2.1). The $B_{\text{max}}$ values corresponded to $1.4 \times 10^5$ receptors/cell and were similar to those previously reported by Reubi et al.¹⁴⁰

Table 2.1

Binding characteristics of $^{111}$In- and $^{177}$Lu-DOTATOC for sstr-2 expressed on HEK-293 cells

<table>
<thead>
<tr>
<th></th>
<th>$^{111}$In-DOTATOC</th>
<th>$^{177}$Lu-DOTATOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (nmoles/L)</td>
<td>$8.4 \pm 2.7$</td>
<td>$11.4 \pm 1.2$</td>
</tr>
<tr>
<td>$B_{\text{max}}$ (receptors/cell x $10^5$)</td>
<td>$1.4 \pm 0.1$</td>
<td>$1.1 \pm 0.1$</td>
</tr>
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</table>

¹ Measured in direct radioligand binding assays fitted to a 1-site binding model using GraphPad Prism® Version 4.0 software (n=4). There were no statistical differences in $K_d$ or $B_{\text{max}}$ values between the two radioligands (P>0.05).

2.2.2 Evaluation of sstr2 expression on cells

The expression of sstr2 on HEK-293 cells transfected with the $sstr2$ gene or on MC-38 cells infected with ddVV encoding the $sstr2$ gene at an MOI of 1 is shown in Fig. 2.1 A and B, respectively. There was a 1.65 unit shift in MFI from 8.70 to 10.35 for non-transfected cells compared to HEK-293 cells transfected with the $sstr2$ gene. There was a 2.0 unit increase in MFI for MC-38 cells infected with ddVV compared to non-infected MC-38 cells (MFI= 10.38 vs. 8.38 respectively).
Fig. 2.1:
Flow cytometry histogram showing fluorescence of anti-somatostatin receptor subtype 2 (sstr2)-associated cells

A: HEK - SSTR2 FITC
B: MC38 - ddVV FITC

Receptor expression on cells measured by cell sorting on a FACScan flow cytometer with 10,000 events recorded. Histogram peaks were generated using CELLQuest software Ver. 3.3. No-fill and fill histograms are the non-expressing and sstr2-expressing cells of the same cell line, respectively.

2.2.3 Measurement of internalization and nuclear importation

Internalization and nuclear importation is important for the cytotoxicity of the subcellular range Auger electrons emitted by $^{111}$In. Approximately 70% of total cell bound $^{111}$In-DOTATOC remained on the cell membrane of HEK-293 cells; 20% was internalized, and 2-5% was imported into the nucleus from 4 to 24 h when incubated with the cells at 37°C (Fig. 2.2). There was similar internalization and nuclear importation of $^{177}$Lu-DOTATOC (22% and 3.6%, respectively).
Fig. 2.2:

Histogram showing the percent of the total cell-bound $^{111}$In-DOTATOC in the different subcellular compartments in HEK-293 cells.

2.2.4 Treatment of HEK-293 cells in monolayer culture

The effect of treatment with $^{111}$In- or $^{177}$Lu-DOTATOC alone or in combination with ddVV at an MOI of 0.1 on the growth of HEK-293 cells, is shown in Fig. 2.3. At amounts of $^{111}$In- or $^{177}$Lu-DOTATOC alone up to 20 ng (120 – 180 kBq) per $5 \times 10^5$ cells, the growth of these cells compared to untreated cells over 5 d was significantly reduced to 63.4 ± 8.8% or 53.6 ± 0.9%, respectively (p = 0.03) (Fig. 2.3A). At a higher amount (60 ng), the growth of HEK-293 cells over 5 d was reduced to 44.5 ± 9.7% by $^{111}$In-DOTATOC and to 29.9 ± 12.3% by $^{177}$Lu-DOTATOC (Fig. 2.3B). However, these differences in growth inhibition for $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC were not significant (p=0.15). Interestingly, culture of HEK-293 cells treated with $^{111}$In-DOTATOC for 7 d resulted in a recovery of the growth of the cells to 66.3 ±
16.3% of that of untreated cells at 7 d (Fig. 2.3B). This was different from the growth of cells treated with $^{177}$Lu-DOTATOC for 7 d at 60 ng 15.8 ± 2.8% (p=0.07). ddVV alone at an MOI of 0.1 appeared to be less effective than $^{177}$Lu-DOTATOC reducing the growth of HEK-293 cells over 7 d to 37.6 ± 20.2% but the difference did not quite reach statistical significance (p=0.06) (Fig. 2.3C). Treatment of HEK-293 cells with unlabeled DOTATOC (20 ng) or $^{111}$In-acetate had only a minor effect on the growth of the cells whereas exposure to $^{177}$Lu-acetate reduced their growth moderately (Fig. 2.3D). The effect of the ddVV infection period (15 h or 48 h) and MOI (0.05 or 0.1) on cell growth was studied for HEK-293 cells grown in monolayer (Fig. 2.4). An MOI of 0.1 was similarly effective as an MOI of 0.05, but a 48 h incubation period appeared to provide greater cell growth inhibition, particularly at later time points (>6 d).

Combining ddVV with $^{111}$In-DOTATOC prevented the apparent growth recovery of HEK-293 cells at 7 d observed with $^{111}$In-DOTATOC alone and significantly decreased the growth of these cells to 22.9 ± 8.0% compared to untreated cells (p<0.001; Fig. 2.3C). The growth of HEK-293 cells treated with the combination of ddVV and $^{177}$Lu-DOTATOC was strongly reduced to 6.6 ± 1.8%. Even greater differences were observed between ddVV treatment alone or in combination with $^{111}$In- or $^{177}$Lu-DOTATOC at earlier culture periods. For example, the growth of HEK-293 cells was reduced only to 98.7 ± 10.7% by ddVV after 1 d of culture, whereas growth was reduced at this same time point to 65.8 ± 2.7% and 23.9 ± 0.2% by ddVV in combination with $^{111}$In- or $^{177}$Lu-DOTATOC, respectively (p=0.01 and p=0.001). ddVV at an MOI = 0.05 or 0.1 similarly reduced the growth of HEK-293 cells in monolayer culture, but an infection period of 48 h was more effective; a 15 h infection period allowed some regrowth of the cells (Fig. 2.4). Toxicity of vaccinia virus at 15hrs and 48hrs were evaluated based on the fact that vaccinia virus replication occurs every 12hrs. In other words, one complete replication cycle of the virus takes 12hrs. Hence, a 15hrs time point shows toxicity after one replication cycle while 48hrs shows toxicity after 4 replication cycles.

### 2.2.5 Treatment of HEK-293 and MC-38 cells in spheroid culture

Both $^{111}$In- and $^{177}$Lu-DOTATOC alone were highly effective in reducing the growth of MC-38 cells in spheroid culture (Fig. 2.5A), decreasing their growth over 8 d to 4.9 ± 0.02% or 4.9 ± 0.4%, respectively (not significantly different; P>0.05). $^{111}$In- and $^{177}$Lu-DOTATOC reduced the growth of HEK-293 spheroids to 17.2 ± 4.9 and 26.5 ± 6.3%, respectively, over 8 d
ddVV alone was significantly less growth-inhibitory to MC-38 spheroids than ¹¹¹In- or ¹⁷⁷Lu-DOTATOC, reducing their growth only to 18.0 ± 2.7% (p=0.003 and p=0.0005, respectively) over 8 d (Fig. 2.5C). ddVV alone was as effective as ¹¹¹In- or ¹⁷⁷Lu-DOTATOC in reducing the growth of HEK-293 cells in spheroid culture (18.5 ± 9.9% over 8 d; p= 0.29 and 0.39, respectively; Fig. 2.5D) but was less effective at earlier time points.

Combining ddVV with ¹¹¹In- or ¹⁷⁷Lu-DOTATOC decreased the growth of MC-38 spheroids to 4.9 ± 0.01% or 5.0 ± 0.6%, but these values were not significantly different than those for radiopharmaceutical treatment alone (p=0.41 and p=0.44) (Fig. 2.5C). Similar to the results for monolayer culture, combining ¹¹¹In- or ¹⁷⁷Lu-DOTATOC with ddVV provided greater anti-proliferative effects at earlier time points. For example, at 3 d the growth of MC-38 cells was reduced to 83.8 ± 17.5% by ddVV alone, but to 68.5 ± 0.7% or 60.6 ± 15.5% (p= 0.003 and 0.02) when combined with ¹¹¹In- or ¹⁷⁷Lu-DOTATOC, respectively. A slight but insignificant reduction in cell growth was obtained for combining ddVV with ¹¹¹In- or ¹⁷⁷Lu-DOTATOC for treatment of HEK-293 spheroids (Fig. 2.5D). ddVV alone decreased the growth of HEK-293 spheroids to 18.5 ± 9.9% over 8 d, but when combined with ¹¹¹In-DOTATOC, cell growth was 13.1 ± 7.1% (p=0.29). The effects of combining ddVV with ¹¹¹In- or ¹⁷⁷Lu-DOTATOC on HEK-293 cell growth at earlier time points were more dramatic, however, than that for MC-38 cells. For example, at 3 d, the growth of HEK-293 cells was reduced to 82.4 ± 12.4% by ddVV alone, but to 30.1 ± 14.1% or 43.5 ± 14.1% (p=0.01 and 0.04) when combined with ¹¹¹In- or ¹⁷⁷Lu-DOTATOC, respectively. ddVV at an MOI = 0.05 or 0.1 reduced the growth of HEK-293 in spheroid culture, but an infection period of 48 h was more effective. A 24 h infection period allowed growth of these HEK-293 cells, particularly at early time points (Fig. 2.6). Similar to monolayer therapy with vaccinia virus alone, toxicity of vaccinia virus at 24hrs and 48hrs were evaluated based on the time it takes the virus to complete a replication cycle which is 12hrs. Hence, a 24hrs time point shows toxicity after two replication cycles while 48hrs shows toxicity after 4 replication cycles.
Graph showing the cytotoxicity of vaccinia virus, $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC in HEK-293 cells grown as monolayers.

Growth of HEK-293 cells after treatment with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC compared to untreated cells using 20 ng (A) or 60 ng (B) of these radiopharmaceuticals. Growth of HEK-293 cells after treatment with ddVV only, ddVV in combination with $^{111}$In-DOTATOC (60 ng) or ddVV with $^{177}$Lu-DOTATOC (60 ng) (C), or after treatment with unlabeled DOTATOC (60 ng), $^{111}$In-acetate or $^{177}$Lu-acetate (D). MOI = 0.1 was used for
ddVV experiments. Cytotoxicity was measured by the WST1 assay. Each data point represents the mean ± standard deviation (n=3) of three independent studies.

Fig. 2.4

Graph showing the growth of HEK-293 cells treated with vaccinia virus and infected for different times and at different MOIs compared to untreated cells.

Growth of HEK-293 cells compared to untreated cells after treatment with ddVV at MOI of 0.05 (A) and an MOI of 0.1 (B) after 15 h (1 replication cycle) or 48 h (4 replication cycles) of infection. Cytotoxicity was measured by the WST1 assay. Each data point represents the mean ± standard deviation (n=3) of three independent studies.
Fig 2.5

Graph showing the cytotoxicity of vaccinia virus, $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC on HEK-293 spheroids and MC-38 spheroids.

Growth after treatment with either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC compared to untreated cells in MC-38 spheroids (A) or in HEK-293 spheroids (B). Growth of spheroids after treatment with ddVV only, ddVV in combination with $^{111}$In-DOTATOC (60ng) and ddVV with $^{177}$Lu-DOTATOC (60ng) in MC-38 spheroids (C), and in HEK-293 spheroids (D). MOI – 0.1 was used for ddVV experiments. Cytotoxicity was measured by the WST1 assay. Each data point represents the mean ± standard deviation (n=3) of three independent studies.
Growth of HEK-293 spheroids compared to untreated spheroids after treatment with ddVV at MOI of 0.05 (A) and 0.1 (B) after 24 h (2 replication cycles) and 48 h (4 replication cycles) of infection. Cytotoxicity was measured by the WST1 assay. Each data point represents the mean ± standard deviation (n=3) of three independent studies.
2.3 Discussion

One of the determining factors in the use of a radiotherapeutic approach exploiting the expression of sstr2 on tumours is the degree of receptor expression. In this study, two different cell lines (HEK-293 and MC-38 cells) with different levels of sstr2 expression were employed. Ujendra et al \(^1\) previously reported that the human embryonic kidney cells (HEK-293) transfected with the sstr2 gene stably overexpressed these receptors. Cell binding assays using \(^{111}\text{In-DOTATOC}\) confirmed the receptor population (\(1.4 \times 10^5\) receptors per cell) reported (Table 2.1). Murine colon cancer cells (MC-38), on the other hand, had low endogenous expression of sstr2. In this report, HEK-293 cells served as control cell lines which had a higher level of expression of the somatostatin receptors, and was consequently used to compare the receptor expression of the vaccinia virus induced somatostatin receptors in MC-38 cells infected with this virus. In addition, the toxicity of both radiopharmaceuticals was compared in both cell lines. McCart et al \(^1\) constructed a strain of vaccinia virus (ddVV) that encodes the sstr2 gene which upon infection of cells expresses the receptors. They demonstrated that the increase in the population of these receptors in MC-38 tumours \(\textit{in vivo}\) following systemic administration of the virus was sufficient for molecular imaging using \(^{111}\text{In-pentetreotide}\) \(^1\). This approach was adapted in our \(\textit{in vitro}\) studies in which we also observed an increase in the sstr2 expression of MC-38 cells measured by flow cytometry using a fluorescein-conjugated anti-sstr2 antibody. The sstr2 expression in MC-38 cells infected with the ddVV was similar to that in the HEK-293 cells (Fig 2.1). Receptor expression mediated by the ddVV is dependent, however, on the multiplicity of infection (MOI) and the virus infection time of the cells. The sstr2 gene is placed under a late synthetic promoter which allows for receptor expression at the end of the virus replication cycle of 12 h. In preliminary cellular uptake studies of \(^{111}\text{In-DOTATOC}\) in HEK-293 cells, sstr2 expression was examined at 12, 24, 39 and 48 h post-infection. Though the levels of receptor expression increased at the later time points, a substantial amount of cell death was often noted due to the cytotoxic effects of the virus. Thus, for evaluating the effects of combined ddVV and targeted radiotherapy, an incubation time of 24 h was chosen to allow viral sstr2 expression for subsequent targeting with the radiopharmaceuticals but not causing high viral cell killing. Similarly, an MOI of 1 reduced the survival of the cells to <5% (not shown) within 48 h. This would have prevented the ability to discern the combined effects of the virus and \(^{111}\text{In- or }^{177}\text{Lu-DOTATOC};\) thus an MOI of 0.05 or 0.1 was used.
In order to evaluate the combined effects of ddVV and either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC, it was necessary to first compare the toxicity caused by these treatments alone on HEK-293 or MC-38 cells. Our results revealed that the potency of antiproliferative effects were greatest for $^{177}$Lu-DOTATOC on cells in monolayer culture or grown as spheroids followed by $^{111}$In-DOTATOC and then ddVV (at an MOI = 0.1). The difference in cytotoxicity between $^{177}$Lu- and $^{111}$In-DOTATOC may be due to the more energetic emissions from $^{177}$Lu ($\beta$-particles with energy of 0.5 MeV) and their longer range (up to 2 mm) which would easily traverse the cell monolayers and spheroids causing lethal DNA damage. In contrast, the very low energy Auger electrons from $^{111}$In (maximum energy 25 keV and 10 µm range) are toxic only when situated in close proximity to the cell nucleus. While cell fractionation experiments in this study showed internalization of $^{111}$In-DOTATOC into the cytoplasm of HEK-293 cells, the proportion of nuclear localization was low (4.5%). Others have similarly reported internalization of $^{177}$Lu-DOTATATE and less nuclear uptake $^{141}$. In addition to the differences in potency on HEK-293 cells in monolayer, there was regrowth of these cells when treated with $^{111}$In-DOTATOC but not those treated with $^{177}$Lu-DOTATOC after 4 or 5 d in culture (Fig 2.3). These results suggest that a proportion of the cells treated with $^{111}$In-DOTATOC received a sublethal dose of radiation which allowed their survival and continued proliferation, presumably after an initial growth arrest delay to allow for DNA repair. This is plausible since targeting of each cell would be required for cytotoxicity from $^{111}$In-DOTATOC (unless there is a bystander effect), whereas $^{177}$Lu-DOTATOC could kill non-targeted cells through a “crossfire” effect due to the longer range of the $\beta$-particles. Nonetheless, the cytotoxicity of $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC was both receptor-mediated because the controls $^{111}$In-acetate, $^{177}$Lu-acetate or unlabeled DOTATOC were much less toxic to the cells. ddVV at an MOI of 0.1 and infection time of 24 h was the least effective at killing HEK-293 cells or HEK-293 or MC-38 spheroids, but this was anticipated since the amount of virus used and infection time were selected to minimize cell killing to allow an examination of the effects in combination with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC, as discussed earlier.

Combination treatment of ddVV with $^{111}$In- or $^{177}$Lu-DOTATOC (20 ng) proved to be more effective at cell growth inhibition than either of these single agents in monolayer culture. However, 60 ng of $^{177}$Lu-DOTATOC alone was as effective at inhibiting cell growth as combination treatment with ddVV and $^{177}$Lu-DOTATOC (20 ng) (Fig 2.3). A few other studies have examined the interaction between oncolytic viruses and external radiation on malignant cells $^{142,143}$, however, none have reported the combination of vaccinia virus in particular with a molecularly targeted radiotherapeutic agent. We determined that combining ddVV with $^{177}$Lu-
DOTATOC provided higher toxicity to cells in monolayers at earlier time points (day 0-4) than the combination of ddVV with $^{111}$In-DOTATOC. This is likely caused by the differences in potency between these two radiopharmaceuticals as explained above. Cytotoxicity from vaccinia virus alone is due to direct lysis of the infected cells which is dependent on the replication of the virus. Cytotoxicity due to radiation is mainly due to direct or indirect DNA damage due to the production of free radicals. The independence of these two cell killing mechanisms makes their combination rational for cancer treatment. After day 4, however, the, toxicity of either $^{111}$In- or $^{177}$Lu-DOTATOC in combination with ddVV were similar (Fig 2.3).

The use of spheroid models in this study was a strategy to mimic the microenvironment observed in tumours. As a result of the similar geometry to solid tumours, the interaction between tumour cells and the behaviour elicited by them after exposure to cytotoxic agents such as ionizing radiation can be observed in spheroids. In addition the resulting toxicity from such agents which would be similar to tumors can also be observed in spheroids. In our spheroid models, both $^{177}$Lu- and $^{111}$In-DOTATOC were equally effective at killing sstr2-expressing HEK-293 or MC-38 cells as single agents (Fig 2.5). Although no difference in the potency of these radiopharmaceuticals was noted in spheroid models, dramatically higher toxicity was found compared to cells treated as monolayers. The spheroids used were relatively small (400-460 µm in diameter) and as a result should be well-perfused with growth medium and oxygen and not expected to have hypoxic or necrotic areas which have been previously observed in larger spheroids. Since $^{111}$In-DOTATOC would require direct targeting of cells as well as internalization and nuclear localization to produce cytotoxic effects from the Auger electron emissions, and it is unlikely that all cells within these spheroids were targeted by the radiopharmaceutical, it is possible that there may be a significant “bystander effect” which compensates and provides overall equivalent cytotoxicity as $^{177}$Lu-DOTATOC. These bystander effects result in the transmission of cell death signals to surrounding viable cells from lethally damaged cells by means of cytokines and chemokines and are known to be more prevalent in spheroid cultures in vitro or in solid tumours in vivo. The crossfire effects from the long-range β-particles emitted by $^{177}$Lu-DOTATOC would not require a bystander effect to kill most including non-targeted cells in these small spheroids. The role of crossfire in the toxicity of $^{177}$Lu-DOTATOC would be minimized however by the short incubation period used (24 h) before the spheroids were disaggregated and cultured for evaluation of the cytotoxic effects, although one may nonetheless exist. Similar to the monolayer experiments, ddVV at an MOI of 0.1 and infection time (48 h) was less toxic to the spheroids than $^{111}$In- or $^{177}$Lu-DOTATOC, but again,
this was due to the low amount of virus and short infection period chosen to evaluate the combined effects of the virus and the radiopharmaceuticals.

Treatment of sstr2-positive HEK-293 or MC-38 spheroids with ddVV at an MOI of 0.1 in combination with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC was only slightly more effective at killing the cells as treatment with the radiopharmaceuticals alone (Fig 2.5). This slight increase in toxicity was possibly due to modestly increased binding of the radiopharmaceuticals to the cells as a result of the expression of additional sstr2 receptors by the virus. In this regard, the combination with ddVV may improve toxicity and could potentially be enhanced further at a higher MOI, but careful selection of the MOI and infection time is needed to determine this effect in the presence of the high cytotoxicity produced by the virus alone or by the radiopharmaceuticals alone.

2.3 Conclusions

We therefore conclude that the combination of ddVV with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC is advantageous compared to either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC as single agents only in monolayer cultures of HEK-293 cells expressing the somatostatin receptor. In spheroids, however, there was no added benefit of combining vaccinia virus with either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC as cytotoxicity observed in the combination treatment was similar to when either radiopharmaceutical was used as single agents in HEK-293 and MC-38 spheroids. Vaccinia virus was found to be the least effective at cell growth inhibition, but this might be due to the low multiplicity of infection used (0.1) and the infection time of the virus in the cells and spheroids.
CHAPTER 3

Summary and Future Directions
In this study, it was found that the combination of ddVV at an MOI of 0.1 and infection time of 48 h with $^{177}$Lu-DOTATOC was more effective at killing HEK-293 cells in monolayer than the combination of ddVV with $^{111}$In-DOTATOC. One of the factors that may be responsible for this difference in toxicity is the greater maximum distance travelled by the $\beta$-particles emitted by $^{177}$Lu (2 mm) compared to the Auger electrons from $^{111}$In (<10 µm). Auger electrons are known to be most effective at causing damage to cells when positioned in close proximity to the nucleus of the cell. Nevertheless internalization and nuclear localization studies of $^{111}$In-DOTATOC in sstr2-positive cells in this study revealed only 4.5% nuclear localization after 15 hours of incubation. To improve nuclear localization of $^{111}$In-DOTATOC, Maechke et al have synthesized a new construct consisting of DOTATOC modified with a nuclear localizing sequence (NLS) peptide attached to the N-terminal of the DOTATOC (NLS-DOTATOC) and have shown that these analogues have superior nuclear localization than the $^{111}$In-DOTATOC in somatostatin receptor-positive cells. Thus, similar studies to the ones described in this thesis could be performed with $^{111}$In-NLS DOTATOC in combination with ddVV to evaluate its toxicity in comparison to $^{177}$Lu-DOTATOC in sstr2-positive cells and spheroids.

Based on the cytotoxicity results found in HEK-293 and MC-38 spheroid cultures, it was proposed that bystander effects may play an important role in the degree of toxicity observed for $^{111}$In-DOTATOC in these models but less so in monolayers. In particular, the degree of cell growth inhibition observed after treatment with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC was equivalent for cells treated in spheroids than in monolayer culture. Further experiments need to be conducted to confirm the presence of these bystander effects. Two different approaches have been described in the literature to examine the bystander phenomenon. One, reported by Boyd et al, would involve growing cells expressing sstr2 with non-sstr2-expressing cells in different ratios as spheroids, then targeting these cells with $^{111}$In-DOTATOC. Clonogenic survival of spheroids containing the different ratios of the two cell types could be then be used as an endpoint to evaluate the additional toxicity inflicted on non-targeted (and receptor-negative) cells by the bystander effects. A different approach to measure bystander effects is discussed in a review by Hei et al. A medium-transfer method could be utilized in which non-irradiated cells are treated with media from previously irradiated cells. This concept takes into consideration the release of cell death mediators from targeted irradiated cells into the medium that, after transfer to the non-irradiated cells, would be lethal.

One important model used in in vitro studies reported in this thesis is the 3-D spheroid model. The encouraging results from the in vitro studies reported in this thesis would serve as a
basis to study \textit{in vivo} treatment of sstr2-positive tumour xenograft mouse models. These spheroids are important and relevant to future \textit{in vivo} tumour xenograft studies because of their geometric resemblance, therefore, the toxicity of the agents to cells in this model provides an insight to what may be expected in \textit{in vivo} tumour models. In the results, it was demonstrated that both $^{111}$In-DOTATOC and $^{177}$Lu-DOTATOC in combination with ddVV were as effective at cell growth inhibition in sstr2-positive cells grown as spheroids compared to either agent alone. In the next step, it would be important to confirm these findings in \textit{in vivo} tumour xenograft models.

McCart et al reported a study in which athymic nude mice bearing MC-38 tumours in their right flank were injected with ddVV and then administered $^{111}$In-DTPA octreotide ($^{111}$In-pentetreotide) for imaging purposes but not for targeted radiotherapy\textsuperscript{116}. This mouse model could be employed for \textit{in vivo} studies to evaluate the effect of combining ddVV with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC. In one possible \textit{in vivo} study design, groups of athymic mice (nu/nu) would be inoculated with MC-38 cells in the right flank. Tumour cells would be allowed to grow to form tumours 5 to 7 mm in size, after which the mice would be injected i.v. (tail vein) with $10^9$ pfu of ddVV. Since the ddVV used can also carry the gene for the Red florescence protein (RFP), the spread of the virus could be confirmed by examining the mice at least 3 days after virus infection using small animal fluorescence imaging. When sufficient RFP is expressed, the mice could be pre-injected with D-lysine to inhibit the accumulation of radiolabeled DOTATOC in the kidneys\textsuperscript{152}, after which they would be injected with increasing doses of the radiopharmaceuticals. Tumour sizes could be measured for a period of 5 to 7 weeks. To evaluate if there is any benefit of combining ddVV with $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC, two different control groups of tumour-bearing mice would be used for comparison. One control group would be injected with ddVV and the other group would be injected with only the radiopharmaceuticals, i.e. $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC. The tumour sizes of these control group mice could be statistically compared to mice treated with the combination therapy of ddVV and either $^{111}$In-DOTATOC or $^{177}$Lu-DOTATOC. Other control groups include mice treated with normal saline alone, mice treated with ddVV alone, mice treated with $^{111}$In or $^{177}$Lu alone, and mice treated with DOTATOC alone. Due to the immunogenic nature of ddVV, additional studies in immunocompetent animals would be required for these \textit{in vivo} studies to provide a clear picture of the potential toxicity of the vaccina virus in humans. Alternatively, for future use in clinical practice, a means of reducing its immunogenicity would be required to provide continued efficacy.
According to the flow cytometry results in this thesis showing the expression of somatostatin receptors, infection of cells with ddVV-sstr2 further increased the receptor population on sstr2-positive but low-expressing MC-38 cells (refer to Fig 2.1). These increased receptor levels were targeted in an imaging study performed by McCart et al.\textsuperscript{116} using \textsuperscript{111}In-pentetreotide, and in the cytotoxicity studies using \textsuperscript{111}In-DOTATOC and \textsuperscript{177}Lu-DOTATOC in this thesis. An interesting concept to evaluate is the ability to populate sstr2 in non-receptor-positive cells and in \textit{in vivo} models by infecting these cells with the ddVV strain used in our study. After viral delivery of the receptors, either \textsuperscript{111}In-DOTATOC or \textsuperscript{177}Lu-DOTATOC could be used to target them in order to study the cytotoxicity \textit{in vitro} and tumour growth inhibition \textit{in vivo}. If this approach is successful, it could serve as a novel strategy in the management of non-sstr2-positive tumours.

A major concern raised in previous clinical trials using vaccinia virus for therapy is the issue of pre-vaccination against the smallpox virus, a virus in the same family as vaccinia\textsuperscript{153}. The injection of vaccinia into such patients might result in an increased immune clearance of the virus as a result of the existence of neutralizing antibodies from the previous vaccination. To make the combination of ddVV with either \textsuperscript{111}In-DOTATOC or \textsuperscript{177}Lu-DOTATOC therapy attractive for use in clinical studies, it would therefore be useful to seek ways of reducing the immunogenic nature of vaccinia virus or the response to the virus in humans. One possibility is the use of cyclophosphamide, an immunomodulator that diminishes the effect of these antibodies against vaccinia\textsuperscript{96,124,154}. Cyclophosphamide has been used in combination with herpes simplex virus and vaccinia virus. Fulci et al demonstrated that cyclophosphamide when combined with HSV1 in \textit{in vivo} studies enhanced the oncolytic effects of HSV by inhibition of immune cells which included CD68+ natural killer cells and CD163+ microglia/microphages\textsuperscript{96,154}. In addition, an increased interferon (IFN-γ) expression was observed with the addition of cyclophosphamide. In a study by Lun et al, where RG2 tumour-bearing Fisher rats were used to study the advantage of pretreatment with cyclophosphamide before vaccinia virus, 60 mg/kg cyclophosphamide was found to enhance the cytotoxicity of the virus based on its anti-immune functions\textsuperscript{124}. As a result, it is possible that pretreatment with cyclophosphamide prior to ddVV would enhance its oncolytic effects as observed in these studies. The targeting of the virally delivered somatostatin receptors on these cells with either \textsuperscript{111}In-DOTATOC or \textsuperscript{177}Lu-DOTATOC would further result in superior cell growth inhibition compared to ddVV only. However, with a reduction in the clearance of vaccinia virus as a result of cyclophosphamide treatment, a higher retention of the virus is expected in the systemic circulation which might increase the risk of exposure to other healthy
tissues. To date, ddVV has been shown to be highly selective for tissues with high proliferating cells and a leaky vasculature. In this respect, only ovarian follicles are most susceptible to the cytotoxic effects. Although studies with wild type vaccinia virus resulted in a substantial amount of damage to the ovaries, the ddVV strain used in this study showed no damage as a result of superior selectivity to tumours as explained previously 113.

Finally, another possibility is to engineer genetically Yaba-like disease virus (YLD) discovered by Hu et al 153 to incorporate the ssTR2 gene instead of vaccinia virus. Yaba-like disease virus has a similar replication capacity to vaccinia virus, but it is not recognized by anti-vaccinia virus antibodies. Consequently it has better efficacy and slower plasma clearance 153.
References


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