Functional DNA Aptamers as Biotherapeutic Molecules

By

Erik William Orava

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

© Copyright by Erik William Orava, 2013
ABSTRACT
Functional DNA aptamers as biotherapeutic molecules

Erik William Orava
Doctor of Philosophy, 2013
Graduate Department of Pharmaceutical Sciences
University of Toronto

Aptamers are single-stranded oligonucleotides, DNA or RNA, which can bind to a myriad of targets such as ions, peptides, proteins, drugs, organic and inorganic molecules with high affinity and specificity. Aptamers are derived using combinatorial libraries comprised of a variable region flanked by two primer regions used for a process termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The central theme of my thesis was to use this technology to develop aptamers able to bind to validated therapeutic targets, specifically the Tumour Necrosis Factor alpha (TNFα) and Carcinoembryonic Antigen (CEA), and block their biological functions. As well, I investigated the use of CEA and MUC1 binding aptamers as targeting agents to guide and detect the delivery of contrast agent-loaded liposomes in tumour-bearing mice using computed tomography (CT) imaging. Aptamer selections successfully identified a 25-base aptamer (VR11) that can bind with high affinity and specificity to TNFα. VR11 blocked TNFα signaling, prevented apoptosis, reduced nitric oxide (NO) production in cultured cells and was non-immunogenic when injected into C57BL/6 mice. As well, aptamers were derived to the IgV-like N-domain of CEA. Two DNA aptamers were isolated containing a 40-base variable region, N54 and N56, bearing anti- CEA homotypic adhesive properties. These aptamers are not cytotoxic or immunogenic and
are able to prevent CEA-mediated homotypic and heterotypic cell adhesion events. In addition, the pretreatment of murine cancer cells expressing CEA with these aptamers prior to their intraperitoneal injection into C57BL/6 mice resulted in the prevention of tumour foci formation. Finally, the in vivo targeting of nanoparticles such as pegylated liposomes to tumour cells was enhanced by introducing tumour marker-specific DNA aptamers on their surface. The CEA-specific aptamer N54 and a 40-base second generation aptamer MUC1-VR1 that recognizes the tumour-associated mucin MUC1 were incorporated into liposomes containing the CT contrast agent Omnipaque350™ and Cy5 to characterize their binding to CEA and MUC1-expressing cancer cells in vitro. Pharmacokinetic studies also revealed that the incorporation of these aptamers into pegylated liposomes significantly lengthened their circulation half-lives to values that paralleled that of untargeted pegylated liposomes.
ACKNOWLEDGEMENTS

I would like to express my sincerest and deepest appreciation to Dr. Jean Gariépy for the opportunity to work and study in his laboratory. I am particularly grateful for his unwavering encouragement, support direction and persistent guidance over the 5 past years. Without his motivation, creativity and assistance my reports, abstracts, patent applications, manuscripts and this dissertation would not have been possible.

I would like to thank my committee members, Dr. Christine Allen, Dr. Robert Macgregor, and Dr. Sachdev Sidhu for their time and expertise in supporting my graduate studies. Their engagement and advice contributed greatly to the success I achieved during my studies. I would like to acknowledge Dr. Christine Allen and former student Mike Dunne and Huang Huang for their assistance in our CIHR Biotherapeutics collaboration, Dr. Sachdev Sidhu and his lab member Nick Jarvik for their help with surface plasmon resonance experiments as well as Dr. Robert Macgregor and Yuen Shek for their advice and technical assistance for circular dichroism experiments.

In addition I would extend my greatest appreciation for my current lab members: Arshiya, Aws, Amirul, Caitlin, Eric, Linda, Marzena, Nick, Nora and former lab members: Andrew and Wei. A special thanks to Dr. Aws Abdul-Wahid for challenging me and engaging in critical discussions that was instrumental in the development of CEA aptamers. I wish everyone the best as they continue to pursue their future studies and career paths.

This research would not have been possible without the financial assistance from the Department of Pharmaceutical Sciences in the form of awards, bursaries and scholarships, particularly the studentship award from the CIHR strategic training
program in biological therapeutics.

Lastly, I would like to thank Sarah and my family for their constant love and support. I could not have pursued and accomplished my goals and attained my education without them.
Table of Contents

ABSTRACT .................................................................................................................. II
ACKNOWLEDGEMENTS ....................................................................................... IV
LIST OF ABBREVIATIONS ........................................................................... IX
LIST OF TABLES .............................................................................................. XIII
LIST OF FIGURES ............................................................................................ XIV

Chapter 1: Introduction ......................................................................................... 1

1.1. Aptamers as therapeutics .................................................................................. 2
    1.1.1. The SELEX procedure: A rapid strategy to identify short single strand
          synthetic oligonucleotides (aptamers) that recognize specific targets .............. 3
    1.1.2. Cell-SELEX ............................................................................................ 7
    1.1.3. RNA versus DNA aptamers ...................................................................... 8

1.1. Aptamers as Inhibitors ..................................................................................... 9
    1.2.1. The role of TNFα in disease progression ................................................. 12
    1.2.2. Inflammation and TNFα ........................................................................ 12
    1.2.3. TNFα as a therapeutic target .................................................................. 15

1.2. Aptamers can serve as intracellular delivery vehicles via their binding to
    known cancer-associated surface antigens ........................................................ 15
    1.3.1. CD33 ..................................................................................................... 18
    1.3.2. Carcinoembryonic antigen (CEA) .......................................................... 21
    1.3.3. CA15-3 antigen, MUC1 peptides and Tn antigens ................................. 24

1.3. Aptamer-guided delivery of payloads into cancer cells ..................................... 26
    1.4.1. Aptamer-drug conjugates ..................................................................... 28
    1.4.2. Aptamer-protein conjugates .................................................................. 28
    1.4.3. Aptamer-radionuclide conjugates .......................................................... 29
    1.4.4. Aptamer-nanostructure conjugates ....................................................... 30
    1.4.5. Challenges facing the in vivo use of aptamers ...................................... 32

1.4. Thesis Hypotheses ......................................................................................... 36
1.5. Specific Aims .................................................................................................. 37
1.6. Chapter Overviews ......................................................................................... 38

Chapter 2 .................................................................................................................. 39

2.1. Abstract ......................................................................................................... 40
2.2. Introduction .................................................................................................... 41

2.1. Materials and Methods .................................................................................. 43
    2.3.1. Expression and purification of human TNFα ......................................... 43
    2.3.2. Aptamer selection screens ..................................................................... 44
    2.3.3. Aptamer-based enzyme linked binding assay ...................................... 45
    2.3.4. NF-κB luciferase reporter assay ............................................................ 45
    2.3.5. Inhibition of TNFα induced cytotoxicity .............................................. 46

VI
2.3.6. Surface plasmon resonance.................................................................46
2.3.7. Determination of aptamer concentration and Circular Dichroism
Spectroscopy. ..............................................................................................47
2.3.8. Inhibition of TNFα induced NO production in macrophages............48
2.3.9. Analysis of VR11 ability to activate an innate immune response .........48

2.4. Results and Discussion............................................................................49

2.4.1. Identification of DNA aptamers directed at TNFα............................49
2.4.2. DNA aptamer VR11 inhibits the binding of TNFα to its receptor .......54
2.4.3. Structural features of DNA aptamer VR11.........................................60
2.4.4. Immunogenicity of DNA aptamer VR11............................................64

Chapter 3......................................................................................................66
Blocking the attachment of cancer cells in vivo with DNA aptamers displaying anti-
adhesive properties against the carcinoembryonic antigen.............................66
3.0. Abstract...............................................................................................67
3.1. Introduction...........................................................................................68
3.2. Materials and Methods.........................................................................71

3.2.1. Generation of recombinant CEA modules.........................................71
3.2.2. Aptamer selection and cloning .........................................................71
3.2.3. Aptamer-based inhibition of CEA homotypic interactions ...............72
3.2.4. Cells lines and growth conditions....................................................73
3.2.5. Aptamer-based inhibition of homophilic cellular adhesion.................73
3.2.6. Aptamer binding to CEA on cells as measured by flow cytometry ....74
3.2.7. Inhibition of MC38.CEA tumour implantation...................................75
3.2.8. Aptamer cytotoxicity assay...............................................................75
3.2.9. Analysis of aptamer-based innate immune responses .......................76
3.2.10. Statistical methods and data analysis ..............................................76

3.3. Results .................................................................................................77

3.3.1. Generation of DNA aptamers displaying inhibitory properties of CEA-
dependent homotypic adhesions.....................................................................77
3.3.2. Aptamers N54 and N56 inhibit homophilic cellular adhesion ..........79
3.3.3. Aptamers N54 and N56 specifically recognize the N domain of CEA ...83
3.3.4. Addition of aptamers N54 and N56 to MC38.CEA cells reduces tumour
implantation in vivo ......................................................................................86
3.3.5. Aptamers specific to the CEA N domain are not cytotoxic and aptamer
N54 does not activate an innate immune response....................................89

3.4. Discussion.............................................................................................91

Chapter 4.....................................................................................................97
4.0. Abstract...............................................................................................98
4.1. Introduction...........................................................................................99
4.2. Materials and Methods.......................................................................103

4.2.1. MUC1 expression, purification and glycosylation .........................103
4.2.2. Identification of MUC1 binding aptamers using the SELEX process ....104
4.2.3. MUC1 Aptamer binding and internalization ....................................105
4.2.4. Binding kinetics of MUC1 aptamers determined by surface plasmon
resonance ....................................................................................................105
4.2.5. Synthesis of Aptamer-PEG_{2000}-DSPE conjugates ............................................ 106
4.2.6. Liposome preparation ................................................................................................. 107
4.2.7. Efficiency of aptamer-DSPE-PEG insertion into liposomes .................................. 108
4.2.8. Aptamer-liposome characterization \textit{in vitro} ......................................................... 108
4.2.9. Physio-chemical characterization of aptamer-liposome formulations .................. 109
4.2.10. Aptamer-liposome pharmacokinetic profile ............................................................. 110
4.2.11. Statistical methods and data analysis ................................................................. 110

4.3. \textbf{Results} .................................................................................................................. 111

4.3.1. Identification and characterization of MUC1-VR1, a MUC1-binding aptamer .................. 111
4.3.2. Optimization of MUC1-VR1 and N54 aptamer-targeted liposomes ......................... 114
4.3.3. Aptamer-Liposome internalization in MDA-MB-231 cells ........................................ 117
4.3.4. Aptamer-Liposome physicochemical properties and stability \textit{in vivo} ..................... 119

4.4. \textbf{Discussion} ......................................................................................................... 120

Chapter 5 ............................................................................................................................. 124
5.1. \textbf{Thesis Conclusions} ............................................................................................. 125
5.2. \textbf{Future Directions} ............................................................................................. 127

5.2.1. Modification of aptamer VR11 for \textit{in vivo} ................................................................. 127
5.2.2. Determine the ability of aptamer N54 as a detection and targeting agent. 128
5.2.3. Determine the ability of MUC1 and CEA aptamer to target liposomes. .... 128

References ......................................................................................................................... 142
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$TC</td>
<td>Technetium-99m</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell cytotoxicity</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C6</td>
<td>six carbon spacer</td>
</tr>
<tr>
<td>cApt</td>
<td>control aptamer</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CD$_{50}$</td>
<td>median curative dose</td>
</tr>
<tr>
<td>CDC</td>
<td>complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen cell adhesion molecule</td>
</tr>
<tr>
<td>CH</td>
<td>cholesterol</td>
</tr>
<tr>
<td>CI</td>
<td>cell index</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-cell antigen-4</td>
</tr>
<tr>
<td>Cy5</td>
<td>cyanine-5</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMARDs</td>
<td>disease-modifying antirheumatic drugs</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>DR5</td>
<td>death receptor 5</td>
</tr>
<tr>
<td>DSPE</td>
<td>distearoyl-phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeability and retention effect</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>full length</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidyl inositol</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>phosphoric acid</td>
</tr>
<tr>
<td>HBS</td>
<td>hank’s buffered saline</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>hNE</td>
<td>human neutrophil elastase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgV</td>
<td>Immunoglobulin variable</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>$k_a$</td>
<td>association rate</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kD</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>$K_D$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$k_d$</td>
<td>dissociation rate</td>
</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAG$_2$</td>
<td>mercapto-acetyl diglycine</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUC1</td>
<td>mucin 1</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>nitrite ion</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLK1</td>
<td>polo-like kinase 1</td>
</tr>
<tr>
<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PTK7</td>
<td>protein tyrosine kinase 7</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SELEX</td>
<td>systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>SRB</td>
<td>sulphorhodamine B</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>TLR9</td>
<td>toll-like receptor 9</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFβ</td>
<td>tumour necrosis factor beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VR</td>
<td>variable region</td>
</tr>
<tr>
<td>α5β1</td>
<td>alpha-5-beta-1</td>
</tr>
<tr>
<td>β-gal</td>
<td>beta-galactosidase</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1. Aptamers to targets of therapeutics interest ......................................................... 10

Table 1.2. Advantages and limitations of aptamers versus antibodies................................. 11

Table 1.3. Examples of aptamers and their cargoes directed at internalized surface markers on cancer cells..................................................................................................................... 17

Table 2.1. List of TNFα specific DNA aptamer sequences identified from the SELEX screen as well as control aptamer [cApt(VR) and cApt(FL)] sequences used in the present study. ................................................................................................................................. 51

Table 4.1. Pharmacokinetics of different Omnipaque encapsulated non-targeted and aptamer targeted liposome preparations in CD1 mice (n=5 per group). ............................... 119
LIST OF FIGURES

Figure 1.1. Isolation of aptamers using the SELEX procedure................................. 4

Figure 1.2. Overlapped crystal structures of a RNA and a DNA aptamer bound to the protein thrombin................................................................. 6

Figure 1.3. Crystal structure of TNF alpha trimer created from the Protein Data Bank.. 14

Figure 1.4. A CD33-specific DNA aptamer binds to and is internalized by CD33+ myeloid leukemia cell lines................................................................. 20

Figure 1.5. A CEA-specific DNA aptamer binds to and is imported into colon carcinoma cells expressing human CEA........................................................................... 23

Figure 1.6. Proposed mechanisms of cellular entry and recycling of DNA aptamers directed at aberrantly glycosylated mucin MUC1 present on the surface of epithelial cancer cells............................................................................................ 25

Figure 1.7. Possible endocytic pathways taken by aptamer-cargoes....................... 27

Figure 2.1. Binding of selected full-length (FL) and variable region (VR) DNA aptamers to TNFα and NfkB assays identify FL11 and VR11 as TNFα inhibitors. ................. 53

Figure 2.2. Aptamer VR11 inhibits TNFα-induced cytotoxicity in murine fibroblasts. .. 55

Figure 2.3. Binding kinetics of TNFα to aptamers VR11 and VR20 as determined by surface plasmon resonance................................................................. 57

Figure 2.4. Inhibition of TNFα-induced NO2-production in macrophages............... 59

Figure 2.5. Structural analysis of aptamers VR11 and VR20 by Circular Dichroism..... 63

Figure 2.6. Aptamer VR11 does not cause an innate immune response in vivo. ........ 65

Figure 3.1. Aptamers selected to bind to the CEA IgV-like N domain inhibit homotypic adhesion events................................................................. 78

Figure 3.2. Addition of aptamers specific to the CEA N domain inhibits homophilic cellular adhesion................................................................. 80

Figure 3.3. Minimal regions required for aptamer inhibition of homophilic cellular adhesion......................................................................................... 82

Figure 3.4. Alignment of the IgV-like N-domains of CEACAM family members. ....... 84

Figure 3.5. Aptamer binding to CEA+ and CEA- cells using Cy5 labelled aptamers by flow cytometry................................................................. 85

Figure 3.6. Pre-treatment of CEA-expressing MC38.CEA cancer cells with CEA-specific
aptamers reduces tumour implantation in vivo. .......................................................... 88

Figure 3.7. Aptamer N54 is noncytotoxic and does not activate innate immune responses.
........................................................................................................................................ 90

Figure 4.1. Proposed action of aptamer targeted liposomes accumulating at the site of the
tumour by virtue of the Enhanced permeability and Retention (EPR) effect and
internalizing into cells........................................................................................................ 102

Figure 4.2. Identification of MUC1-specific DNA aptamers and the ability of MUC1+
MDA-MB-231 cells to internalize FITC-labeled aptamer MUC1-VR1. ..................... 113

Figure 4.3. Liposome internalization into CEA+/MUC1+ MDA-MB-231 cells is
dependent on the number of DNA aptamers present on the surface of liposomes. ...... 116

Figure 4.4. Binding and internalization of targeted liposome formulations in vitro. ..... 118
Chapter 1 : Introduction

A version of this chapter has been previously published:
Delivering cargoes into cancer cells using DNA aptamers targeting internalized surface portals (Biochim Biophys Acta. 2010 Dec;1798(12):2190-200)
Erik W. Orava, Nenad Cicmil and Jean Gariépy

Author Contributions:
Erik Orava and Dr. Jean Gariépy wrote the review

Erik Orava contributed to Tables 1.3 and Figures 1.4.,1.5. and 1.6.
Nenad Cicmil contributed to Figures 1.2., 1.3. and 1.7.
1.1. Aptamers as therapeutics

Many classes of oligonucleotides such as siRNAs, microRNAs and antisense oligonucleotides represent potential therapeutic agents in view of their ability to selectively block the expression or transcription of genes and mRNAs inside diseased cells. Unfortunately, their anionic character makes them cell-impermeant and thus will not reach their intracellular targets unless they are conjugated or complexed to a cell-penetrating peptide, a polymeric vector, a protein ligand (hormones, cytokines, monoclonal antibodies), a nanoparticle or a liposome favoring their import into cells or are delivered using a viral vector. A more recent and potentially simpler solution to this challenge is to derive short synthetic oligonucleotides known as DNA and RNA aptamers which themselves specifically bind to internalized surface markers (cellular portals) and thus can act as delivery vehicles for therapeutic oligonucleotides and other therapeutic cargoes. As well, these aptamers can be generated to bind to validated therapeutic targets and act as antagonists (Keefe et al., 2010). Although in its infancy, the field of aptamer development for therapeutic and diagnostic purposes is rapidly growing and their utility is becoming even more evident (Soontornworajit and Wang, 2011; Yang et al., 2011).
1.1.1. The SELEX procedure: A rapid strategy to identify short single strand synthetic oligonucleotides (aptamers) that recognize specific targets

Cancer cells typically harbor multiple oncogenic mutations leading to the aberrant display and/or overexpression of molecular signatures on their surface. Classical approaches to target such signatures have made use of peptides, proteins and mainly antibodies. However, recent studies suggest that oligonucleotides known as aptamers can be utilized in the same capacity. Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) that can form specific and complex three-dimensional structures which can bind with high affinity to specific targets. The term ‘aptamer’ is derived from the Latin word *aptus* meaning “to fit” (Ellington and Szostak, 1990). Two groups reported a PCR-based strategy termed SELEX (an acronym for *Systematic Evolution of Ligands by EXPonential enrichment*; (Tuerk and Gold, 1990)) to derive aptamers that specifically recognized targets ranging from small molecules to large proteins (Figure 1.1). SELEX is an iterative panning procedure where combinatorial libraries composed of a random oligonucleotide element flanked by constant primer regions are allowed to bind to an immobilized target. The bound oligonucleotides are then recovered and amplified by PCR to generate a sub-library of aptamers able to recognize a given target. The binding/amplification cycle is then repeated several times on enriched pools of aptamers until one recovers ssDNA or RNA aptamers displaying $K_d$s in the nanomolar to picomolar range for their respective targets. So far, thrombin represents the only protein that does not normally bind nucleic acids and for which crystals structures of its complexes with aptamers have been obtained (Long et al., 2008; Padmanabhan et al., 1993). Interestingly, the two available structures (thrombin complexed to a DNA and a RNA aptamer) indicate that each aptamer binds to a distinct
Figure 1.1. Isolation of aptamers using the SELEX procedure. A combinatorial library of DNA oligonucleotides is chemically synthesized using standard solid phase methods. The library is composed of a random oligonucleotide element sequence (typically 20 to 50 nucleotides in length) flanked by two distinct constant sequences for the subsequent enrichment of target-binding library elements by PCR. The synthetic DNA oligonucleotide pool is directly mixed with the immobilized target for the purpose of retaining bound DNA aptamers (step 1). In the case of an RNA aptamer selection, a random library of RNA aptamers is initially derived from a double stranded DNA library by in vitro transcription, in which case the 5’ constant sequence includes a T7 promoter region. Aptamers in each library will adopt different three-dimensional structures based on their random sequence element with some oligonucleotides able to bind to a target immobilized on beads or other solid supports (step 1). The crystal structure of a thrombin-binding RNA aptamer (Padmanabhan et al., 1993) is displayed (center) to emphasize the presence of bulges and hairpins in such structures. Following a washing step (step 2), the bound oligonucleotides are eluted from the solid support (step 3) and amplified by PCR using the constant flanking sequences acting as primer sites (step 4). The selection cycle is repeated (step 5) typically 10-15 times with increasing stringency (lower target concentration for example) until tight-binding aptamers for a given target are identified, sequenced and synthesized for subsequent
region on the protein located on opposite sides of each other on the molecule (Figure 1.2). This finding suggests that the process of identifying aptamers using the SELEX procedure does not necessarily favor a unique epitope on a given target. Specifically, the DNA aptamer was shown to contact a region of thrombin that normally binds to fibrinogen (exosite 1), while the RNA aptamer binds to a domain associated with heparin binding (exosite 2)(Sheehan and Sadler, 1994). Interactions between these aptamers and thrombin are mostly electrostatic since both of the exosites are positively charged interfaces (Bode et al., 1992; Padmanabhan et al., 1993; Sheehan and Sadler, 1994). These structural features highlight the fact that aptamers may recognize these targets mostly through electrostatic interactions in contrast to dominant hydrophobic interactions typically observed in protein-protein interactions. It also suggests that the number of surface elements on a given target that could serve as recognized interfaces for aptamers is finite and potentially predictable.
Figure 1.2. Overlapped crystal structures of a RNA and a DNA aptamer bound to the protein thrombin (Long et al., 2008; Padmanabhan et al., 1993). The SELEX process has identified aptamers that interact with two distinct sites on thrombin (grey-colored contour surface). The 25-nucleotide long RNA aptamer Toggle-25t [purple, top] was found to bind near exosite 2 (heparin-binding site on thrombin) while the 15-base long DNA aptamer [blue, bottom] bound near the thrombin exosite 1 (fibrinogen-binding domain). Both interacting interfaces on thrombin displayed positively charged surfaces.
1.1.2. Cell-SELEX

Aptamers can be selected against recombinant forms of surface markers and still recognize the native forms on cells. Most notably are the aptamers to the MUC1 peptide, the extracellular domain of prostate-specific membrane antigen (PSMA), cell adhesion molecule P-selectin and protein tyrosine phosphotase 1B (PTP1B) (Ferreira et al., 2009; Gutsaeva et al., 2011; Lupold et al., 2002; Townshend et al., 2010). However, the recombinant form of proteins can be different in a physiological context resulting in a loss of aptamer binding. A case in point is the RNA aptamers to the EGFRvIII ectodomain which bound with high affinity in vitro however showed no binding to this target displayed on cells (Liu et al., 2009b). A process termed Cell-SELEX (also called Whole-Cell SELEX and cell-based SELEX) was recently devised to prevent the selection of aptamers to protein targets lacking their native conformation in vitro (Ohuchi et al., 2005; Zhang et al., 2010).

Established SELEX techniques required the immobilization of a protein or target on an affinity sorbent such as beads, resins, membranes, columns or plates. Washing steps, altering buffer solutions and reducing the amount of target represent SELEX stringencies that can remove DNA aptamer species that non-specifically associate with a given target from the selected pool of aptamers. In the Cell-SELEX procedure, non-specific species are removed by including a counter selection stage using cells that do not express any of the markers of interest. This step allows for a pool of aptamers to be generated that most likely recognize unique surface markers on the cell line of interest and possibly unknown or uncharacterized surface markers. A case in point is Cell-SELEX derived aptamers that were able to distinguish between rat glioblastoma and microglial cells (Blank et al., 2001). This approach may prove useful to derive aptamers as
biosensors to detect and discern populations of cells as well as deliver therapeutic cargos into cells.

### 1.1.3. RNA versus DNA aptamers

A large number of RNA aptamers have now been reported against different targets. The versatility of RNA molecules as functional ligands is well documented in regards to the frequent occurrence of modified nucleotides within their structure, their base pairing properties and their tendency to form intricate three-dimensional structures (Maas, 2011). For instance, all natural riboswitches (which bind to small molecules) are RNA molecules (Wakeman et al., 2007). The derivation and use of RNA aptamers does present some important practical challenges. For instance, the SELEX process requires the synthesis of random oligonucleotide libraries and the chemical synthesis of random RNA oligonucleotide pools remains expensive. Therefore, an *in vitro* transcription step is introduced in the SELEX procedure to obtain the initial RNA pool. Secondly, RNA oligonucleotides are more susceptible to hydrolysis than their DNA counterparts and thus their manipulation requires RNAse-free conditions. DNA tertiary structures have been observed in nature (Chou et al., 2003). These structures, rich in guanine, are found in telomeres and promoter regions (Neidle and Parkinson, 2003; Siddiqui-Jain et al., 2002). Guanine-rich sequences form various G-quadruplexes that appear to be major structural elements found in DNA aptamers as exemplified in the thrombin DNA aptamer (Figure 1.2). Examples of DNA aptamers have been reported and include an anti-HIV aptamer (Phan et al., 2005) and the anti-nucleolin aptamer AS1411(Teng et al., 2007). Catalytically-active DNA aptamers have also been derived using the SELEX approach (Liu et al., 2009a; Silverman, 2009). The
selection procedure for DNA aptamers is simpler than for RNA aptamers. Specifically, inexpensive pools (libraries) of DNA oligonucleotides can be chemically synthesized and contain only single stranded sequences as opposed to the initial double stranded pool of DNA sequences required for the in vitro transcription step used for RNA-based aptamer selection. Furthermore, reverse transcription is not required and an asymmetric PCR step is sufficient to recover the sub-library of ligand-binding aptamers needed to proceed to the next round of selection. In summary, the advantages of DNA aptamers stem from the simpler enrichment procedure involved and the lower cost and stability of the final aptamers while the benefit of selecting for RNA aptamers is the higher level of structural diversity possible with RNA templates.

1.1. Aptamers as Inhibitors

In theory, aptamers that function as inhibitors in blocking a given protein from interacting with another protein, receptor or ligand can be identified for the purpose of treating any disease. Thus, disease-promoting or persisting protein-protein and protein-ligand interactions can be disrupted (White et al., 2000). Most of the aptamers already developed as inhibitors tend to act as antagonists in blocking proteins to their receptors. Although there have been reports of aptamers that can acts as agonists, namely to HER3 and isoleucyl tRNA synthetase, these aptamers have not yet proved to have any clinical or therapeutic advantage (Chen et al., 2003; Hale and Schimmel, 1996). Since the invention of the SELEX process in the early 1990’s, researchers have been able to identify aptamers that bind to currently validated therapeutic targets (Table 1.1). As well, modifications of the SELEX approach have been reported that allow for the selection of aptamers with higher affinities (by decreasing off-rates) which would allow the aptamers
to be bound to their targets and in the case of inhibitors, provide a prolonged blockage (Keefe and Cload, 2008). Aptamers also possess considerable advantages to their antibody counterparts with some of their limitations being overcome such as synthesis costs and the optimization of protocols for their chemical modifications (Table 1.2).

Table 1.1. Aptamers to targets of therapeutics interest (Keefe et al., 2010).

<table>
<thead>
<tr>
<th>Target (alternative name)</th>
<th>$K_D$ (nM)</th>
<th>Therapeutic applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-thrombin</td>
<td>25</td>
<td>Prevent thrombosis</td>
</tr>
<tr>
<td>HIV-1 reverse transcriptase</td>
<td>1</td>
<td>Inhibit viral replication</td>
</tr>
<tr>
<td>HIV-1 Rev</td>
<td>&lt;1</td>
<td>Inhibit viral replication</td>
</tr>
<tr>
<td>Fibroblast growth factor Z, basic</td>
<td>0.35</td>
<td>Prevent angiogenesis</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>40</td>
<td>Prevent infection</td>
</tr>
<tr>
<td>HIV-1 integrase</td>
<td>10</td>
<td>Inhibit viral replication</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>0.14</td>
<td>Prevent neovascularization</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>0.1</td>
<td>Prevent tumour development</td>
</tr>
<tr>
<td>Immunoglobulin E</td>
<td>10</td>
<td>Prevent allergies</td>
</tr>
<tr>
<td>ε-Selectin</td>
<td>3</td>
<td>Modulate inflammation</td>
</tr>
<tr>
<td>α-Adenosine</td>
<td>1,100</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acetylcholine-specific auto-antibodies</td>
<td>60</td>
<td>Treat myasthenia gravis</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>68</td>
<td>Modulate inflammation and immune response</td>
</tr>
<tr>
<td>Keratinocyte growth factor</td>
<td>0.0002</td>
<td>Treat epithelial hyperproliferative disease</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>n/o</td>
<td>Modulate inflammation</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0.04</td>
<td>Inhibit viral adhesion</td>
</tr>
<tr>
<td>Acetylcholine receptor</td>
<td>2</td>
<td>Control neurotransmission</td>
</tr>
<tr>
<td>Phospholipase $\Lambda_2$</td>
<td>118</td>
<td>Treat ARDS, septic shock</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td>18</td>
<td>Inhibit oncogenesis, viral regulation</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>110</td>
<td>Prevent thrombosis</td>
</tr>
<tr>
<td>CD4</td>
<td>0.5</td>
<td>Modulate immune response</td>
</tr>
<tr>
<td>Nuclear factor κB</td>
<td>1</td>
<td>Treat chronic inflammatory disease</td>
</tr>
<tr>
<td>Lymphocyte function-associated antigen 1</td>
<td>500</td>
<td>Prevent tumour development, modulate inflammation</td>
</tr>
<tr>
<td>Cytohesin 1</td>
<td>5</td>
<td>Modulate cytoskeletal reorganization</td>
</tr>
<tr>
<td>αβ33 integrin</td>
<td>2</td>
<td>Prevent tumour development</td>
</tr>
</tbody>
</table>
Table 1.2. Advantages and limitations of aptamers versus antibodies (Keefe et al., 2010).

<table>
<thead>
<tr>
<th>Advantages of antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Pharmacokinetic and other systemic properties of antibodies are often sufficient to support product development</td>
</tr>
<tr>
<td>• Large size prevents renal filtration and together with binding to neonatal Fc receptors can give extended circulating half-lives</td>
</tr>
<tr>
<td>• Not susceptible to nuclease degradation</td>
</tr>
<tr>
<td>• Antibody technologies are widely distributed because the early intellectual property either never existed or has expired</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Limitations of antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Antibodies are produced biologically in a process that is difficult to scale up without affecting product characteristics</td>
</tr>
<tr>
<td>• Viral or bacterial contamination of manufacturing process can affect product quality</td>
</tr>
<tr>
<td>• Often immunogenic</td>
</tr>
<tr>
<td>• Large size limits bioavailability or prevents access to many biological compartments</td>
</tr>
<tr>
<td>• Limited ability to utilize negative selection pressure or to select against cell-surface targets not available in functional recombinant form</td>
</tr>
<tr>
<td>• Susceptible to irreversible denaturation; limited shelf life</td>
</tr>
<tr>
<td>• Chemistries required for the attachment of conjugation partners are stochastic and lead to product mixtures and reduced activity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Advantages of aptamers</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Aptamers are produced chemically in a readily scalable process</td>
</tr>
<tr>
<td>• Chemical production process is not prone to viral or bacterial contamination</td>
</tr>
<tr>
<td>• Non-immunogenic</td>
</tr>
<tr>
<td>• Smaller size allows more efficient entry into biological compartments</td>
</tr>
<tr>
<td>• Able to select for and against specific targets and to select against cell-surface targets</td>
</tr>
<tr>
<td>• Can usually be reversibly denatured, and phosphodiester bond is extremely chemically stable</td>
</tr>
<tr>
<td>• Conjugation chemistries for the attachment of dyes or functional groups are orthogonal and can be readily introduced during synthesis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Limitations of aptamers</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Pharmacokinetic and other systemic properties are variable and often hard to predict</td>
</tr>
<tr>
<td>• Small size makes them susceptible to renal filtration and they therefore have a shorter half-life</td>
</tr>
<tr>
<td>• Unmodified aptamers are highly susceptible to serum degradation</td>
</tr>
<tr>
<td>• Aptamer technologies are currently largely covered by a single intellectual property portfolio</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strategies to overcome aptamer limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Aptamers can be optimized for activity and persistence under physiological conditions during selection or during structure–activity relationship and medicinal chemistry studies conducted after discovery</td>
</tr>
<tr>
<td>• Addition of conjugation partners such as polyethylene glycol or cholesterol can increase circulating half-life</td>
</tr>
<tr>
<td>• Chemical modifications incorporated into the sugars or internucleotide phosphodiester linkages enhance nuclease resistance</td>
</tr>
<tr>
<td>• Original intellectual property covering the SELEX (systematic evolution of ligands by exponential enrichment) technique will soon expire</td>
</tr>
</tbody>
</table>
1.2.1. The role of TNFα in disease progression

Despite the importance of immune responses in controlling infections, it is now clear that uncontrolled reactions for such responses are intimately associated with degenerative diseases such as atherosclerosis, arthritis, encephalitis, and tumours (Feldmann et al., 2005). The primary pro-inflammatory cytokine, tumour necrosis factor alpha (TNFα) plays a critical regulatory role in enhancing these responses (Borish and Steinke, 2003). In the past decade, disease modifying antirheumatic drugs (DMARDs) that target underlying immune responses processes have improved treatment outcomes in patients with chronic immune response disorders. Anti-TNF antibody-based therapies represent the dominant categories of DMARDs (Feldmann et al., 1998; Maini et al., 1993). These protein therapeutics remain expensive to produce and ~40% of patients still display moderate to high levels of disease activity after treatment, suggesting a substantial need for improved therapies (Mierau et al., 2007).

1.2.2. Inflammation and TNFα

While the cause of many chronic inflammatory diseases remains unknown, most are characterized by dysregulation of cytokine networks, which often leads to the overproduction of proinflammatory cytokines, causing a perturbation in the equilibrium between pro- and anti-inflammatory cytokines (Feldmann et al., 2005). At the apex of this cytokine network is the Tumour necrosis factor alpha (TNFα), which acts as a primary trigger for the inflammatory response by promoting the synthesis of other proinflammatory cytokines (Borish and Steinke, 2003; van den Berg, 2001). TNFα was first identified for its ability to induce rapid haemorrhagic necrosis of experimental cancers (Carswell et al., 1975). TNFα is a type 2 transmembrane protein with an
intracellular amino terminus. It is produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges. It is synthesized as a 26-kD membrane bound protein (pro-TNFα) that is cleaved to release a soluble 17-kD TNFα molecule. TNFα has the ability to signal as a membrane bound protein and the soluble form is only active as a non-covalently associated homotrimer (Figure 1.3) (Hehlgans and Pfeffer, 2005; Locksley et al., 2001). Sufficient levels of TNFα as well as other mediators are critical for sustaining normal immune responses (Ehlers, 2005; Furst et al., 2006; Wallis and Ehlers, 2005). TNFα can initiate host defence at the site of a local injury but its sustained presence can also cause acute and chronic tissue damage (Beutler, 1999). Elevated levels of TNFα have been detected in patients with rheumatoid arthritis (RA), psoriasis and Crohn’s disease (Komatsu et al., 2001; Mussi et al., 1997; Robak et al., 1998). The evidence for the effects of sustained production of TNFα in inflammatory and autoimmune diseases sparked investigations to develop TNFα antagonists as anti-inflammatory agents (Brennan et al., 1989; Feldmann et al., 1992).
Figure 1.3. Crystal structure of TNF alpha trimer created from the Protein Data Bank (PDB: 1TNF). Each colour represents a monomer. It is believed that the highlighted area is responsible for binding to its receptors.
1.2.3. **TNFα as a therapeutic target**

The need for new therapies for the treatment of inflammatory diseases has arisen during an era where clinicians have realized that chronic inflammatory conditions are far more ominous than was previously thought. Inhibition of TNFα alone has been shown to downregulate the proinflammatory cascade leading to two findings: the discovery of an inter-linkage between different cytokines and the ability of one cytokine to orchestrate inflammation (Feldmann et al., 2001). It was realized by the mid 1990’s that inhibitors of TNFα would be successful therapeutic agents for a range of human chronic inflammatory diseases (Feldmann and Maini, 2008). Although pro-inflammatory cytokines aid in protecting the host against infectious agents, anti-TNFα protein therapeutics can reduce systemic and local inflammation in patients with rheumatoid arthritis (RA), psoriasis and Crohn’s disease. As of 2012, the global market for anti-TNFα protein therapeutics was valued at US$26.5 billion with Humira, Remicade and Enbrel representing the top 3 selling drugs in the world (2012).

1.2. **Aptamers can serve as intracellular delivery vehicles via their binding to known cancer-associated surface antigens**

The main purpose of this review is to highlight the potential of membrane-impermeant oligonucleotides to serve as intracellular delivery agents if they can be engineered to target internalized surface markers on cancer cells. The best described surface determinant used for this purpose (Table 1.3) has been the prostate-specific membrane antigen (PSMA), a membrane protein overexpressed on the surface of prostate cancer cells. PSMA is internalized by such cells via clathrin-coated pits (Chang et al., 1999; Liu et al., 1998; Lupold et al., 2002; Ross et al., 2003). From a drug delivery perspective,
antibody studies have shown that the rate of PSMA internalization was promoted by the binding of an antibody to its extracellular domain (Liu et al., 1998). The PSMA antigen is also differentially expressed on prostate cancer cells with normal prostate cells displaying an alternatively spliced cytosolic form of the protein while malignant cells express the full length surface protein (Su et al., 1995). The extracellular domain of PSMA served as a target for developing the first RNA aptamers known to bind a tumour-associated antigen (Lupold et al., 2002). The selective delivery and uptake properties of such aptamers by prostate cancer cells led to the subsequent design of an RNA chimera incorporating a PSMA-specific aptamer (delivery vehicle) and a therapeutic siRNA that targets Polo-like kinase 1 (PLK1) and BCL2. This RNA aptamer-siRNA construct was shown to cause tumour regression in a xenograft model of prostate cancer (McNamara et al., 2006). These findings suggested that by choosing appropriate internalized surface markers on cancer cells, one may be able to develop aptamers that can serve as both cell targeting agents and intracellular delivery vehicles. We will now focus our discussion on recent evidence from our laboratory suggesting that DNA aptamers can indeed be generated against membrane-bound tumour markers that are recycled inside cells.
Table 1.3. Examples of aptamers and their cargoes directed at internalized surface markers on cancer cells

<table>
<thead>
<tr>
<th>Reference</th>
<th>Aptamer</th>
<th>Internalized Target</th>
<th>Cargoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tong et al., 2009)</td>
<td>sgc8c</td>
<td>PTK7</td>
<td>Viral capsid</td>
</tr>
<tr>
<td>(Bagalkot et al., 2006)</td>
<td>A10</td>
<td>PSMA</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>(Huang et al., 2009)</td>
<td>sgc8c</td>
<td>PTK7</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>(Ferreira et al., 2009)</td>
<td>5TR1</td>
<td>MUC1 tandem repeat</td>
<td>Chlorin e6</td>
</tr>
<tr>
<td>(Ferreira et al., 2009)</td>
<td>5TRG2</td>
<td>Tn antigen</td>
<td>Chlorin e6</td>
</tr>
<tr>
<td>(Ferreira et al., 2009)</td>
<td>GalNAc3</td>
<td>N-acetylgalactosamine</td>
<td></td>
</tr>
<tr>
<td>(Chu et al., 2006a)</td>
<td>A9</td>
<td>PSMA</td>
<td>Gelonin</td>
</tr>
<tr>
<td>(Huang et al., 2008)</td>
<td>sgc8c</td>
<td>PTK7</td>
<td>Nanostructures</td>
</tr>
<tr>
<td>(Cheng et al., 2007; Dhar et al., 2008; Farokhzad et al., 2006; Farokhzad et al., 2004; Zhang et al., 2007)</td>
<td>A10</td>
<td>PSMA</td>
<td>Nanorod</td>
</tr>
<tr>
<td>(Cao et al., 2009)</td>
<td>AS1411</td>
<td>Nucleolin</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>(Chu et al., 2006b)</td>
<td>A9</td>
<td>PSMA</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>(Bagalkot et al., 2007)</td>
<td>A10</td>
<td>PSMA</td>
<td>Quantum dot - doxorubicin</td>
</tr>
<tr>
<td>(Hicke et al., 2006)</td>
<td>TTA1</td>
<td>Tenascin-C</td>
<td>Radioisotopes (99mTc)</td>
</tr>
</tbody>
</table>
1.3.1. CD33

The CD33 antigen is a 67-kDa type 1 transmembrane glycoprotein that belongs to the superfamily of sialic acid-binding immunoglobulin-related lectins (siglecs; siglec-3) (Freeman et al., 1995). CD33 is expressed on early multilineage hematopoietic progenitors, myelomonocytic precursors, as well as more mature myeloid cells, monocytes, macrophages and dendritic cells (Andrews et al., 1986; Andrews et al., 1983; Griffin et al., 1984). Most adult and pediatric acute myeloid leukemia (AML) cases as well as 15–25% of acute lymphoblastic leukemia (ALL) cases are CD33-positive (Dinndorf et al., 1986; Putti et al., 1998; Scheinberg et al., 1989; Terstappen et al., 1992). The presence of CD33 on AML blasts has led to the development of monoclonal antibody treatments that have been approved for AML patients that have relapsed. One of these anti-CD33 antibodies was conjugated to calicheamicin, a potent cytotoxic antibiotic that cleaves double-stranded DNA at unique sites. The resulting antibody-drug conjugate is commonly known as Gemtuzumab ozogamicin or Mylotarg (Wyeth Laboratories, PA, USA) (Bernstein, 2000; Hamann et al., 2002). Antibody-bound CD33 has been shown to be rapidly internalized by myeloid cells, a process that is largely modulated by its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (van der Velden et al., 2004; Walter et al., 2005). A 26% response rate has been observed for AML patients treated in first relapse with gemtuzumab ozogamicin as a monotherapy with a median disease-free-survival of 6.4 months in patients achieving complete remission (Tsimberidou et al., 2006). Surprisingly, there is no major loss of surface CD33 expression on leukemic blasts at relapse after Gemtuzumab treatment suggesting that alternate therapies targeting CD33-positive cell populations would be feasible and safe (Chevallier et al., 2008; van der Velden et al., 2004). This finding would suggest the
development and use of smaller and less immunogenic CD33-specific aptamers carrying less toxic cargoes than calicheamicin (hepatotoxicity) into CD33+ cells. As a proof-of-concept, our group recently developed 25-base long synthetic DNA aptamers against a recombinant form of CD33 to examine their ability to be internalized by myeloid (CD33+) cell lines. As shown by flow cytometry and confocal microscopy (Figure 1.4), one such CD33-specific Cy5-labeled DNA aptamer binds to (4°C) and is internalized (37°C) by CD33+ cells within 90 minutes of exposing cells to this oligonucleotide. In contrast, no binding or cellular uptake was observed for a control aptamer (25-base long repeat of the sequence GATC) identically modified with a Cy5 probe exposed to the same set of cell lines. Finally, neither aptamers bound to the CD33- cell line LP1. The dissociation constant (K_d) of this monomeric CD33-specific aptamer was calculated to be 17.3 nM suggesting that it is only ~10 fold less avid for its target than modified forms of the established bivalent-binding CD33-specific monoclonal antibody HuM195 (Chen et al., 2006). These results suggest that DNA aptamers evolved to bind to the antigen CD33 can mimic the properties of anti-CD33 antibodies in terms of binding and being imported into CD33-positive cells.
Figure 1.4. A CD33-specific DNA aptamer binds to and is internalized by CD33+ myeloid leukemia cell lines. FACS histograms (top) and fluorescence confocal images (bottom) of CD33+ myeloid leukemia cell lines HL60, OCI-AML5 and THP1 exposed to a 25-base long synthetic, Cy5-labeled CD33-specific DNA aptamer at 4°C and 37°C for 90 minutes. The flow cytometry profiles indicate that the CD33-specific DNA aptamer binds specifically to CD33+ cells at 4°C (green curves) with increased fluorescence intensities being observed at 37°C (red curves) as a consequence of internalization. In contrast, the labeled aptamer did not recognize the CD33- myeloma cell line LP1. A 25-base long Cy5-labeled control DNA aptamer composed of GATC repeats displayed fluorescence profiles at 4°C (black curves) and 37°C (blue curves) that were comparable to the autofluorescence profiles (grey areas) of unstained cells.
1.3.2. Carcinoembryonic antigen (CEA)

The human carcinoembryonic antigen (CEA) is a 180 kDa GPI-linked cell glycoprotein and a member of an immunoglobulin cell adhesion molecule superfamily (CEACAMs). CEA was originally identified as a surface marker on adenocarcinomas of the human gastrointestinal tract as well as on cells of the fetal digestive system (Gold and Freedman, 1965a). Other CEACAM members have since been identified in an array of tumours including breast, lung, pancreas, stomach, thyroid, ovaries and melanomas (Hammarstrom, 1999). CEA is aberrantly overexpressed on the surface of colorectal tumour cells in relation to normal colonic cells (Boucher et al., 1989). As the tumour progresses and invades the basal lamina, elevated levels of CEA can be detected in sera. For this reason, CEA has been used as a serum marker for recurrence of colorectal cancer despite its low sensitivity and specificity (Goldstein and Mitchell, 2005). CEA has often being referred to as a non-internalizing or as a shed antigen, yet studies have shown that anti-CEA antibodies are endocytosed at a rate consistent with the metabolic turnover of CEA (Ford et al., 1996; Schmidt et al., 2008; Shih et al., 1994; Stein et al., 1999) Anti-CEA antibody targeted therapies have been reported to date (Behr et al., 2002; Goldenberg, 2002). As in the case of antibody therapies aimed at solid tumours, poor tumour penetration remains an issue and in the specific cases of high affinity CEA antibodies, their rapid clearance due by free circulating antigen (Adams et al., 2001; Graff and Wittrup, 2003). In order to assess the potential of CEA as an internalizing antigen on cancer cells, DNA aptamers were developed specifically to recognize a recombinant form of the N-terminal Ig domain of human CEA using the SELEX approach. The binding of one such 25-base long DNA aptamer (and a control DNA aptamer) to the mouse colon adenocarcinoma cell line MC-38 (CEA⁺) and its related cell
line transduced to express the human CEA gene, MC-38.cea (CEA\textsuperscript{+}) (Robbins et al., 1991) was monitored by flow cytometry. Specifically, these cells were incubated with a Cy5 conjugated CEA-specific DNA aptamer at 4\textdegree{}C (surface binding only) and at 37\textdegree{}C (binding and internalization). As shown in Figure 1.5, MC-38 (CEA\textsuperscript{−}) MC38 cells showed no significant binding of the CEA-specific aptamer at both temperatures (Figure 1.5B). In contrast, the CEA-specific aptamer strongly associated with the CEA-positive cell line MC-38.cea, with a significant increase in mean fluorescence intensity being observed after 2 hours at 37\textdegree{}C in relation to 4\textdegree{}C (Figure 1.5B). The higher fluorescence signal observed at 37\textdegree{}C is attributed to the CEA aptamer being internalized during this time period. The irrelevant Cy5-labeled DNA aptamer (control; GATC repeats) did not bind to either cell lines at both temperatures. Thus, CEA may represent a powerful portal for aptamer-directed conjugates to selectively reach and be imported into colon cancer cells.
Figure 1.5. A CEA-specific DNA aptamer binds to and is imported into colon carcinoma cells expressing human CEA. FACS histograms confirmed the binding (4°C, green curves) and internalization (37°C, red curves) of a 25-base long synthetic, Cy5-labeled CEA-specific DNA aptamer binding to the human CEA⁺ MC38.cea cell line after a 2-hour incubation period. No binding or internalization was observed for the Cy5 aptamer to the parent CEA⁻ MC38 cell line or for a control aptamer (as described in Figure 1.4; black (4°C) and blue (37°C) curves) to both cell lines.
1.3.3. CA15-3 antigen, MUC1 peptides and Tn antigens

The mucin MUC1 is a membrane glycoprotein that is highly expressed and is aberrantly glycosylated [shortened O-glycans structures] in greater than 90% of all primary and metastatic breast cancers (Hanisch and Muller, 2000; McGuckin et al., 1995; Spicer et al., 1995; Taylor-Papadimitriou et al., 1999). The mucin MUC1 extracellular domain largely consists of 30 to 100 copies of a 20-amino acid long tandem repeat (Gendler et al., 1988). Serine and threonine residues within the tandem repeat represent sites of O-glycosylation. The pattern of O-glycosylation at such sites is altered in cancer cells giving rise to truncated short sugar chains known as the T, Tn and sialyl-Tn antigens as well as exposing antigenic sites on the peptide chain itself (Hanisch et al., 1996). MUC1 peptide domains and its associated truncated carbohydrate epitopes are clinically referred to as the CA15-3 antigen. Increasing serum levels of the CA15-3 antigen correlate with poor prognosis. In terms of drug delivery, mucin MUC1 glycoforms are endocytosed and recycled by cells in order to complete their glycosylation pattern prior to returning to the cell surface (Altschuler et al., 2000; Ceriani et al., 1992; Henderikx et al., 2002; Litvinov and Hilkens, 1993). Any ligands binding to such structures will thus be imported into MUC1+ cells and in particular through Golgi compartments. Our group has recently derived short 25-base long, synthetic DNA aptamers that specifically recognize either the MUC1 peptide backbone or its Tn antigens (GalNAc sugars linked to serine and threonine hydroxyl side chains on the MUC1 peptide tandem repeat) on epithelial cancer cells with binding affinities (Kd) for their targets ranging from 18 to 85 nM (Ferreira et al., 2009). Confocal microscopy and flow cytometry studies have shown that these labeled aptamers circulate from the cell surface and into endosomal and Golgi compartments upon binding to underglycosylated mucins (Figure 1.6). These DNA
Aptamers were subsequently derivatized at their 5′ end with the photodynamic therapy agent chlorin e₆ and shown to deliver chlorin e₆ to cellular compartments and cause cytotoxicity at concentrations 2- to 3-orders of magnitude lower than the concentration needed for the free drug (Ferreira et al., 2009).

Figure 1.6. Proposed mechanisms of cellular entry and recycling of DNA aptamers directed at aberrantly glycosylated mucin MUC1 present on the surface of epithelial cancer cells. Aptamers bind to membrane-bound, underglycosylated MUC1 mucin (branched structures). These mucin structures are recycled from the cell surface into endosomes that are either sent back to the cell surface or routed to the Golgi network where they are further glycosylated before returning to the cell surface.
1.3. **Aptamer-guided delivery of payloads into cancer cells**

In theory, aptamers represent simpler antibody-like mimics in terms of their ability to recognize tumour markers. Therapeutic agents can be directly coupled to aptamers or packaged into particles modified with aptamers in order to exploit recycling pathways associated with internalized cancer markers. However, the optimal efficacy of an aptamer-based intracellular delivery agent will depend in part on the recycling properties of their target and the possible induction of a receptor-mediated internalization event upon binding to a surface marker. In addition, the intracellular routing of aptamers is influenced by the abundance of the cell surface target itself, the macroscopic nature of the aptamer conjugate being delivered (size and nature of the cargo) and the dominant endocytic pathways associated with a given tumour cell type. The known cellular import mechanisms that lead to the vesicular trafficking of ligands bound to cell surface receptors are illustrated in Figure 1.7 and include (1) macropinocytosis and (2) phagocytosis, distinguished by the size of their endocytic vesicles, (3) clathrin-mediated, (4) caveolae (caveolin-based lipid rafts) and (5) clathrin-independent pathways. Recently designed aptamer-cargoes complexes do exploit import pathways, although few studies have explored their mode of cellular delivery. Most reported examples of internalized aptamer conjugates (Table 1.3) have either made use of the RNA aptamers A9 and A10 directed at the prostate-specific membrane antigen (PSMA) or the DNA aptamer sgc8c recognizing the tyrosine kinase 7 (PTK7).
Figure 1.7. Possible endocytic pathways taken by aptamer-cargoes. The nature and size of a cargo, the membrane-cycling property and abundance of a targeted surface portal on a given cell type as well as the number of portal-directed aptamers attached to a cargo (valency) can influence which import mechanism(s) may dominate in routing an aptamer-containing conjugate into cells. Large multivalent aptamer-targeted nanoparticles, polymer aggregates and liposomes would favor actin-filament-mediated uptake mechanisms such as macropinocytosis (1) or phagocytosis (2) while smaller monomeric aptamer conjugates involving a drug, a radionuclide, an siRNA or a protein as examples of payloads may enter cells via receptor-mediated events involving clathrin-dependent (3), caveolin-dependent (4) and/or clathrin-independent (5) endocytic pathways.
1.4.1. Aptamer-drug conjugates

Aptamer-drug conjugates have been constructed by chemically coupling a chemotherapeutic drug to the aptamer via a linker or by intercalating the drug into the aptamer folded structure creating a physical complex (Bagalkot et al., 2006; Huang et al., 2009). The drug is then imported into target cells while reducing its toxicity towards other cells (oligonucleotides including aptamers are cell-impermeant). Drugs can be conjugated to aptamers during solid-phase synthesis or post-synthesis by incorporating an amino or thiol group at one end of the oligonucleotide during their assembly. For instance, doxorubicin, an anthracycline used in the treatment of various cancers, has been coupled via an acid-labile hydrazone linker to a 41-nucleotide long tyrosine kinase 7 PTK7-specific DNA aptamer (sgc8c) to release the drug in endosomes (Willner et al., 1993). This aptamer-drug conjugates has been shown to prevent the nonspecific internalization of the drug as well as decrease its cellular toxicity towards non-target cells. The conjugate is selectively internalized by CCFR-CEM cells (T-cell acute lymphoblastic leukemia cells) with no apparent reduction in aptamer affinity for its target (Huang et al., 2009). As mentioned in section 1.3.3, DNA aptamers targeting known tumour-associated antigens such as mucin MUC1 peptides and mucin Tn antigens have also been modified with a photodynamic therapy agent chlorin e₆ and delivered to epithelial cancer cells. These aptamer-chlorin e₆ conjugates exhibited a >500-fold increase in toxicity upon light activation as compared to the drug alone and were not cytotoxic to cells lacking these mucin markers (Ferreira et al., 2009).

1.4.2. Aptamer-protein conjugates

Previous work with antibody-toxin conjugates has suggested that the most important determinant of cellular cytotoxicity of immunotoxins is the efficiency of their import into
cells (Goldmacher et al., 1989). The coupling of aptamers to cytotoxic as well as therapeutic proteins can facilitate them reaching their intracellular substrates. A case in point is the anti-PSMA RNA aptamer (A9) conjugated to gelonin, a ribosome-inactivating protein toxin. As mentioned in section 1.2, the prostate-specific membrane antigen (PSMA) is internalized by prostate cancer cells and thus provides a portal for the directed entry of the cytotoxic PSMA-specific aptamer-gelonin construct into such cells. Gelonin is an enzyme that inactivates ribosomes when deposited in the cytosol of intoxicated cells. The construct displayed a 600-fold increase in toxicity towards PSMA$^+$ LNCaP cells as compared to non-PSMA-expressing PC3 cells and ~180-fold increase in toxicity towards LNCaP cells relative to free gelonin (Chu et al., 2006a).

1.4.3. **Aptamer-radionuclide conjugates**

Few aptamers to date have been modified to incorporate radionuclides or metal chelators with a view to image or kill cancer cells *in vivo*. Hicke and coworkers have reported the introduction of the metal chelator mercapto-acetyl diglycine (MAG$_2$) at the 5’ end of TTA1, a Tenascin-C-specific aptamer (Hicke et al., 2001). TTA1 is a 40-nucleotide long RNA aptamer that incorporates 2-fluoro-pyrimidines and binds to the protein Tenascin C with a $K_d$ of 5 nM (Hicke et al., 2001). Tenascin is a large, hexameric glycoprotein associated with the extracellular matrix and is expressed during tissue remodeling events linked to angiogenesis and tumour growth. The MAG$_2$-containing TTA1 aptamer chelates $^{99m}$Tc and was used to determine its biodistribution *in vivo* in the context of nude mice harboring a human glioblastoma U251 xenograft. $^{99m}$Tc-TTA1 showed rapid blood clearance and tumour uptake, reaching a tumour-to-blood ratio of 50 within 3 hours. In addition, good scintigraphy images of a breast and glioblastoma tumour xenograft in nude mice were recorded using this labeled aptamer (Hicke et al., 2006). The success of
this particular chelator-aptamer complex also highlighted the empirical nature of the
design process as an alternate choice of a chelator and radionuclide does result in
significant changes in the uptake and clearance patterns of this aptamer \textit{in vivo}. Nevertheless, the use of radiolabeled aptamers for imaging purposes \textit{in vivo} is feasible.

1.4.4. Aptamer-nanostructure conjugates

The recent creation of aptamer conjugated nanostructures suggests that they may represent a promising class of new agents for targeted cancer imaging and therapy. These targeted structures include nanorods, quantum dots, as well as soft and hard nanoparticles. Nanorods for example, can be viewed as an alternate scaffold for assembling and immobilizing aptamers to nanomaterials in order to generate multivalent conjugates. Huang and colleagues were able to show that up to 80 aptamers could be covalently linked to the surface of Au-Ag nanorods via a 5’end thiol group introduced into the structure of the fluorescein-labeled DNA aptamer sgc8c (section 1.4.1). The avidity of the resulting aptamer-nanorods towards the tyrosine kinase 7 PTK7 transmembrane protein on CCFR-CEM cells was shown to be 26-fold higher than the affinity of the unconjugated fluorescein-labeled aptamer sgc8c for the same cells. The fluorescence intensity signal observed by flow cytometry was also 300-fold greater for the aptamer-nanorod labelled cells than the signals observed for CCFR-CEM cells labelled with the unconjugated fluorescein-labeled aptamer (Huang et al., 2008).

RNA aptamers directed at the prostate-specific membrane antigen (PSMA) have been used in the design of numerous nanostructures. Streptavidin-coated quantum dots (QD; semiconductor nanocrystals) have also been decorated with a biotinylated, 70-nucleotide long PSMA-specific RNA aptamer termed A9 and the resulting conjugates used for cellular imaging. Specifically, the photostability and small size of quantum dots
was shown to improve the visualization of PSMA-positive cells (LNCaP) as adherent cell monolayers, in suspension preparations and embedded in a collagen matrix (Chu et al., 2006b). Aptamer particles have also been designed to serve the dual purpose of acting as a tumour-targeted agent and as a particle capable of controlled drug release. For example, the FITC-labeled PSMA-specific RNA aptamer A10 was coupled to a poly(lactic acid)-block-polyethylene glycol (PEG) copolymer nanoparticles that have been derivatized with a terminal carboxylic acid functional group (PLA-PEG-COOH). Rhodamine-labelled dextran was encapsulated (as a model drug) into these polymeric particles. The nanoparticles including their cargo were selectively imported into PSMA-positive LNCaP cells as confirmed by fluorescence microscopy (Farokhzad et al., 2004). Farokhzad and colleagues subsequently loaded docetaxel, a chemotherapeutic drug into the aptamer-conjugated nanoparticles and injected a single intratumoural dose of the construct in nude mice harboring a LNCaP xenograft (Farokhzad et al., 2006). Significant tumour regression was observed with no apparent immunogenicity. More recently, the same aptamer-nanoparticle conjugates were loaded with docetaxel and doxorubicin or with cisplatin although the overall improvement in survival in the treated tumour-bearing animals was modest in relation to the non-aptamer targeted drug loaded nanoparticles (Dhar et al., 2008; Zhang et al., 2007). Finally, the creation of a conjugate composed of the PSMA-specific RNA aptamer A10-doxorubicin-quantum dot was recently reported by Jon and Farokhzad groups (Bagalkot et al., 2007). Again, this nanostructure is imported into PSMA+ LNCaP prostate cancer cells by PSMA-mediated endocytosis. The construct offers the dual advantages of specifically delivering doxorubicin intercalated into the A10 aptamer structure to prostate cancer cells as well as
imaging the delivery process through a FRET event arising from interactions of the released doxorubicin and the QD itself (Bagalkot et al., 2007).

To date, liposomes remain one of the most successful drug delivery systems (Kaneda, 2000). Liposome formulations of many of the most frequently prescribed chemotherapeutic drugs have been approved and are currently used in clinical practice (Wagner et al., 2006). Liposomes have been shown to increase the circulation time of aptamers while these aptamers aid in targeting liposomes to their desired site of action (Farokhzad et al., 2006; Willis et al., 1998). Liposomal drug delivery strategies have focused on developing long-circulating liposomes that target areas of increased vascular permeability via the enhanced permeation and retention (EPR) effect (Matsumura and Maeda, 1986). The EPR effect however remains a passive tumour localization strategy that can lead to detrimental systemic consequences and suboptimal antitumour efficacy (Mamot et al., 2003; Woo et al., 2008). Aptamer-labeled liposomes can thus increase the delivery of encapsulated therapeutic agents to cancer cells.

1.4.5. Challenges facing the in vivo use of aptamers

The concept of using aptamers as therapeutic agents was initially tested by selecting aptamers to thrombin with a view to preventing blood clotting (Bock et al., 1992). The rationale for creating thrombin-selective aptamers was to generate heparin mimics that did not form complexes with platelet factor 4 which reacts with platelet-activating antibodies leading to heparin-induced thrombocytopenia (Stribling et al., 2007). Larry Gold`s group selected aptamers against the targeted HIV reverse transcriptase (Schneider et al., 1995). Since virus transcriptases normally bind nucleic acids, they represent excellent aptamer targets. Other parts of the virus are also being targeted by aptamers, some of which are DNA aptamers (Chou et al., 2005; Zhou et al., 2009). In spite of their
large therapeutic potential, aptamer drugs are still not a commonplace treatment mostly due to the previously mentioned challenges associated with translating small scale in vitro laboratory experiments into medical practice. Currently, the only aptamer approved by the FDA is Macugen (OSI Pharmaceuticals and Pfizer), an aptamer used to treat age-related macular degeneration (AMD). Macugen is a PEGylated 29-nucleotide long RNA aptamer with a modified backbone that significantly increases its circulating half-life (Ng et al., 2006). Macugen recognizes the vascular endothelial growth factor isoform VEGF165 but does not bind to VEGF121. In contrast, the antibody against VEGF marketed by Genentech under the name Ranibizumab shows specificity towards both isoforms (Kourlas and Abrams, 2007).

Aptamer structures can be evolved to recognize minor structural differences within a given target and typically bind to their targets with affinities comparable to those of antibodies (Conrad et al., 1994; Schneider et al., 1995). Practical advantages of aptamers over antibodies include their lower mass, low cost of synthesis, long shelf-life and consistent quality. However, aptamers do face challenges as potential therapeutic or delivery agents. Firstly, nucleic acids are small, charged molecules. As such, they cannot passively traverse a cell membrane. Secondly, oligonucleotides are rapidly degraded by nucleases in plasma and cleared from circulation, resulting in short in vivo half-lives (Chu et al., 2006a; White et al., 2000). Thirdly, oligonucleotides are typically not immunogenic. Yet, immune responses mediated by Toll-like receptor family members have been reported as exemplified by unmethylated CpG sequences (Wagner et al., 2006). Solutions to these challenges are available. There are several approaches for increasing the circulating time (half-life) of aptamers in plasma. One of them is PEGylation, the process of conjugating polyethylene glycol (PEG) groups to such
molecules. The coupling of a cholesterol group or a cell-penetrating peptide can also reduce their systemic clearance (Healy et al., 2004; Willis et al., 1998). Another approach is by using chemically modified nucleotides shown to increase the half-life of aptamer sequences by more than 40-fold (Peng and Damha, 2007). Such changes can be introduced during the SELEX process by using modified nucleotides that are incorporated by the T7 polymerase at the in vitro transcription step when RNA aptamers are being selected. In the case of DNA aptamers, modified nucleotides are simply introduced during library synthesis (Aurup et al., 1992; Latham et al., 1994). Possible modifications compatible with the SELEX protocol include substitution of the 2' OH group with a 2' fluoro or 2' amino group (Jellinek et al., 1995; Padilla and Sousa, 1999). Besides the sugar component of the molecule, various groups such as aromatic and alkyl moieties can be attached to the C5 position of UTP (Schoetzau et al., 2003). Other modifications termed “post SELEX” have been introduced after a useful sequence is identified (Eaton et al., 1997). One form of post-SELEX modification is Locked Nucleic Acid (LNA) (Barciszewski et al., 2009). The LNAs can have one or more nucleotides with a methylene linkage between the 2' oxygen and the 4' carbon, which results in the “locked” conformation of the sugar. This modification provides an increased affinity for the complementary strand, higher thermal stability, and resistance to nuclease degradation (Vester and Wengel, 2004). Multivalency represents another factor that can increase the avidity and potency of aptamers, as demonstrated by the oligomerization of an RNA aptamer against the Drosophila protein B52 (Santulli-Marotto et al., 2003). The tetravalent RNA aptamer recognizing the cytotoxic T-cell antigen-4 (CTLA-4) has also shown a therapeutic advantage over its monomeric counterpart in prolonging the survival of C57BL/6 mice implanted with the B16/F10.9 murine melanoma (McNamara et al.,
Among other aptamers selected to target tumour specific proteins, the first one to enter clinical trials is an unmodified DNA aptamer termed AS1411 (Antisoma). It was shown that its G rich sequence binds nucleolin present on the surface of cancer cells and can inhibit NF-kB pathways (Bates et al., 1999; Girvan et al., 2006). This aptamer is currently in Phase II clinical trials and shows activity towards many types of hematological cancers (clinical trials.gov identifier NCT00512083; NCT00740441). Interestingly, this 26-nucleotide long unmodified DNA aptamer is stable in serum, which indicates that the sequence of the aptamers results in a three dimensional structure that is not easily susceptible to nuclease degradation (Dapic et al., 2002). Thus, the need to further modify DNA aptamers to increase their stability in vivo may not be necessary in all cases.

Finally, Figure 1.7 outlines how aptamer-cargoes can reach several intracellular vesicular compartments. The illustration is also meant to highlight the fact that the cytosolic release of cargoes entrapped in vesicles remains an inefficient process and a common challenge confronting other drug delivery strategies involving polymer formulations, antibody conjugates and cell-penetrating peptides. Aptamer-targeted cargoes such as radionuclides (acting within a cell diameter or via a bystander effect), hydrophobic drugs, gold particles and liposomes may reach the cytosol or have their therapeutic effect enhanced by simply residing or cycling through vesicles. Other charged cargoes such as siRNAs, plasmids and proteins will be inefficiently released from endosomal compartments and may require the use of endosomolytic agents.
1.4. Thesis Hypotheses

To date, many protein-based therapeutics have established themselves as the gold standard for the treatment of an array of diseases. However, the use of proteins as therapeutic agents comes with challenges such as immunogenicity [even for humanized forms] and production costs. Many of the protein-based therapies involve the binding and blocking of targets associated with the persistence or occurrence of a disease. Therefore, new biotherapeutics are needed that exhibit reduced immunogenicity and are less costly than proteins while acting as potent agonists or antagonists to clinically relevant targets. My overall objective was to develop DNA aptamers able to target and block the function of validated therapeutic targets or to act as specific delivery agents.

The hypotheses of this thesis were:

1) DNA aptamers can be developed to behave as inhibitors of TNFα activity.

2) DNA aptamers can be developed to act as anti-adhesives of carcinoembryonic antigen (CEA).

3) DNA aptamers recognizing the unique cell surface biomarkers CEA and MUC1 can be used as targeting agents on liposomes containing a contrast agent for Computed Tomography (CT) imaging of breast cancer tumours.
1.5. Specific Aims

To test these hypotheses, the specific aims were:

1) To develop a robust SELEX protocol that can be used to screen and identify DNA aptamers to TNFα, CEA and MUC1.

2) Employ the SELEX methodology to select and identify DNA aptamers to TNFα and evaluate their ability to block binding to the TNF receptor by monitoring the inhibition of apoptosis, downstream signaling and reduction of nitric oxide (NO) and characterize the level of immunogenicity.

3) Employ the SELEX methodology to select and identify DNA Aptamers to CEA and characterize their ability to behave as anti-adhesive in blocking homotypic binding and prevent peritoneal tumour foci formation.

4) To evaluate whether DNA aptamers to CEA and MUC1 can target liposomes encapsulated with Omnipaque350™ to the breast cancer cell line MDA-MB-231 and characterize their effect on tumour accumulation and internalization.
1.6. Chapter Overviews

The studies addressing the specific aims outlined are described in Chapters 2-4 of the thesis. Chapter 2 describes the selection and identification of aptamer VR11 and characterizes its ability to block TNFα-induced apoptosis in murine cells as well as prevent the production of nitric oxide (NO) in macrophage cells. This study also showed that aptamer VR11 has a high binding affinity and is specific to TNFα while exhibits no immunogenicity in C57BL/6 mice. Chapter 3 demonstrates that DNA aptamers identified to bind CEA with high affinity and specifically bound to the IgV-like N domain of CEA. Two such aptamers prevented the adhesion properties of CEA expressing cells and prevented the formation of peritoneal tumour foci without eliciting an immune response. Chapter 4 describes the characterization of CEA and MUC1 targeted liposomes containing the contrast agent Omnipaque350™ and their ability to internalize in MDA-MB-231 cells. As well, conjugation of these aptamers did not significantly change the circulatory half-life of the liposomes formulations in mice. In Chapter 5 the overall conclusions are presented and discussed and future research is proposed.
Chapter 2
A short DNA aptamer that recognizes TNF-alpha and blocks its activity in vitro

A version of this chapter has been previously published:
A short DNA aptamer that recognizes TNF-alpha and blocks its activity in vitro
Erik W. Orava, Nick Jarvik, Yuen Lai Shek, Sachdev S. Sidhu, Jean Gariépy

Author Contributions:
Erik Orava and Dr. Jean Gariépy designed the experiments, analyzed data and wrote the paper.

Erik Orava contributed Table 2.1 and Figures 2.1, 2.2, 2.3A-B, 2.4, 2.6, 2.7, 2.8 and 2.9.
Nick Jarvik helped perform SPR experiments for Figure 2.3 C-G in the laboratory of Dr. Sachdev S. Sidhu

Yuen Lai Shek contributed by performing CD experiments for Figure 2.5.
2.1. Abstract

Tumour necrosis factor-alpha (TNFα) is a pivotal component of the cytokine network linked to inflammatory diseases. Protein-based, TNFα inhibitors have proven to be clinically valuable. Here, we report the identification of short, single stranded DNA aptamers that bind specifically to human TNFα. One such 25-base long aptamer, termed VR11, was shown to inhibit TNFα signalling as measured using NF-κB luciferase reporter assays. This aptamer bound specifically to TNFα with a dissociation constant of 7.0±2.1nM as measured by surface plasmon resonance (SPR) and showed no binding to TNFβ. Aptamer VR11 was also able to prevent TNFα-induced apoptosis as well as reduce nitric oxide (NO) production in cultured cells for up to 24 hours. As well, VR11, which contains a GC rich region, did not raise an immune response when injected intraperitoneally into C57BL/6 mice when compared to a CpG oligodeoxynucleotide (ODN) control, a known TLR9 ligand. These studies suggest that VR11 may represent a simpler, synthetic scaffold than antibodies or protein domains upon which to derive nonimmunogenic oligonucleotide-based inhibitors of TNFα.
2.2. Introduction

Unregulated immune responses are intimately associated with degenerative diseases such as atherosclerosis, arthritis, encephalitis, and tumours (Borish and Steinke, 2003; Feldmann and Maini, 2001; van den Berg, 2001). The primary pro-inflammatory cytokine, tumour necrosis factor alpha (TNFα) plays a critical regulatory role in enhancing these responses (Furst et al., 2006). In the past decade, disease-modifying antirheumatic drugs (DMARDs) that target underlying immune responses processes have improved disease outcomes in patients with chronic immune response disorders (Smolen et al., 2007). Anti-TNFα protein-based therapies (Enbrel, Humira, InfliximAb) have emerged as a dominant category of DMARDs (Mount and Featherstone, 2005). However, ~ 40% of patients still display moderate to high levels of disease even after treatment with protein therapeutics suggesting a substantial need for improved therapies (Mierau et al., 2007).

TNFα is a pro-inflammatory cytokine that is produced by an array of cell types such as macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges (Carswell et al., 1975). TNFα is a type 2 transmembrane protein with an intracellular amino terminus and is synthesized as a 26-kD membrane-bound protein (pro-TNFα) that is cleaved to release a soluble 17-kD TNFα molecule. TNFα has the ability to signal as a membrane bound protein as well as a soluble cytokine. As a soluble cytokine, TNFα is only active as a non-covalently associated homotrimer (Hehlgans and Pfeffer, 2005; Locksley et al., 2001). Sufficient levels of TNFα as well as other mediators are critical for sustaining normal immune responses. TNFα can initiate host defence mechanisms to
local injury but can also cause acute and chronic tissue damage (Beutler, 1999; Feldmann and Maini, 2008).

Despite advances in antibody and protein engineering, the major drawbacks of protein-based TNFα inhibitors are their immunogenicity arising from their chronic use and their production costs resulting in expensive therapies for patients. Simpler, synthetic, non-immunogenic classes of TNFα inhibitors could be derived from short, single-stranded nucleic acid oligomers (ssDNA or RNA) known as aptamers. These molecules adopt a specific tertiary structure allowing them to bind to molecular targets with high specificity and affinities comparable to that of monoclonal antibodies (Ellington and Szostak, 1990). In some cases, aptamers will display functional properties beyond just binding to their target. For instance, an aptamer to the inflammation factor human neutrophil elastase (hNE) was shown to significantly reduce lung inflammation in rats and displayed greater specificity for their target than an antielastase IgG control (Bless et al., 1997). Examples of other aptamers exhibiting functional attributes include a DNA aptamer to anti-HIV reverse transcriptase and RNA aptamers to the basic fibroblast growth factor and vascular endothelial growth factor (Jellinek et al., 1994; Jellinek et al., 1993; Schneider et al., 1995). Finally, a single-stranded DNA aptamer selected to bind to thrombin has been shown to inhibit thrombin-catalyzed fibrin-clot formation in vitro using either purified fibrinogen or human plasma (Bock et al., 1992).

In the present study, we constructed a 25-nucleotide variable region DNA library to perform SELEX [Systematic Evolution of Ligands by Exponential enrichment] (Tuerk and Gold, 1990) selections using recombinant TNFα as our target. Several ssDNA aptamers shown to specifically bind to TNFα were further evaluated for their ability to inhibit TNFα signalling in several independent in vitro assays. These analyses led to the
identification of VR11, a DNA aptamer capable of inhibiting TNFα functions in vitro.

2.1. Materials and Methods

2.3.1. Expression and purification of human TNFα.

The expression plasmid (RCA-093) encoding a 157-amino acid TNFα his-tag recombinant protein (residues 77-233) was obtained from Bioclone Inc. (San Diego, CA). LB broth supplemented with kanamycin (50µg/ml) was inoculated with a glycerol stock and grown at 37°C until the A600 reached 0.8. The expression of the TNFα fragment was induced using a final concentration of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubated overnight. Soluble TNFα was purified by nickel affinity chromatography under non-denaturing conditions. Cells were harvested by centrifugation at 8,000 rpm for 30 min and resuspended for 1 hour in BugBuster Reagent (Novagen), 1 U Benzonase (Novagen) and a complete EDTA-free protease inhibitor tablet (Roche). The lysate was then centrifuged at 8,000 rpm for 30 min and supernatant was loaded on a Ni-NTA agarose column (Sigma-Aldrich) pre equilibrated with Buffer A (50 mM phosphate buffer, pH 8, 300 mM NaCl, and 10 mM imidazole). After several washes with buffer A the protein was eluted with 3 column volumes of Buffer B (50 mM phosphate buffer, pH8, 300 mM NaCl and 250 mM imidazole). The eluate was concentrated by ultrafiltration into a 100 mM Tris-HCl buffer (pH 6.0) using an Amicon ultrafiltration unit (Millipore; 10 kDa MWCO). Purified TNFα was characterized by SDS-PAGE and quantified using the Bradford assay (Bradford, 1976). To verify that the protein was properly folded and functionally active, a cytotoxicity assay was performed using the L929 cell line sensitized with Actinomycin D and compared to a commercial recombinant human TNFα purchased from Bioclone Inc. (Appendix 3).
2.3.2. Aptamer selection screens.

The initial ssDNA library contained a central randomized sequence of 25 nucleotides flanked by T3 and SP6 primer regions respectively. Briefly, the sequence of the starting library was 5’ AAT TAA CCC TCA CTA AAG GG-(25N)-CTA TAG TGT CAC CTA AAT CGTA. The forward primer 5’AAT TAA CCC TCA CTA AAG GG 3’ and reverse primer 5’ TAC GAT TTA GGT GAC ACT ATA G 3’ were used for selection and cloning (IDT Technologies, Inc.). A 25 nucleotide-long aptamer (cApt) comprised of AGTC repeats [which has no predicted secondary structures] served as a control for all experiments. To begin the selection, 50 nmol aliquot of the library representing ~3.0 $\times$ $10^{16}$ molecules consisting of ~25 copies of each possible unique sequence was first counter-selected against a 6-His peptide (HHHHHH) loaded onto Ni-NTA magnetic agarose beads. The resulting sub-library was then exposed to 10 µg of His-tagged TNFα immobilized onto Ni-NTA beads suspended in Selection Buffer (PBS, 0.005% (v/v) Tween-20) at 37°C for 1 hour. Unbound DNA sequences were washed away (PBS, 0.005% (v/v) Tween-20) and DNA-protein complexes were eluted from the recovered beads using an imidazole buffer (PBS, 0.005% (v/v) Tween-20, 240 mM imidazole). The ssDNA component was precipitated with sodium perchlorate/isopropanol and recaptured using a silica membrane-based purification system (Qiagen Inc.). The DNA aptamers were then amplified by asymmetrical PCR using a 100-fold excess of forward primer. After every three subsequent rounds of selection, the amount of target was reduced in half to increase the selection pressure to capture the tightest binding species. After 12 rounds of selection, the bound sequences were amplified by PCR, cloned into a pCR™4-TOPO® TA vector (Invitrogen) and sequences were analyzed using BioEdit sequence alignment editor software (Ibis Therapeutics).
2.3.3. **Aptamer-based enzyme linked binding assay.**

A 96-well ELISA microtiter-plate (BD Falcon) was coated overnight at 4°C with 100µl of human TNFα (10µg/ml) prepared in coating buffer (0.2 M carbonate/bicarbonate, pH 9.4). The plate was then washed 3 times with PBS-T (0.1M phosphate, 0.15M sodium chloride, pH 7.0 containing 0.05% Tween-20) and blocked overnight at 4°C in 150 µl of blocking solution (PBS-T + 1% (w/v) BSA). The plate was washed 3 times with PBS-T and incubated overnight at 4°C with 100µl (10µg/ml) of 5’ biotinylated aptamers dissolved in PBS, 0.005% (v/v) Tween-20. The plates were washed and incubated for 1 hour at 4°C with 100 µl of streptavidin-HRP (1:2000). Plates were then read on a plate reader at 450 nm following washing and dispensing of 100µl of TMB substrate [1 TMB tablet (Sigma-Aldrich Inc.) dissolved in 1ml of DMSO in 10 ml of 0.05M phosphate citrate, pH 5.0 and 2 µl sodium peroxide] per well. Color development was stopped by adding 50µl of 1M sulphuric acid per well.

2.3.4. **NF-κB luciferase reporter assay.**

An NF-κB luciferase reporter plasmid was transfected into cells as a marker of TNFα binding as previously described (Mamaghani et al., 2009). Briefly, 0.1µg/well TA-LUC NF-κB and 10ng/well β-gal CMV were co-transfected to the cells. PANC1 (ATCC) cells (1.0×10⁴) and HEK293T (ATCC) (5.0×10³) were seeded in a 96-well plate 24 hours before the transfection steps. DNA was transfected into cells using Lipofectamine 2000 (Invitrogen Canada Inc.). Cells were incubated 4-6 hours in Opti-mem medium (Promega) and transferred to 10% FBS DMEM overnight. Aptamers were added to the medium (1 µM final concentration for PANC-1 cells or 2 µM, 200nM and 20nM for HEK293T) 30 minutes prior to treating the transfected cells with recombinantly
expressed human TNFα (100 ng/ml). Luciferase activity was measured after 6 hours using the Dual-Light® System luciferase assay from Applied Biosystems according to the manufacturer’s protocol and read on a Luminoskan Ascent luminometer (ThermoLab Systems). The results were normalized to the values obtained for β-galactosidase activity.

2.3.5. Inhibition of TNFα induced cytotoxicity.

L929 cells (ATCC) were seeded 24 hours before experiments in 96-well flat-bottom microtiter plates at a density of 1.0×10⁴ cells/well in DMEM medium containing 10% FBS. Aptamers (2µM) or 20µg/ml anti-TNFα mAb (R&D systems) were incubated with human TNFα for 2 hours in PBS prior to incubation with cells. Subsequently, aptamer-TNFα samples were added to the cells for 2 hours. Cells were then washed with warm PBS and incubated in complete medium for another 48 hours. The viability of adherent cells was subsequently determined using the sulforhodamine B assay (Skehan et al., 1990).

2.3.6. Surface plasmon resonance.

All experiments were performed using the ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories, Inc.) and one ProteOn NLC sensor chip, coated with NeutrAvidin for coupling of biotinylated molecules. Biotinylated aptamer VR11, VR20 and rApt(VR) were synthesized with a 5’ biotin with a standard C6 spacer and HPLC purified (Integrated DNA Technologies, Inc.). Subsequently 2.5nM of aptamers were diluted in PBS-T and injected for 30 sec at 30ul/min. Aptamer loading on the chip yielded approximately 10–12 response units which represented ~1ng of aptamer. Human
TNFα and TNFβ stock solutions were diluted as a series of two-fold dilutions ranging from 2µM to 3.9nM in PBS-T (0.05% (v/v) Tween-20) pH 7.4 (Figure 3C-F). Protein concentrations and a buffer control were injected in the analyte channel with a contact time of 120 sec, dissociation time of 800 sec, and a flow rate of 100 µl/min. The ligand channels were regenerated with a 30 sec injection of 1M H₃PO₄ followed by a 30 sec injection of 1 M NaCl. All experiments were performed at 25°C and repeated in triplicate. Sensorgrams were then double-referenced by subtracting the buffer response and using the interspot reference. The sensorgrams were fitted globally to a 1:1 Langmuir binding model and the kinetic parameters for the association (kₐ), dissociation rates (k₅), and binding constant (Kₐ) were derived from the fitted curves.

2.3.7. Determination of aptamer concentration and Circular Dichroism Spectroscopy.

The oligonucleotide concentration of each aptamer solution was determined from its absorbance at 260 nm as measured at 25 °C with a Cary 300 Bio spectrophotometer (Varian Canada, Inc.) using molar extinction coefficients of 244 400 M⁻¹ cm⁻¹, 239 400 M⁻¹ cm⁻¹ and 251 800 M⁻¹ cm⁻¹ for VR11, VR20 and cApt respectively. Circular dichroism (CD) spectra were recorded at 25 °C using an Aviv model 62 DS spectropolarimeter (Aviv Associates). The buffer used for CD measurements contained 10 mM phosphate, 0.1 mM EDTA (free acid) and 100 mM of either sodium or potassium ions. Either KOH or NaOH was added to the buffers until pH 7 was reached and the ionic strengths of the buffers were adjusted to the desired level by adding known amounts of NaCl or KCl. Dry and desalted DNA aptamers were dissolved in the buffers until a concentration of ~0.03 mM was achieved. The DNA aptamer solutions were heated to
100°C for 5 minutes to transform the DNA aptamer into their unfolded forms and slowly allowed to cool to 25°C. Ellipticity values [reported as θ] were measured from 320 nm to 200 nm in 1 nm increments using a quartz cuvette with an optical path length of 0.1 cm.

2.3.8. Inhibition of TNFα induced NO production in macrophages.

Experiments were modified using a previous protocol (Ding et al., 1988). Briefly, RAW 264.7 cells (ATCC) were seeded at a density of 1.0 × 10⁵ cells/well in a 12-well plate in RPMI 1640 + 10% FBS. Cells were pre-treated for 1 hour with 2U/ml IFN-γ (Peprotech) then treated with 100ng/ml of human TNFα in 1ml of medium with aptamers VR11, VR20 or the control aptamer cApt(VR) at a final concentration of 2µM or with 10µg/ml anti-TNF mAb. Aliquots of the medium [100µl] were removed at each time point and the NO₂⁻ level determined using the Griess reagent kit for nitrite determination (Invitrogen).

2.3.9. Analysis of VR11 ability to activate an innate immune response.

The aptamer VR11 and cApt were dissolved in sterilized saline at a concentration of 500 µg/ml and CpG ODN (5’-TCCATGACGTTCCTGACGTT-3’; type B murine, ODN 1826, Invivogen) was dissolved at a concentration of 50 µg/ml. Intraperitoneal injections (200 µl) were administered in C57BL/6 mice. Untreated group received an injection of 200 µl of saline alone. Three hours after injection, mice were sacrificed and their serum was collected for analysis. Protein cytokine concentrations were determined using the DuoSet ELISA development kits for mouse CXCL1/KC (murine IL-8, R & D systems Inc.) and murine TNFα (R & D systems Inc.) as per manufacturer’s protocols. Animals were kept under standard pathogen-free conditions at the Ontario Cancer Institute animal
facility. Experiments were performed in accordance with the rules and regulations of the Canadian Council for Animal Care.

2.4. Results and Discussion

2.4.1. Identification of DNA aptamers directed at TNFα.

Traditional treatments for chronic inflammation include NSAIDs, glucocorticoids, cytostatic drugs and DMARDs. Due to the increased understanding of the molecular pathology of several inflammatory disorders, immunosuppressants, such as those directed at TNFα, have moved to the forefront of anti-inflammatory drugs. The current anti-TNFα therapy market is dominated by protein therapeutics, namely etanercept (Enbrel®), adalimumAb (Humira®) and infliximAb (Remicade®). These TNFα inhibitors have been shown to be therapeutically non-equivalent in terms of their structure, binding to TNFα and their therapeutic effects (van Vollenhoven, 2007). More importantly, these protein-based therapeutics are large macromolecules that are costly to produce, immunogenic when used in a chronic therapy setting, with close to 40% of patients still showing disease symptoms even after treatment.

Using TNFα as a target, we perform SELEX searches with a view to identify short, synthetic DNA aptamers able to recognize this key cytokine and potentially mimic the inhibitory action of an anti-TNFα antibody (Figure 2.1). After 12 rounds of selection, 60 clones were sequenced and found to be enriched for 3 specific variable region (VR) sequences. Specifically, the 25-base long sequences VR1, VR2, and VR6 accounted for 20%, 13% and 10% of the observed sequences respectively (Table 2.1). Aptamers VR11
and VR20 represented unique sequences identified in this search that also selectively bound to TNFα. Aptamer VR11 was the only aptamer that inhibited human TNFα functions on cells. Interestingly, VR11 incorporates the sequence 5′ – CAGTCGGCGA-3′ which was identical to a region of aptamer VR12 with the exception of a single G base insertion [5′ – CAGGTGGCGA – 3′]. This 10-base long conserved span represents a 40% coverage of the variable region element of our library and despite the homology between these two sequences, VR12 displayed no inhibitory activity. This finding suggests that even a single base insertion can eliminate the functional properties of such aptamers.

In order to identify and confirm that observed sequences were specifically binding to TNFα, 96-well ELISA microtitre plates were coated with human TNFα, and subsequently exposed to synthetic full-length (FL; a sequence that includes a specific VR sequence as well as the two flanking primer regions) and variable region (VR) only DNA aptamers harbouring a 5’ biotin group. Aptamer binding to TNFα was confirmed with streptavidin–HRP for 20 unique sequences tested (Figure 1A) with most of them showing an ELISA signal at least double that of the control aptamers (cApt(VR), cApt(FL)).
Table 2.1. List of TNFα specific DNA aptamer sequences identified from the SELEX screen as well as control aptamer [cApt(VR) and cApt(FL)] sequences used in the present study.

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR1</td>
<td>ACAACCGACAAATTATCGCACTTAC</td>
</tr>
<tr>
<td>VR2</td>
<td>ATCACAGGGGTTACGAATTGAGT</td>
</tr>
<tr>
<td>VR4</td>
<td>ACAGCTACGGGACTCTCAAACGCC</td>
</tr>
<tr>
<td>VR5</td>
<td>AAGAACTGGCAGGCCACCACCGGT</td>
</tr>
<tr>
<td>VR6</td>
<td>CCGTCCGCTTTGAGTCTCGAAAGGG</td>
</tr>
<tr>
<td>VR8</td>
<td>ATACCCATGTTACGGCCGCCCATTC</td>
</tr>
<tr>
<td>VR11</td>
<td>TGGTTGAATGGCCAGTCTCGGCAAA</td>
</tr>
<tr>
<td>VR12</td>
<td>CTCGTCAGTTCAGTCGGCGCATG</td>
</tr>
<tr>
<td>VR16</td>
<td>AGTGAGCGCTTATGCTCGCAGTGG</td>
</tr>
<tr>
<td>VR20</td>
<td>TCCTCATATAGAGTCGGGGCGT</td>
</tr>
<tr>
<td>cApt(VR)</td>
<td>AGTCAGTCAGTCAGTCAGTCAGTC</td>
</tr>
<tr>
<td>FL1</td>
<td>AATTAACCTCCTAAGGGAACTATCGCAATTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL2</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL4</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL5</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL6</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL8</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL11</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL12</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL16</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>FL20</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
<tr>
<td>cApt(FL)</td>
<td>AATTAACCTCCTAAGGGAACTATCGCCTTACCTATAGTGTCACCTAAATCGTA</td>
</tr>
</tbody>
</table>
Figure 2.1. Binding of selected full-length (FL) and variable region (VR) DNA aptamers to TNFα and NfκB assays identify FL11 and VR11 as TNFα inhibitors.

(A) Histogram of ELISA signals confirming the binding of biotinylated FL (grey bars) and VR aptamers (black bars) to immobilized recombinant TNFα. The control DNA aptamers cApt(VR) and cApt (FL) gave ELISA signals comparable to wells treated with streptavidin-HRP alone (open bar). (B) Histogram of fold increase in luciferase activity in PANC-1 cells transiently transfected with a DNA plasmid construct expressing the luciferase gene under the control of NfκB DNA binding promoter. Cells were treated with TNFα (100ng/ml), FL (dark grey bars) and VR aptamers (black bars) or an inhibitory anti-TNFα mAb (light grey bar). Only aptamer VR11 and its full-length variant FL11 displayed inhibitory activities comparable to a control anti-TNFα mAb. (C) Histogram of TNFα induced, NF-κB luciferase activity in HEK293T cells. Cells were treated with 2 µM (black bars), 200 nM (grey bars) and 20 nM (white bars) of the control aptamer cApt (VR), the TNFα specific DNA aptamers VR11, VR20 as well as 20µg/ml of the inhibitory anti-TNFα mAb. Each histogram bar represents the average enhancement in observed luciferase activity + SEM (n=9) normalized to that of untreated samples.
2.4.2. DNA aptamer VR11 inhibits the binding of TNFα to its receptor.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a downstream eukaryotic transcription factor that is activated by various intra and extra cellular cytokines, including TNFα (Sen and Baltimore, 1986). We selected a cell-based assay using an NF-κB luciferase reporter assay in order to define aptamer sequences that inhibited human TNFα from binding to its receptor, leading to the loss of a cell signalling event. Our preliminary screen was conducted using the human pancreatic cancer cell line PANC-1 transiently transfected with the NF-κB luciferase reporter (Mamaghani et al., 2009). The luciferase activity was measured 6 hours post-transfection with only two DNA aptamers, namely VR11 and FL11, displaying an ability to inhibit TNFα-dependent NF-κB expression (Figure 2.1B). We subsequently used a more sensitive TNFα-mediated NF-κB assay employing HEK293T cells [higher transfection efficiency] and confirmed the inhibitory ability of VR11 in a dose-dependent manner (Figure 2.1C). The inhibition of TNFα-mediated NF-κB signal was observed down to a VR11 aptamer concentration of 200nM which represented a 35-fold molar excess of aptamer over TNFα. As expected, control aptamers VR20 and cApt(VR) did not show any inhibition of TNFα-mediated signalling suggesting that the inhibitory effects of VR11 were not due to a concentration-dependent, non-specific binding event.

The L929 mouse fibroblast cell line was subsequently employed to define the ability of aptamer VR11 to inhibit human TNFα-induced cytotoxicity (Matthews et al., 1987). The TNFα CD₅₀ value for L929 mouse fibroblast cells is approximately 3 nM (Figure 2.2). When L929 mouse fibroblast cells were treated with human TNFα in the presence of aptamer VR11 or an inhibitory TNFα mAb, the observed CD₅₀ values were
Figure 2.2. Aptamer VR11 inhibits TNFα-induced cytotoxicity in murine fibroblasts. L929 cells were treated with increasing concentrations of TNFα alone (▲) or in the presence of either the inhibitory anti-TNFα mAb (△), inhibitory aptamer VR11 (○), the control aptamer cApt (VR) (□) or aptamer VR20 (■). Each point represents the average % survival value ± SEM (n=12).
shifted to approximately 100 nM and 260 nM respectively representing a 33-fold and 86-fold decrease in toxicity (Figure 2.2). These CD50 values for aptamer VR11 and TNFα mAb, correspond to approximately a 24-fold and 5-fold molar excess over TNFα respectively. The control aptamer cApt(VR) as well as the TNFα-specific DNA aptamer VR20 showed no effect on the toxicity of TNFα (Figure 2). Besides the removal of the 3’ terminal A base of VR11, the truncation of bases from either the 3’ or 5’ ends of VR11 resulted in the loss of its inhibitory activity towards human TNFα (Appendix 1).

The binding properties of two TNFα-binding DNA aptamers, namely VR11 and VR20, were further characterized by surface plasmon resonance (SPR) with their binding constants determined to be in the low nanomolar range [respective Kd values of 7.0±2.1nM and 8.7±2.9nM]. To assess the specificity of VR11 and VR20 for human TNFα, we also used human TNFβ as an analyte. TNFβ was chosen since it binds to TNF receptors and is the closest known homolog to TNFα having highly similar structures (Figure 3B) despite having low sequence homology (Figure 2.3A). It was subsequently observed that aptamers VR11 and VR20 showed no binding to TNFβ by SPR (Figure 3E and 3F). One feature of aptamer VR11 which may contribute to its inhibitory function may be its slow on- and off-rates (Figure 2.3). Once bound to TNFα, VR11 was able to block TNFα-enhanced NO production in macrophages over a period of 24 hours (Figure 2.4B; as referred to as NO2- levels). Specifically, one of the consequences of unregulated overproduction of TNFα is the persistence of inflammation. Nitric oxide (NO) is a key mediator of inflammation stimulated by TNFα that is released by macrophages (Figure 4). We thus used the measurement of nitrite ions [NO2-] in solution as a marker of TNFα-
Figure 2.3. Binding kinetics of TNFα to aptamers VR11 and VR20 as determined by surface plasmon resonance. (A) Sequence alignment of soluble TNFα and the closest known homolog TNFβ. (B) Alignment of ribbon diagrams derived from the crystal structures of monomeric TNFα (orange, PDB: 1TNF) and TNFβ (blue, PDB: 1TNR). Fitted binding curves of immobilized inhibitory aptamer VR11 associated with increasing concentrations of (C) TNFα and (E) TNFβ (n=3). Binding curves of immobilized aptamer VR20 associated with increasing concentrations of (D) TNFα and (F) TNFβ (n=3). (G) Calculated dissociation constants (K_D) for TNFα binding to aptamer VR11 and VR20.
induced inflammatory signalling in the macrophage cell line RAW264.7. Human TNFα alone does not elicit the release of nitrite ions from macrophages. However, a synergistic effect occurs when macrophages are concomitantly treated with interferon-gamma (IFN-γ) and TNFα (Paludan, 2000). At the 12 and 24 hour time periods, aptamer VR11 was able to inhibit ~54% and ~45% of the TNFα-dependent NO release signal respectively (Figure 2.4). In comparison, the TNFα mAb blocked ~79% and ~74% of the NO release signal over the same two time periods (Figure 2.4). No inhibition of TNFα-induced NO release by RAW264.7 cells was observed when treated with control aptamers cApt(VR) and VR20. This sequence thus represents a template that may be useful for modifications that may yield a suitable product for use in vivo.
Figure 2.4. Inhibition of TNFα-induced NO2- production in macrophages. RAW264.7 cells were stimulated for (A) 6 hours or (B) 24 hours with IFNγ, IFNγ and TNFα or IFNγ, TNFα in the presence of either the inhibitory anti-TNFα mAb, inhibitory aptamer VR11, control aptamer cApt(VR) and aptamer VR20. The concentration of NO2- produced in culture medium was determined using the Griess assay. Each point represents the average amount of NO2- produced ± SEM (n=9).
2.4.3. Structural features of DNA aptamer VR11.

The site targeted by VR11 on TNFα was not defined in the present study. The epitope recognized by the mAb to TNFα has not been identified to date and as such this probe could not be used to locate the VR11 binding site on TNFα. In addition, the size and high avidity of this anti-TNFα mAb was able to block the binding of VR4, VR11 and VR20 TNFα-directed aptamers to their target (see Appendix 2). A structural feature of aptamer VR11 that may contribute to its inhibitory activity is its predicted G-quadruplex structure as determined by the QGRS Mapper software (Kikin et al., 2006). G-quadruplexes arise from the association of four G-bases into a cyclic Hoogsteen H-bonding arrangement. Identifying G-quadruplex structures within aptamers has been the focus of recent interest in view of the therapeutic efficacy of previously identified G-quadruplex forming ssDNA and RNA (Bates et al., 1999; Marchand et al., 2002; Xu et al., 2001). A case in point is the 15-mer DNA thrombin aptamer which folds into a G-quadruplex structure (Russo Krauss et al., 2011). Structurally, VR11 incorporates 11 Guanine bases which accounts for 44% of its sequence. G-quadruplexes typically occur when 3 or more consecutive G bases are present within a sequence (Randazzo et al., 2012). However, the G-rich content of VR11 sequence displays an 18-base long stretch containing 4 GG repeats (5’-\textbf{GGTGGATGGCGCAGTCGG} -3’) which may stabilize the aptamer (Collie and Parkinson, 2011). Interestingly, the DNA aptamer KS-B, specific for thrombin, also incorporates 4 GG repeats within its sequence (\textbf{GGTGGATGGCGCAGTCGG}), and adopts an intramolecular G-quadruplex structure (monomeric chair form) has proven from its crystal structure (Macaya et al., 1993; Padmanabhan et al., 1993; Wang et al., 1993). Thus, the projected G-quadruplex for VR11 may exist in solution. An intramolecular G-quartet structure (Gilbert and Feigon, 1999; Williamson, 1994) within VR11 would
support our SPR data which suggest a 1:1 association of VR11 with TNFα. The circular dichroism spectra of VR11, VR20 and the control aptamer cApt were thus recorded in order to monitor for the possible occurrence of a G-quadruplex structure within these DNA aptamers. The sequence of VR20 harbours 4 consecutive G bases [Table 1; 5’- CGGGGC-3’ motif] and its CD spectrum displays a negative ellipticity band centered around 240 nm as well as a positive band near 260 nm. This spectrum indicates that the GGGG motif present within VR20 adopts a characteristic G quadruplex structure in solution with a stacking pattern involving guanosines having identical glycosidic bond angles giving rise to a four stranded parallel quadruplex structure typically observed for the motif 5’-TGGGG-’3 (Hardin et al., 1991; Hardin et al., 1992). The CD spectrum of control aptamer cApt did not reveal any known spectral features indicative of G-quadruplexes. As for VR11, its CD spectrum harbours a broad positive band around 290 nm suggesting that guanosines with different glycosidic bond angles within its sequence are stacked in the presence of sodium or potassium (Figure 2.5A and 2.5B). Unlike the CD spectrum observed for VR20, the VR11 spectrum does not provide conclusive proof that the guanosine elements within its 25-bp sequence are arranged in a traditional G-quadruplex structure. Nevertheless, a structural arrangement involving the stalking of guanosine bases is present in VR11 and may stabilize its structure. Aptamer stability is crucial for targeting TNFα since the effectiveness of current protein-based therapies can partly be attributed to their long circulating half-lives such as Etanercept which is linked to the Fc portion of an IgG1 to help extend its half-life to 4-5 days or the PEGylation of anti-TNF antibodies (Choy et al., 2002; Mohler et al., 1993). Recently, some of the most common modifications of aptamers investigated have been their conjugation to polyethylene glycol (PEG) groups for increased stability (Healy et al., 2004; Willis et al.,
In addition, to increase the *in vivo* circulating half-life of DNA aptamers, modified nucleotides could be introduced into the libraries during synthesis such as 2’ OH group with 2’fluoro or 2’ amino, aromatic or alkyl moieties or introduction of Locked Nucleic Acid (LNA) (Barciszewski et al., 2009; Schoetzau et al., 2003). Also, aptamer libraries with longer variable regions may identify sequences with a higher G-rich content which in turn could adopt different G-quadruplex structures and yield improved inhibitors to TNFα (Burge et al., 2006).
Figure 2.5. Structural analysis of aptamers VR11 and VR20 by Circular Dichroism.

CD spectra of the 25-bp aptamers VR11, VR20 and cApt in the presence of either a) Sodium or b) Potassium ions.
2.4.4. Immunogenicity of DNA aptamer VR11.

One therapeutic obstacle that DNA aptamers may encounter is their possible activation of an immune response similar to that of oligodeoxynucleotides containing CpG motifs. These CpG motifs can promote a Th1 response by signaling through TLR9 which is expressed on human B cells and plasmacytoid dendritic cells leading to an innate immune response (Krieg, 2002; Krug et al., 2001). VR11 contains two possible CpG motif sequences (Figure 2.6A). To test whether this DNA aptamer can induce an innate immune response, we injected 100 µg of aptamer VR11 and cApt as well as 10 µg of a known TLR9 ligand CpG ODN in the intraperitoneal cavity of C57BL/6 mice and quantified the amount of TNFα and mouse IL-8 present in their serum after 3 hours. The CpG ODN positive control yielded a 35-fold increase in TNFα and a 24-fold increase in murine IL-8 serum levels while the control aptamer cApt (which contains no CpG motifs) and the TNFα-specific VR11 DNA aptamer yielded statistically non-significant increases in both TNFα and murine IL-8 cytokine levels as compared to untreated mice (Figure 2.6B and 2.6C).
Figure 2.6. Aptamer VR11 does not cause an innate immune response in vivo. (A) Potential CpG motifs located in the variable region of aptamer VR11. C57BL/6 mice (n=3) were given an intraperitoneal injection of 10 µg of CpG ODN, 100 µg of aptamer VR11 or cApt and the amount of (B) TNFα or (C) KC in serum 3 hours after injection were determined by ELISA.
Chapter 3

Blocking the attachment of cancer cells in vivo with DNA aptamers displaying anti-adhesive properties against the carcinoembryonic antigen

A version of this chapter has been accepted for publication in the journal

*Molecular Oncology*

Blocking the attachment of cancer cells in vivo with anti-adhesive DNA aptamers

(Molecular Oncology 2013)

Erik W. Orava, Aws Abdul-Wahid, Eric H.B. Huang, Amirul Islam Mallick, Jean Gariépy

Author Contributions:

Erik Orava, Dr. Aws Abdul-Wahid and Dr. Jean Gariépy designed the experiments, analyzed the data and wrote the paper.

Erik Orava contributed Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.7.
Dr. Aws Abdul-Wahid contributed Figure 3.6

Eric Huang performed selection experiments that identify DNA aptamers in this project as well as helped perform animal studies.
Dr. Amirul Islam Mallick helped perform animal studies.
3.0. Abstract

The formation of metastatic foci occurs through a series of cellular events, initiated by the attachment and aggregation of cancer cells leading to the establishment of micrometastases. We report the derivation of synthetic DNA aptamers bearing anti-adhesive properties directed at cancer cells expressing the carcinoembryonic antigen (CEA). Two DNA aptamers targeting the homotypic and heterotypic IgV-like binding domain of CEA were shown to block the cell adhesion properties of CEA, while not recognizing other IgV-like domains of CEACAM family members that share strong sequence and structural homologies. More importantly, the pre-treatment of CEA-expressing tumour cells with these aptamers prior to their intraperitoneal implantation resulted in the prevention of peritoneal tumour foci formation. Taken together, these results highlight the effectiveness of targeting the cell adhesion properties of cancer cells with aptamers in preventing tumour implantation.
3.1. Introduction

Metastatic forms of cancer account for 90% of all cancer-related deaths (Sporn, 1996). Cellular processes associated with tumour cell implantation and expansion of micrometastases at sites distal from a primary tumour site are linked to altered growth signals, deregulation of proliferative potential, evasion of apoptosis/anoikis and cell and matrix adhesion events that create and support the formation of metastatic foci (Fidler, 2003; Hanahan and Weinberg, 2000). As such, surface molecules which mediate intercellular adhesion represent candidate targets for engineering antiadhesive or antiaggregative therapies. One such surface marker is the carcinoembryonic antigen (CEA, CEACAM5 and CD66e), a member of the CEACAM family, an oncofetal antigen over-expressed on the surface of breast, colon, lung and a range of other epithelial cancer cells and an important cancer biomarker (Gold and Freedman, 1965b; Goldenberg et al., 1976; Hammarstrom, 1999; Thompson et al., 1991). Under normal physiological conditions, cells lining the colon express CEA in a polarized manner, with low levels of this antigen being detected in the intestinal lumen and blood. In contrast, higher levels of shed CEA are detected in the blood of 95% of patients with colorectal cancer (Chevinsky, 1991; Zhu et al., 2000). The deregulated overexpression of CEA has been linked to tumour implantation and metastasis (Hammarstrom, 1999).

Membrane-bound CEA is comprised of a 108-amino acid IgV-like N domain followed by 6 Ig-C like domains (A₁, B₁, A₂, B₂, A₃ and B₃) and a 27-amino acid C-terminus region which includes a glycosylphosphatidyl inositol (GPI) anchor signal sequence. The precise biological function of CEA has not been determined, but its deregulated overexpression by cancer cells is associated with a vast array of functional roles such as cooperating with novel oncogenes in cellular transformation, inhibition of
anoikis, differentiation inhibition via its GPI anchor, protection of tumour cells to apoptotic stimuli, immunomodulation as well as functioning as an intercellular adhesion molecule displaying both homotypic and heterotypic cell adhesion properties (Benchimol et al., 1989; Jessup et al., 2004; Kitsuki et al., 1995; Ordonez et al., 2000; Screaton et al., 2000; Screaton et al., 1997; Soeth et al., 2001).

A common denominator in CEA-dependent adhesion events is its IgV-like N-domain which can lead to cellular aggregation through by binding to other N-domains (defined as homotypic binding and homophilic interactions) or CEA IgC-like A3B3 domains on distinct tumour cells (defined as heterotypic binding and homophilic interactions) or its association with extracellular markers (defined as heterophilic interactions) such as its association with α5β1 integrins in binding to fibronectin (Jessup et al., 1993a; Nicholson and Stanners, 2006; Taheri et al., 2000; Zhou et al., 1993). Clinically, CEA is used to monitor patients with metastatic disease during active therapy, as increasing levels of CEA in serum correlate with treatment failure and poor prognosis (Duffy, 2006; Harris et al., 2007). Importantly, mounting a sustained antibody response directed at an altered self form of the CEA N domain results in the prevention of tumour implantation and formation of metastatic tumour foci in CEA transgenic mice (Abdul-Wahid et al., 2012). This effect has been assigned to the blockage of CEA-dependent adhesion properties by circulating antibodies as well as by immune mechanisms such as antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Similarly, targeting the CEA IgV-like N domain with cyclised peptides or monoclonal antibodies result in modest blockage of CEA-specific cell adhesion, migration and invasion in vitro as well as impeding the metastatic potential in mouse models (Blumenthal et al., 2005; Taheri et al., 2000; Zheng et al., 2011). These findings
suggest that CEA-specific, anti-adhesive agents may represent a successful treatment for metastatic cancers linked to the overexpression of CEA.

Aptamers represent an emerging alternative to protein-based ligands. Specifically, Aptamers are short single-stranded DNA or RNA oligonucleotides that adopt complex secondary and tertiary structures that allow for their specific and high affinity binding to a range of targets that include metal ions, proteins, bacterial cells and tumour cells (Hamula et al., 2008; Hicke et al., 2001; Morris et al., 1998; Rajendran and Ellington, 2008). Aptamers are derived through an iterative selection process, termed systematic evolution of ligands by exponential enrichment (SELEX), using a synthetic library containing a randomized region of 25 to 80 nucleotides flanked by two constant regions for PCR amplification (Tuerk and Gold, 1990). Alternatively, RNA aptamers are more labile than DNA oligonucleotides and the cost as well as time required to perform RNA SELEX searches are greater. More stable variants of RNA aptamers can be assembled with a modified T7 polymerase to incorporate 2’-fluoro and 2’-O-Me nucleotides.

Our group has reported the expression and purification of a folded recombinant form of the IgV-like N domain able to elicit an immune response as well as recapitulate the binding property of glycosylated full length CEA with CEA-expressing cells and purified human CEA from cancer patients. Importantly, the un-glycosylated form of the CEA N domain represents a suitable target for identifying aptamers since this domain has few putative glycosylation sites and that glycosylation of the N domain does not contribute to the adhesive properties between CEA N domain molecules (Charbonneau and Stanners, 1999; Krop-Watorek et al., 2002). We report the isolation of two functional DNA aptamers selected to bind this recombinant form of the IgV-like N domain of CEA and show its ability to block CEA-mediated cellular interactions and inhibit peritoneal
tumour nodule formation from CEA-expressing tumour cells \textit{in vivo}.

3.2. Materials and Methods

3.2.1. Generation of recombinant CEA modules

Recombinant CEA (rCEA) modules N, FLAG-N and FLAG-A_{3}B_{3} were expressed and purified as previously reported (Abdul-Wahid et al., 2012). Briefly, recombinant CEA domains were purified from inclusion bodies in \textit{E. coli} under denaturing conditions using urea (8M). The protein was subsequently purified by nickel-NTA chromatography and treated with Detoxi-gel (endotoxin removing gel; Thermo Fisher Scientific Inc.) to remove remaining traces of bacterial lipopolysaccharides (LPS).

3.2.2. Aptamer selection and cloning

The initial ssDNA library contained a central randomized sequence of 25 nucleotides flanked by two primer regions with the sequence 5’ GAC GAT AGC GGT GAC GGC ACA GAC G-(25N)-CGT ATG CCG CTT CCG TCC GTC GCT C 3’. The forward primer 5’ GAC GAT AGC GGT GAC GGC ACA GAC G 3’ and reverse primer 5’ GAG CGA CGG ACG GAA GCG GCA TAC G 3’ were used for selection and cloning (IDT Technologies, Inc.). A 50 nmol aliquot of the library was first counter-selected against Ni-NTA magnetic beads prior to selection against rCEA N in order to reduce non-specifically bound DNA species. The resulting sub-library was then exposed to 10 \mu g of His-tagged rCEA N domain immobilized onto Ni-NTA beads suspended in 1 mL of Selection Buffer (150 mM NaCl, 50mM Tris pH 8.0) at 37°C for 1 hour. Unbound DNA oligonucleotides were washed away with a 10-fold excess of selection buffer and DNA-
protein complexes were eluted from the recovered beads using an imidazole containing buffer (Selection buffer with 240mM imidazole). The ssDNA component was precipitated with sodium perchlorate/isopropanol and recaptured using a silica membrane-based purification system (Qiagen Inc., Mississauga, Ontario). The DNA aptamers were then amplified by asymmetrical PCR using a 10-fold excess of forward primer. After every three subsequent rounds of selection, the amount of target was reduced in half to increase the selection pressure to capture the tightest binding species. After 12 rounds of selection, the bound sequences were amplified by PCR to produce double stranded products, cloned into a pCR4-TOPO TA vector (Invitrogen) and sequences were analyzed using BioEdit sequence alignment editor software (Ibis Therapeutics, Carlsbad, USA).

3.2.3. Aptamer-based inhibition of CEA homotypic interactions

An enzyme-linked immunosorbent assay (ELISA)-based binding assay was employed to identify aptamers capable of inhibiting homotypic interactions between FLAG-tagged rCEA N domain and either rCEA A3B3 or rCEA N. Briefly, 96-well flat-bottomed Falcon microtiter plates (Becton-Dickinson Biosciences, Franklin Lakes, NJ) were coated with either N or A3B3 domain (1 µg/well in 100µl) in coating buffer (0.2 M carbonate/bicarbonate, pH 9.4) at 37ºC. Plates were then blocked with BSA (1% in PBS) and salmon sperm DNA (200 µl; 10 µg/mL) for 1 hour at room temperature then aptamers were added (200 µl; 25 µg/ml, 1µM) overnight at 4 ºC in PBS-T (0.05% Tween-20). Plates were then washed three times with PBS-T and FLAG-tagged rCEA N domain was added (100 µl; 10 µg/ml, 670 nM) for 1 hour at room temperature after being incubated with a given aptamer (100 µl; 25 µg/ml , 1µM) for 1 hour at room
temperature. After wash steps with PBS-T, the remaining bound FLAG-tagged rCEA N was detected by incubating the plates for 1 hour at room temperature with horseradish peroxidase (HRP) coupled anti-FLAG monoclonal antibody M2 (1:2500) dilution; Sigma-Aldrich). All experiments were performed in quadruplicate.

3.2.4. Cells lines and growth conditions

The murine colonic carcinoma cell lines MC38.CEA and MC38 were kindly provided by Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, Maryland). The cervical adenocarcinoma cell line HeLa (ATCC No CCL-2) as well as transfected cell lines HeLa^{CEACAM1}, HeLa^{CEACAM3}, HeLa^{CEACAM5}, HeLa^{CEACAM6} and HeLa^{CEACAM8} used for flow cytometry experiments were a gift from Dr. Scott Gray-Owen (University of Toronto, Toronto, Canada). All cell lines were cultured at 37ºC, 5.0% CO$_2$ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and dihydrostreptomycin (100 µg/mL).

3.2.5. Aptamer-based inhibition of homophilic cellular adhesion

The ability of aptamers to inhibit CEA-dependent cellular adhesion was measured in real-time using an xCELLigence RTCA SP label-free, impedance-based cell sensing device (Roche Applied Sciences, Laval, Canada). The inhibition of CEA-dependent cellular adhesion was monitored using MC38.CEA and MC38 cells (2.5 × 10$^4$ cells per well) grown in complete medium as described above. Cell suspensions were dispensed alone, with aptamers (100 µl, 250 µg/ml, 10µM) or in the presence of the rCEA N domain acting as a positive control (100 µl, 50 µg/ml, 3.3 µM) into wells of a 96-well
microtiter plate incorporating a sensor electrode array (E-plates) that had been precoated with the rCEA N domain, rCEA A$_3$B$_3$ domain or BSA (1µg/well). Cell attachment was quantified as a change in relative impedance, termed cell index (CI) (Matrone et al., 2010). The adhesion of MC38.CEA cells in the absence of aptamers served as a positive control. Data was collected after 3 hours to allow cells to fully adhere to protein-coated plates but before the start of cell proliferation. All experiments were performed in duplicate and were repeated three times.

3.2.6. Aptamer binding to CEA on cells as measured by flow cytometry

The binding specificity of aptamers N54 and N56 to the N domain of CEA was assessed by flow cytometry using the cell lines MC38.CEA (CEA$^+$) and MC38 (CEA$^-$) as well as HeLa cells expressing different members of the CEACAM family. Aptamers N54, N56 and the control aptamer cApt were synthesized with a 5’end Cy5 fluorophore (IDT Technologies, Inc., Coralville, Iowa). Cells were grown to mid-log phase and detached using an enzyme-free EDTA based cell dissociation buffer (Sigma-Aldrich, St. Louis, MO) washed with PBS (-CaCl$_2$, -MgCl$_2$) and resuspended at a concentration of $10^6$ cells/mL in cold PBS. Aptamers were then added to $1.0\times10^6$ cells at a final concentration of 200 nM in 1 mL. The expression of CEACAMs on HeLa transfected cells lines was confirmed using a FITC-labeled polyclonal anti-CEACAM antibody (Gift from Dr. Gray-Owen, University of Toronto, Toronto, Canada). CEA expression was confirmed with a CEA-specific COL-1 antibody (Invitrogen Inc.). Aptamers and antibody were allowed to bind for 2 hours at 4ºC. Cells were then washed three times in cold PBS and subsequently analysed using a FACScan (BD Biosciences, Franklin Lakes, NJ).
3.2.7. Inhibition of MC38.CEA tumour implantation

For tumour implantation studies, 5.0 × 10^5 MC38.CEA cells were co-injected in the intraperitoneal cavity of C57BL/6 mice with either aptamers N54, N56, N71, cApt or no aptamer (saline) (200 µl; 2.5mg/mL, 100 µM). After 21 days, mice were sacrificed and the number of nodules and their volumes were recorded following dissection, as previously described (Abdul-Wahid et al., 2012). Specifically, the length and width of tumour nodules were measured using microcallipers. Tumour volumes were calculated using the modified formula where the volume of the tumour (mm^3) equals [(x^2 × y)/2]; where x and y represent the transverse and longitudinal diameters of the tumour respectively. Each group consisted of 3 females and 2 male mice. All animals were kept under standard pathogen-free conditions at the Ontario Cancer Institute animal facility. Experiments were performed under the approval of the local animal welfare committee and in accordance with the rules and regulations of the Canadian Council for Animal Care.

3.2.8. Aptamer cytotoxicity assay

MC38.CEA cells were seeded for 24 hours before cell viability experiments were performed in 96-well flat-bottom microtiter plates at a density of 5.0×10^3 cells/well in DMEM medium containing 10% FBS. Aptamers at a concentration of either 250 µg/ml (10 µM) or 2.5 mg/ml (100 µM) were incubated with the cells in medium for 24 hours at a volume of 100 µl. Cells were then washed with warm PBS and incubated in complete medium for another 24 hours. The viability of adherent cells was subsequently determined using a sulforhodamine B assay (Skehan et al., 1990). The absorbance of the sulforhodamine B signal in each well was read at 570 nm using a plate reader. Each
3.2.9. Analysis of aptamer-based innate immune responses

The aptamers N54 (200 µl; 500 µg/ml, 20µM) and cApt (200 µl; 500 µg/ml, 20µM) as well as a TLR9 ligand CpG ODN (5’-TCCATGACGTTCTGACGTT-3’; type B murine, ODN 1826, Invivogen, CA; (200 µl; 50 µg/ml, 500nM) were dissolved in sterile USP saline. These oligonucleotides (200µl) were administered intraperitoneally to 6-8 weeks old C57BL/6 mice. An untreated group of mice received an injection of 200 µl of saline alone. Three hours after injection, mice were sacrificed and their serum was collected for analysis. Serum IL-8 and TNFα concentrations were determined using the DuoSet ELISA development kits for mouse CXCL1/KC (murine IL-8, R & D systems Inc.) and murine TNFα (R & D systems Inc.) as outlined by the manufacturer.

3.2.10. Statistical methods and data analysis

Data sets derived from individual groups of mice were compared using student’s t-test and grouped data sets were analyzed by ANOVA. Statistical analyzes and graphs were assembled using GraphPad PRISM (version 5.01, GraphPad software for Science, San Diego, CA). P values ≤ 0.05 were considered significant unless otherwise indicated. Flow cytometry statistics were analyzed using WinMDI (version 2.8, Windows multiple document interface for flow cytometry, Scripps Research Institute).
3.3. Results

3.3.1. Generation of DNA aptamers displaying inhibitory properties of CEA-dependent homotypic adhesions

Short, 25-base long DNA aptamer sequences specifically recognizing a recombinant protein coding for residues 1-132 of mature CEA (referred to as rCEA N domain) were identified by the SELEX approach (Figure 3.1). Specifically, twelve iterative rounds of PCR-based selection were performed yielding six unique DNA aptamer sequences (Figure 1A). These six sequences, labelled N54, N56, N57, N59, N65 and N71, as well as a control aptamer (cApt) were synthesized with their primer regions and subsequently tested using an ELISA-based assay to assess their ability to directly block the binding of the IgV-like N domain to either rCEA IgC-like A3B3 domains (Figure 3.1B) or to itself (to rCEA N domain; Figure 1C). Aptamers N54 and N56 were the only aptamers found to display inhibitory properties of both types of binding events, where N54 inhibited 39% of the rCEA N→rCEA A3B3 signal and 45% of the rCEA N→rCEA N signal relative to control wells (in the absence of aptamer. Aptamer N56 inhibited 32% of the signal associated with either homotypic interactions (Figure 3.1B and 3.1C). Interestingly, aptamer N65 shares a nearly identical sequence to N54 with the exception of two bases flanking the ends of the sequence and a C to T substitution in the sequence [5’ GCTGAC 3’]. This finding suggests that the antiadhesive property of N54 is sensitive to even a single base change in its sequence.
Figure 3.1. Aptamers selected to bind to the CEA IgV-like N domain inhibit homotypic adhesion events. (A) Aptamer sequences identified using the SELEX procedure. CEA N-domain-specific aptamers inhibit homotypic binding events. ELISA plates (96-well) were pre-coated with (B) rCEA A3B3 or with (C) the rCEA N domain. Aptamers selected to the N-domain and control aptamer (cApt) were added to the wells, incubated overnight at 4°C, washed followed by the addition of FLAG-tagged rCEA N incubated with aptamers for 1 hour and added to the appropriate wells. Bound FLAG-tagged rCEA N was detected using an anti-FLAG HRP-coupled M2 mAb. Each bar represents the average percent of binding ± SEM (n=4).
3.3.2. Aptamers N54 and N56 inhibit homophilic cellular adhesion

CEA-dependent homophilic interactions are a prerequisite to the expansion of metastatic tumour foci. As such, the inhibitory capacity of aptamers N54 and N56 was determined using the CEA-expressing murine cell line MC38.CEA and its parental CEA-negative cell line MC38. MC38.CEA cells adhere to the wells of impedance-based plates (E-plates) pre-coated with the rCEA N and A$_3$B$_3$ domains, but only weakly to wells coated with BSA (Figure 3.2A). Addition of aptamers N54 and N56 resulted in a loss of 59% and 49% of the signal arising from homophilic cellular adhesion between the immobilized CEA N domain and MC38.CEA cells respectively after 3 hours. This time point was chosen as it was observed that cells were able to fully adhere without the presence of inhibitors (Appendix 4). Similarly, a 45% and 51% decrease in signal was observed for MC38.CEA cells interacting with the immobilized rCEA A$_3$B$_3$. In contrast, the control aptamers cApt and N71 showed no significant inhibition of homophilic interactions (Figure 3.2A). As a positive control, the soluble rCEA N domain was pre-incubated with MC38.CEA cells and resulted in a 59% and 44% loss of binding signal of these cells to the immobilized rCEA N or A$_3$B$_3$ domain respectively. As expected, aptamer treatments as well as BSA and rCEA N domain showed no ability to inhibit MC38 cells from adhering to rCEA N- or BSA-coated plates (Figure 3.2B). Subsequently, the inhibitory effects of aptamers N54 and N56 (as 75-base long aptamers) on cell adhesion were titrated in a dose-dependent manner (Figure 3.2C). A significant inhibition of MC38.CEA adhesion was observed starting at an aptamer concentration of 1 µM. Control aptamers cApt and N71 showed no effect suggesting that the inhibition of homophilic cellular adhesion by aptamers N54 and N56 was not due to a concentration dependent non-specific effect.
Figure 3.2. Addition of aptamers specific to the CEA N domain inhibits homophilic cellular adhesion. (A) Addition of aptamers N54 and N56 significantly inhibited CEA-dependent binding of MC38.CEA cells to wells coated with rCEA N domain and rCEA A₃B₃ while not affecting their binding to BSA coated wells. (B) Addition of aptamers N54 and N56 had no effect on CEA MC38 cells adhering to wells coated with rCEA N, rCEA A₃B₃ or BSA. (C) Dose-dependent inhibition of CEA-mediated cellular adhesion of MC38.CEA to rCEA N coated wells was monitored in the presence of increasing concentrations of specific and control aptamers. Each bar in Panels A and B represents the average cell index values observed ± SEM (n=6). In panel C, each bar represents the average percentage of cell binding ± SEM (n=6).
To further characterize the inhibitory properties of aptamers N54 and N56, we constructed series of truncated forms to determine the minimal binding regions required to retain their inhibition of CEA-dependent homophilic adhesion (Figure 3.3A). Aptamer N54 did not retain its ability to inhibit cellular adhesion after a total of 18 bases were removed from both ends of its sequence (aptamer N54-57; Figure 3B). Interestingly, although full length N56 was not as effective as N54 in inhibiting MC38.CEA cell adherence (36% compared to 48% inhibition respectively), N56 did retain its inhibitory ability when truncated down to 32 bases with no significant decrease in the adherence of cells as compared to the full length sequence (Figure 3.3B). Further truncations of N56 however yielded inactive inhibitors of cell adhesion.
Figure 3.3. Minimal regions required for aptamer inhibition of homophilic cellular adhesion. (A) List of aptamer sequences synthesized to determine the minimum aptamer regions needed to inhibit CEA-dependent binding of cells to immobilized CEA N domain. Bold italic letters represent the variable region of aptamer sequences identified by SELEX searches. (B) Inhibition of CEA-dependent binding of MC38.CEA cells to rCEA N with full length and truncated aptamer N54 and N56 sequences. Each bar represents the average percentage of cell binding to wells ± SEM (n=6).
3.3.3. **Aptamers N54 and N56 specifically recognize the N domain of CEA**

The IgV-like N domain of CEA is homologous in sequence to that of other CEACAM members. Specifically, the alignment of CEACAM1, CEACAM3, CEACAM5 (CEA), CEACAM6, and CEACAM8 IgV-like N domain primary structures indicate that 61% of residues along their sequences are identical with up to 84% of residues being similar (Figure 3.4A). In addition, the known structures of the CEACAM1, CEACAM5 and CEACAM8 N domains also indicate that these IgV-like N domains adopt the same folded structure (Figure 3.4B) (Fedarovich et al., 2006; Korotkova et al., 2008). Accordingly, the ability of aptamers N54 and N56 to specifically recognize the N domain of CEA and not IgV-like N domains of related CEACAMs was assessed by monitoring the binding of Cy5-labelled aptamers to CEACAM+ and CEACAM− cells by flow cytometry. Specifically, HeLa cells were stably transfected to express CEACAM1, CEACAM3, CEACAM5, CEACAM6 or CEACAM8.

Analysis of the CEACAM-expressing HeLa cells as well as the CEA+ MC38.CEA cells, CEA− HeLa and MC38 cells demonstrated that aptamers N54 and N56 specifically bound to MC38.CEA and HeLa<sub>CEACAM5</sub> cells while the irrelevant cApt control aptamer showed no binding to any of the cells tested (Figure 3.5). The FITC-labeled polyclonal anti-CEACAM antibody confirmed the expression of individual CEACAMs in all transfected HeLa cell lines and the CEA antibody COL-1 confirmed the presence of CEA (Figure 3.5). Aptamer N56 binding to MC38.CEA and HeLa<sub>CEACAM5</sub> resulted in a ~7-fold increase in mean fluorescence signal intensity (Figure 3.5B,D). Aptamer N54 binding to MC38.CEA and HeLa<sub>CEACAM5</sub> resulted in a greater increase in binding as shown by a ~16-fold and ~14-fold increase in mean fluorescence intensities respectively (Figure 3.5B,D). Similar binding patterns were seen for the endogenously
Figure 3.4. Alignment of the IgV-like N-domains of CEACAM family members. (A) Sequence alignment of the N-domain of CEACAM family members involved in cell-cell interactions. (B) Structural alignment of the N-domains of CEACAM1 (red; PDB: 2GK2), CEA (blue; PDB: 2QSQ) and CEACAM8 (yellow; PDB: 2DKS). (C) The ABED face (red) and the CFG face (blue) of CEA. Residues 42 to 46 (NRQII) and residues 80 to 84 (QNDTG) are shown in yellow. Structures were obtained from the protein data bank (PDB) with accession numbers shown.
Figure 3.5. Aptamer binding to CEA+ and CEA- cells using Cy5 labelled aptamers by flow cytometry. Cy5-labelled aptamers N54, N56 and cApt were incubated with CEA- cells (A) MC38, (C) HeLa or CEA+ (B) MC38.CEA and (D) HeLa<sub>CEACAM5</sub>. Aptamers N54 and N56 did not detect the presence of other CEACAM family members as shown by low mean fluorescence intensities observed for (E) HeLa<sub>CEACAM1</sub>, (F) HeLa<sub>CEACAM3</sub>, (G) HeLa<sub>CEACAM6</sub>, (H) HeLa<sub>CEACAM8</sub> transfected cell lines. A FITC-labeled polyclonal anti-CEACAM antibody was used to confirm the surface expression of CEACAM proteins and an anti-CEA mAb to monitor for the presence of CEA. The auto-fluorescence signal arising from unlabelled cells is shown for cells alone. (I) Binding of Cy5-labelled aptamers N54, N56 and cApt to MC38.CEA cells. Data points represent average mean fluorescence intensity values ± SEM (n=3).
CEA expressing cell lines MCF-7, HT29 and BxPC3 (Appendix 5). The binding of Cy5-labeled aptamers N54 and N56 to MC38.CEA cells was also determined as a function of concentration (Figure 3.5, panel I). Using a single site binding model, it was calculated that aptamers N54 and N56 display binding constants ($K_d$) of $45 \pm 11$ nM and $78 \pm 24$ nM respectively to their CEA target on MC38.CEA cells. Together, these findings suggest that the derived N54 and N56 aptamer sequences specifically bind their cognate target with high affinity.

3.3.4. Addition of aptamers N54 and N56 to MC38.CEA cells reduces tumour implantation in vivo

The ability of aptamers N54 and N56 to inhibit CEA-dependent tumour implantation and subsequent metastasis was addressed by monitoring their ability to interfere with the implantation of murine MC38.CEA tumour cells in the peritoneal cavity of C57BL/6 mice. Briefly, murine MC38.CEA cells were pretreated for 30 minutes at 37°C with 500 µg (200 µl of a 100 µM concentration) of control aptamers cApt, N71, inhibitory aptamers N54, N56 or untreated and co-injected directly into the peritoneal cavity of mice (Figure 3.6A). Mice were then euthanized after 21 days to assess tumour implantation by recording the number of tumour nodules as well as their volumes. Post-mortem analyses of the dissected mice showed that tumour masses were limited to the peritoneal cavity, and that tumour nodules were numerous in the control animal groups given either no aptamer, cApt or N71 (Figure 3.6B,C). In contrast, implanted MC38.CEA cells treated with aptamers N54 and N56 generated significantly fewer tumour nodules (Figure 3.6B). Specifically, aptamer N56 reduced tumour implantation as seen by an average decrease of 48% of cumulative tumour volume while N54 had a significant
decrease of 57% compared to untreated mice (Figure 3.6C). Control aptamers cApt and N71 showed no significant decrease in tumour implantation in relation with the control group that was just implanted with MC38.CEA cells (untreated). The inhibitory effect of aptamers N54 and N56 was even more dramatic in terms of implantation when tumour nodules were enumerated and compared to control groups (Figure 3.6D). Specifically, in the group pretreated with aptamer N54, four of five mice did not develop a secondary tumour nodule compared to untreated groups which had an average of ~6 tumour nodules. Mice treated with control aptamer cApt and N71 showed no significant decrease in the number of tumour foci when compared to the aptamer-untreated group.
Figure 3.6. Pre-treatment of CEA-expressing MC38.CEA cancer cells with CEA-specific aptamers reduces tumour implantation \textit{in vivo}. (A) Experimental design of implantation studies. MC38.CEA cells (5 × 10^5) were co-administered intraperitoneally into C57BL/6 mice with aptamers. Untreated cells served as a positive control for tumour implantation. Animals were sacrificed 21 days post implantation. (B) Photographs illustrating the presence of MC38.CEA tumour nodules in the peritoneal cavity at Day 21 post-implantation. (C) Cumulative tumour volumes in each group of mice (n=5) after 21 days. (D) The number of tumour nodules in the peritoneal cavity in each treatment group (n=5) after 21 days post-implantation.
3.3.5. **Aptamers specific to the CEA N domain are not cytotoxic and aptamer N54 does not activate an innate immune response**

Cytotoxicity studies on MC38.CEA cells treated with aptamers and rCEA N were conducted using the sulforhodamine B cell viability assay to determine if the decrease in tumour implantation seen was due to a cytotoxic effect of the aptamers (Matthews et al., 1987). MC38.CEA cells were incubated in DMEM complete media for 24 hours in the presence of either 250 µg/ml of aptamer (100 µl; 10 µM, same as used for in vitro experiments) or 2.5 mg/ml (100 µl; 100 µM), which represented the concentration of aptamer pretreated MC38.CEA cells prior to tumour implantation (Figure 3.7A). None of the aptamers incubated with MC38.CEA cells led to a loss of cell viability at these concentrations, which suggests that the reduction in tumour implantation observed for N54 and N56 was not due to aptamer-based cytotoxicity towards MC38.CEA cells.

In view of its potency as an inhibitor of CEA-mediated cell adhesion, we also tested whether aptamer N54 could induce an innate immune response similar to oligodeoxynucleotides containing CpG motifs, which promote Th1 responses by signaling through TLR9. Mice received an i.p. injection of 100 µg (200 µl; 20 µM) of control aptamer (cApt), aptamer N54 or 10 µg (100 µl; 500 nM) of a known TLR9 ligand CpG ODN and the amount of TNFα and mouse IL-8 present in their serum was quantified after 3 hours. Activation of TLR9 with the CpG ODN increased the serum levels of mouse IL-8 by ~45-fold while aptamers cApt N54 yielded statistically non-significant increases in serum IL-8 (Figure 3.7B). Similar results were observed in terms of increased production of serum TNFα by CpG ODN but not by aptamers cApt and N54 (Figure 7C). These results suggest that N54 does not activate an in vivo innate immune response associated with the secretion of inflammatory cytokines.
Figure 3.7. Aptamer N54 is noncytotoxic and does not activate innate immune responses. (A) A sulfhorhodamine B cell viability assay was performed on MC38.CEA cells treated with DNA aptamers or rCEA N at the concentration used for in vivo studies (white bars) or at a 10-fold higher concentration (black bars). Aptamer N54, cApt or CpG (positive control) were injected intraperitoneally into C57/BL6 mice. The animals were sacrificed after 3 hours for analysis of TLR-9 dependent activation of (B) IL-8 and (C) TNF-α secretion (n=3).
3.4. Discussion

Cancer cells typically display molecular signatures on their surface which have been exploited more recently as targets for aptamers to chaperone therapeutic cargos into cells (Orava et al., 2010). In the present study, functional DNA aptamers were developed against the key homotypic binding domain of CEA generated from E. coli. We selected DNA aptamers, as opposed to RNA aptamers, because they are more stable [less susceptible to hydrolysis], less expensive and more easily derived by SELEX approaches than RNA aptamers. Functional aptamers acting as mimics of molecules linked to cellular signalling pathways have been reported and in some instances shown to block events as diverse as angiogenesis, thrombosis, viral replication and inflammatory responses (Bless et al., 1997; Bock et al., 1992; Boiziau et al., 1999; Chou et al., 2005; Muller et al., 2009; Ng et al., 2006; Paborsky et al., 1993; Schneider et al., 1995). In the context of adhesion, several aptamers have previously been reported which bind to a group cell surface glycoproteins known as selectins (Huang et al., 1997; Schmidmaier and Baumann, 2008).

Specifically, a phosphorothioate-modified aptamer to E-selectin named ESTA-1 bound with nanomolar affinity and inhibited over 75% of sialyl lewis X positive cells from adhering to endothelial cells overexpressing E-selectin in vitro (Mann et al., 2010). As well, an RNA aptamer to P-selectin known as ARC5690 which contained 2’-fluoro pyrimidine and 2’-methoxy purines inhibited the adhesion of sickle red blood cells and leukocytes to endothelial cells by 90% and 80% respectively in a sickle cell disease model in vivo (Gutsaeva et al., 2010). Furthermore, a DNA aptamer to L-selectin inhibited L-selectin-mediated rolling of lymphocyte and neutrophils on activated endothelial cells in vitro as well as blocking lymphocyte trafficking to the lymph nodes in
vivo (Hicke et al., 1996). The present study highlights functional DNA aptamers able to specifically inhibit CEA-expressing tumour cells from forming metastastic tumour foci. Importantly, CEA plays a key role in tumour progression and the establishment of metastatic foci by CEA-expressing tumours (Berinstein, 2002). CEA has been shown to function as an intercellular adhesion molecule, as a function of reciprocal homophilic binding between N and A$_3$ domains (Jessup et al., 1993a; Taheri et al., 2000; Zhou et al., 1993), an attribute that contributes to its tumourigenicity (Camacho-Leal and Stanners, 2008; Samara et al., 2007). Specifically, its involvement in homotypic and heterotypic interactions correlates with the level of implantation and proliferation of CEA-expressing tumours at distal sites such as the lungs, liver and peritoneal cavity (Asao et al., 1991; Samara et al., 2007; Zhou et al., 1993; Zimmer and Thomas, 2001). CEA molecules bind in a homotypic manner by virtue of the interaction between their N and A$_3$ domains of opposing CEA molecules, events which results in the formation of a network of homophilic cellular contacts between CEA-expressing cells causing cell aggregation, implantation and invasion of organs (Hostetter et al., 1990; Jessup et al., 1993b; Zhou et al., 1993; Zimmer and Thomas, 2001). CEA has also been shown to heterotypically interact with additional binding partners (Benchimol et al., 1989; Oikawa et al., 1989). We hypothesized that blocking the homotypic binding of CEA would represent an effective strategy for inhibiting its adhesive behaviour.

Using the CEA IgV-like N domain as a target for aptamer selection using the SELEX process, we identified two unique 25-base long aptamers, termed N54 and N56 that possess the ability to inhibit CEA-mediated homotypic interactions (Figure 3.1). Of the two aptamers, N54 displayed a moderately greater ability to inhibit these interactions that was comparable to tumour-neutralizing antisera derived from mice vaccinated with
the rCEA N domain (Abdul-Wahid et al., 2012). Addition of N54 significantly inhibited the binding of murine CEA-expressing MC38.CEA cells to wells coated with rCEA N domain yet had no effect on CEA MC38 cells adhering to plates coated with rCEA N (Figure 3.2A and 3.2B). These results demonstrate that aptamers N54 and N56 are able to effectively block the homotypic interaction between the CEA N domains and CEA N domain to rCEA A3B3.

Aptamers N54 and N56 differ greatly in the minimal regions needed to inhibit homophilic cellular adhesion. Aptamer N54 requires both its primer and variable regions as the deletion of a total of 18 bases from its 3’ and 5’ ends resulted in a loss of its inhibition of CEA-mediated cell adhesion from ~50% to ~16% relative to the full length sequence (Figure 3A and 3B). However, aptamer N56 can be truncated from 75 bases down to 32 bases while retaining its inhibitory function (Figure 3.3A and 3.3B). Further deletions to N56 however resulted in a loss of its inhibition of CEA cell adhesion function.

Several members of the CEACAM family have been shown to be involved in cell-cell interactions sharing a high level of sequence identity within their N domain with CEA. Yet, aptamers N54 and N56 were able to uniquely bind to the N domain of CEA (Figures 3.4 and 3.5). Structurally, the N domain of CEACAM1, CEAMCAM3, CEA, CEACAM6 and CEACAM8 adopt an identical Ig V-like fold displaying defined faces. The N domain of CEACAMs displays two faces: an ABED face and an opposite CFG face (Korotkova et al., 2008; Taheri et al., 2000). The CFG interface of CEACAMs has been shown to mediate CEACAM-CEACAM interactions (Figure 3.4 C) (Markel et al., 2004). Furthermore, peptides to residues 42 to 46 (NRQII) and residues 80 to 84 (QNDTG) on CEA were found to modestly block CEA-mediated cellular aggregation
(Taheri et al., 2000) at concentrations >150-fold higher than aptamers N54 and N56. Both of these peptide sequences are found on the CFG face of CEA (Taheri et al., 2000). Interestingly, the peptide NRQII corresponding to residues 42 to 46 is unique to CEA while the sequence QNDTG is found in CEACAM1 and CEACAM6. In view of the specificity of aptamers N54 and N56 in binding to CEA, it would suggest that these aptamers might interact with residues in the vicinity of peptide NRQII on the CFG face of the CEA N domain.

One issue facing the use DNA aptamers as therapeutics is their short half-life *in vivo* due to nuclease degradation and their rapid clearance through the kidneys (based on their low molecular weights). The circulation half-lives of aptamers can be increased by conjugating them to polyethylene glycol (PEG) moieties. As well, the stability in serum can be increased by substituting nucleotides with modifications of either sugar residues (eg. 2’ OH group with 2’-amino, 2’-fluoro 2’-O-methyl), the phosphate (eg. Phosphorothioate) or of the base (2’-thiopyrimidine, methyl or trifluoromethyl) (Healy et al., 2004; Latham et al., 1994; Mayer, 2009; Schoetzau et al., 2003; Willis et al., 1998).

Also, Locked Nucleic acids (LNA) have been introduced within their structures to increase stability (Barciszewski et al., 2009). However, the *in vivo* tumour implantation mouse model used in this study to assess the ability of aptamers N54 and N56 to inhibit the adhesion and proliferation of murine MC38.CEA cells in the peritoneal cavity of mice were performed with no modification to the aptamer structure. Both of these aptamers were effective in inhibiting tumour implantation in the peritoneal model (Figure 3.6B). Interestingly, an RNA aptamer to CEA has recently been reported that prevents hepatic metastasis (Lee et al., 2012). However, this RNA aptamer differs from the present DNA apamers N54 and N56 in two key aspects. First, this RNA aptamer prevents the
binding of CEA to death receptor 5 (DR5), thus contributing to the prevention hepatic metastasis by inducing anoikis. In contrast, aptamers N54 and N56 are non-cytotoxic towards MC38.CEA cells and the treatment of MC38.CEA cells with aptamers N54 and N56 did not lead to growth arrest/reduction (SRB cell viability assay) in relation to untreated cells (Figure 3.7A). Secondly, the reported RNA aptamer to CEA was shown to bind the PELPK motif present at residues 108-122 of CEA. This motif is present on CEACAM1 and CEACAM 6: two broadly-expressed CEACAMs on normal tissues, suggesting that this aptamer may not be specific for CEA (possible off-target effects). In contrast, aptamers N54 and N56 showed specific binding to CEA suggesting that their anti-adhesive effects focus on the specific inhibition of homotypic CEA interactions (Figure 3.4). Importantly, the murine cell line MC38.CEA used for both in vitro and in vivo studies, does not express other human CEACAM members on its surface. In view of the specificity of aptamer N54 and N56 for the N domain of CEA only, these aptamers are not expected to inhibit possible homophilic cellular interactions involving other CEACAM members present on tumour cells.

Finally, DNA aptamers are generally considered to be non-immunogenic (Foy et al., 2007; Yu et al., 2009). However, to confirm that aptamer N54, as an example, did not generate an inflammatory innate immune response as seen for CpG ODN (a ligand for TLR-9), mice were given via an intraperitoneal injection, a bolus of either an irrelevant aptamer (cApt), aptamer N54 or a positive control CpG ODN, and their sera analyzed after 3 hours for the production of inflammatory cytokines IL-8 and TNFα (Figure 3.7B and C). As projected, aptamer N54 did not generate a serum increase in CpG ODN-associated cytokines suggesting that this aptamer is non-immunogenic.

In summary, this study reports the identification of two DNA aptamers that are
able to specifically recognize the N domain of the cancer-associated antigen CEA and block its homophilic adhesive properties. These aptamers specifically bound to the IgV-like N domain of CEA, with a dissociation constant in the nanomolar range and significantly inhibited tumour implantation of murine MC38.CEA cells by virtue of their antiadhesive properties. As well, aptamer N54 displayed no cytotoxicity towards MC38.CEA cells and did not trigger a TLR-9 dependent innate immune response.
Chapter 4

Aptamer-targeted liposomes for the computed tomography (CT) imaging of breast cancer tumours

Erik W. Orava, Michael Dunne, Jinzi Zheng, Huang Huang, Christine Allen, Jean Gariépy

Author Contributions:
Erik Orava, Dr. Jean Gariépy and Dr. Christine Allen designed experiments, analyzed the data and prepared a manuscript.

Erik Orava contributed Figure 4.1, 4.2, 4.3, 4.4

Michael Dunne and Dr. Jinzi Zheng helped prepare the liposomes used in this study as well as perform the animal imaging study at STTARR (Table 4.1). Huang Huang was involved in preliminary experiments in characterizing aptamer liposome conjugation.
4.0. Abstract

The *in vivo* targeting of nanoparticles such as pegylated liposomes to tumour cells can be enhanced by introducing tumour marker-specific DNA aptamers on their surface. We have recently generated DNA aptamers that specifically recognize either the mucin MUC1 protein tandem repeat (40-base long MUC1-VR) or the N domain of the carcinoembryonic antigen CEA (75-base long N54), both markers being aberrantly and abundantly expressed on the surface of a broad range of epithelial cancer cells (breast, colon, lung, ovarian, pancreatic and prostate). These DNA aptamers were conjugated via an amino group to cyanur-PEG2000-DPSE and incorporated into preformed liposomes (~50 aptamers/liposome) containing the contrast agent Omnipaque 350™. The level of incorporation of aptamer was quantified using Cy5-labeled aptamers and found to be ~95%. MUC1 and CEA surface antigens are internalized by cancer cells at different rates. We report that a previously developed and characterized multimodal pegylated liposomes loaded with the contrast agent for use in computed tomography (CT) imaging modified to display these aptamers can either increase the binding to cells and internalization. *In vivo* studies in mice have shown that the incorporation of these aptamers on the liposome surface does not significantly alter the pharmacokinetic profile of these nanoparticles. Current studies are underway to determine the extent of tumour accumulation and internalization of the MUC1 and CEA targeted liposomes using an MDA MB 231 orthotopic tumour model.
4.1. Introduction

A number of approaches are being employed to increase the selective toxicity of anticancer agents. The discovery and application of single pathway-based cancer-targeted drugs have showed some clinical promise, although their overall antitumour efficacy being often temporary (Hanahan and Weinberg, 2011). One approach that is being extensively investigated is the identification and use of interactome-based drugs to interfere with newly identified cancer specific pathways (Roukos, 2012). Multimodal, nanomaterial-based formulations offer the potential of delivering anticancer drugs and contrast agents in a more directed fashion for guided in vivo imaging and therapeutic approaches (Huang et al., 2012).

In theory, the best approach to improve the quality of life in patients with cancer is to detect diseases as early as possible so that the timely initiation of therapy can maximize the efficacy of the treatment. In the past decade, the field of nanomaterial-based technologies has made fundamental advancements in the detection of diseases in early stages as well as the delivery of drugs (Ryu et al., 2012; Taruttis and Ntziachristos, 2012). Magnetic nanoparticles incorporating inorganic nanocrystals as their magnetic cores and composed of metals, alloys and metal oxides have been reported (Laurent et al., 2008). The surfaces of these nanoparticles have also been modified with layers of organic polymer or inorganic metal or oxide surfaces making them suitable for the conjugation to biomolecules (Berry et al., 2003) with the resulting nanoparticles being used for magnetic resonance imaging (MRI) and drug delivery (Fang and Zhang, 2009; Veiseh et al., 2010). Metal nanoparticles have also been used by generating gold and silver nanoparticles, which display excellent plasmonic properties advancing the use of surface-enhanced Raman-spectroscopy (Ando et al., 2011; Sathuluri et al., 2011). Silica-
based nanoparticles have also been useful for encapsulating proteins such as the green fluorescence protein (Cai et al., 2011) for cellular applications, in light of their nonporous structure which protects GFP in terms of photo-stability and their versatile surface for conjugating targeting moieties (Qhobosheane et al., 2001; Wittenberg and Haynes, 2009).

Hydrogels represent another category of nanomaterials composed of crosslinked polymer structures that can retain large amounts of water or biological fluids within their matrix and have found numerous medical and pharmaceutical applications due to their ability to provide a slow controlled release of their cargo (Kashyap et al., 2005; Miyata et al., 1999). Carbon-based nanomaterials, such as carbon nanotubes, fullerenes and recently graphene and graphene oxide have shown promise in the field of cellular imaging and drug delivery (Hong et al., 2012; Liu et al., 2011; Peng et al., 2010). More recently, studies have shown these compounds are toxic at higher concentrations as MTT assays failed to predict toxicity of graphene oxide and graphene sheets because of the reduction of MTT by graphene resulting in a false positive signal (Liao et al., 2011). These nanomaterials have been investigated due to their beneficial characteristics such as their high surface area, mechanical strength, electrical and thermal conductivity and photoluminescence. As such they have shown promise as sensors but also for imaging applications (Ajayan, 1999).

Liposomes have had the greatest clinical impact as they have been used as a drug delivery system for over 40 years (Bangham et al., 1965). These lipid-based nanomaterials allow for retention of their cargo until they reach their intended target organ. They can be modified with the addition of polyethylene glycol (PEG), to enhance their circulation time (Gregoriadis, 1995) and will accumulate at newly vascularized tumour sites as a consequence of the enhanced permeability and retention (EPR) effect.
(Maeda, 2001). At least twelve liposome-based drugs have now been approved for clinical use the US Food and Drug Administration (FDA), four of which are for the treatment of cancers and over twenty more liposomal formulations are currently in clinical trials (Chang and Yeh, 2012). Importantly, antibodies, peptides, aptamers and small molecular weight ligands have been introduced on the surface of nanoparticles to target them to diseased tissues (Kumar et al., 2012).

In the present study, we report the incorporation of aptamer-lipid conjugates into pegylated liposomes loaded with a contrast agent with a view to guide their delivery and imaging of MDA-MB-231 tumour xenografts implanted in athymic CD-1 mice (Figure 4.1). Aptamers are short single-stranded DNA or RNA oligonucleotides selected using a PCR-based *in vitro* iterative process termed SELEX (Ellington and Szostak, 1990; Tuerk and Gold, 1990). The identified aptamers bind to their target with high specificity and affinity. DNA aptamers have been derived that can behave as sensors, therapeutics, regulators of cellular processes or as cellular delivery agents (Holthoff and Bright, 2007; Kaur and Roy, 2008; Orava et al., 2010; Toulme et al., 2004). DNA aptamers, unlike antibodies, are not immunogenic, are easy to synthesize and to couple to nanoparticles, and can be reversibly denatured (Yang et al., 2011). There are currently a dozen aptamers in clinical trials with the anti-VEGF aptamer Pegaptanib (Macugen) approved since 2004 for age-related macular degeneration (Bunka et al., 2010; D'Amico et al., 2006). Aptamer-functionalized nanomaterials are now being investigated in multiple fields of research (Xing et al., 2012; Yang et al., 2011).
Figure 4.1. Proposed in vivo routing of mucin MUC1 directed DNA aptamers coupled to pegylated liposomes filled with the CT contrast agent Omnipaque 350. The liposomes accumulate within the interstitium of a solid tumour by virtue of the Enhanced Permeability and Retention (EPR) effect. The aptamer-decorated liposomes are then subsequently internalized into MUC1-expressing tumour cells (modified from Peer et al. 2007).
The DNA aptamers N54 and MUC1-VR used in the present study were respectively derived against two established tumour markers, namely the carcinoembryonic antigen (CEA) and the mucin MUC1. CEA is a 180 kDa GPI-linked cell glycoprotein that is also a member of the immunoglobulin cell adhesion molecule superfamily (CEACAMs) (Gold and Freedman, 1965a). It is aberrantly over-expressed on the surface of colon, breast, lung and other epithelial cancer cells (Hammarstrom, 1999). Antibodies against CEA are slowly internalized by CEA-expressing cells at a rate comparable to their metabolic turnover rate suggesting that CEA may be a suitable target for tumour targeting (Schmidt et al., 2008). The derivation of the CEA-specific aptamer N54 was described in Chapter 3. In contrast to CEA, the mucin MUC1 membrane glycoprotein is highly expressed and aberrantly glycosylated in more than 90% of primary and metastatic breast cancers (McGuckin et al., 1995) with mucin MUC1 underglycosylated forms such as the Tn antigen being rapidly endocytosed by cells (Altschuler et al., 2000). Our group has previously identified aptamers to the Tn antigen of MUC1 (Ferreira et al., 2009). A second generation, 40-base long DNA aptamer MUC1-VR1 was derived for the present study.

4.2. Materials and Methods

4.2.1. MUC1 expression, purification and glycosylation

A recombinant mucin MUC1 peptide composed of five 20-residue long MUC1 tandem repeats was expressed in *E.coli* and purified to homogeneity by Ni-NTA chromatography as previously reported (Brokx et al., 2003). The purity and sequence of the MUC1 peptide were confirmed by SDS-PAGE and mass spectrometry. The peptide was stored at
-20°C. Fifteen GalNac sugars (Tn antigens) were enzymatically introduced on the hydroxyl side chain group of serines and threonines within each MUC1 tandem repeat using a recombinant secreted form of the human glycosyltransferase ppGalNAc-T1 expressed in *Pichia pastoris* as previously described (Brokx et al, 2003). The level of glycosylation was confirmed by SDS PAGE and mass spectrometry (Appendix 6).

### 4.2.2. Identification of MUC1 binding aptamers using the SELEX process

The initial ssDNA library contained a central randomized sequence of 40 nucleotides flanked by two primer regions with the sequence 5’ GAC GAT AGC GGT GAC GGC ACA GAC G-(25N)-CGT ATG CCG CTT CCG TCC GTC GCT C 3’. The forward primer 5’ GAC GAT AGC GGT GAC GGC ACA GAC G 3’ and reverse primer 5’ GAG CGA CGG ACG GAA GCG GCA TAC G 3’ were used for selection and cloning (IDT Technologies, Inc.). A 50 nmol aliquot of the library was first counter-selected against Ni-NTA agarose beads prior to selection against the GalNAc-modified MUC1 peptide (Tn antigen) in order to remove DNA species that non-specifically bound to Ni-NTA agarose itself. The resulting sub-library was then exposed to 5 µg of His-tagged glycosylated MUC1 peptide dissolved in 200 µl of PBS, 10 µg/ml salmon sperm DNA and 10 µg/ml BSA (selection buffer) for 30 min then added to a column containing 200 µl of Ni-NTA agarose beads (Qiagen Inc.). Unbound DNA sequences were washed away with a 10-fold column volume excess of the selection buffer. DNA-protein complexes were eluted from the beads with selection buffer containing 240mM imidazole. The ssDNA component was precipitated with sodium perchlorate/isopropanol and recaptured using a silica membrane-based purification system (Qiagen Inc., Mississauga, Ontario). The DNA aptamers were then amplified by asymmetrical PCR using a 10-fold excess of
forward primer. After every second round of selection, the amount of target (Tn antigen MUC1 peptide) was reduced in half to capture the tightest binding aptamers against the MUC1 target. The bound sequences were amplified by PCR after 10 rounds of selection to produce double stranded products, cloned into a pCR4-TOPO TA vector (*Invitrogen*) and sequences were analyzed using BioEdit sequence alignment editor software (Ibis Therapeutics, Carlsbad, USA).

**4.2.3. MUC1 Aptamer binding and internalization**

To determine if the identified MUC1 aptamers were able to specifically bind MUC1 on MUC1⁺ cells, FITC-labeled MUC1-VR1, MUC1-VR4, MUC1-VR7 and MUC1-VR were synthesized (IDT technologies) and incubated for 1 hour in 1 ml of DMEM + 10% FBS with the human breast cancer cell line (MUC1⁺) MDA-MB-231 (Gilbey et al., 2004; McGuckin et al., 1995) or the MUC1⁻ human cell line HeLa at a density of 10⁶ cells/ml at 4°C or 37°C with or without the addition of trypan blue to quench aptamer fluorescence. Cells were subsequently placed on ice, washed three times with cold PBS (-MgCl₂/-CaCl₂) and analyzed by flow cytometry for the binding of fluorescent MUC1 aptamers (FACScan, BD Biosciences, Franklin Lakes, NJ). Experiments were repeated in triplicate and averages were reported as mean fluorescence intensity ± S.E.M.

**4.2.4. Binding kinetics of MUC1 aptamers determined by surface plasmon resonance**

All experiments were performed using the ProteOn XPR36 surface plasmon resonance instrument (Bio-Rad Laboratories, Inc.). The amine reactive ProteOn GLC sensor chip was washed and activated according to manufacturer’s protocols. MUC1 and
glycosylated MUC1 peptides were diluted to 100 µg/ml in PBS-T (0.05% (v/v) Tween-20), pH 7.4 and injected for 120 seconds at 30 µl/min. After the loading phase of protein ~100 and 150 RUs of protein was bound respectively. Control aptamer (cApt) used for subsequent experiments was a 40 base sequence of AGTC repeats, 5’ AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG TCA GTC AGT C 3’. Subsequently aptamers MUC1-VR1, MUC1-FL1 and cApt were diluted as a series of two-fold dilutions ranging from 2µM to 3.9nM in PBS-T (0.05% (v/v) Tween-20) pH 7.4. Aptamer concentrations and a buffer control were injected in the analyte channel with a contact time of 200 sec, dissociation time of 800 sec, and a flow rate of 100 µl/min. The ligand channels were regenerated with a 30 sec injection of 1M H₃PO₄ followed by a 30 sec injection of 1 M NaCl. All experiments were performed at 25°C and repeated in duplicate. Sensorgrams were then double-referenced by subtracting the buffer response and using the interspot reference. The sensorgrams were fitted globally to a 1:1 Langmuir binding model and the kinetic parameters for the association (kₐ), dissociation rates (kₐ), and binding constant (Kᵃ) were derived from the fitted curves (MUC1-VR1 and MUC1-FL1 fitted to MUC1 and glycosylated MUC1 had χ² > 0.93).

4.2.5. **Synthesis of Aptamer-PEG₂₀₀₀-DSPE conjugates**

Aptamers were synthesized with a C6 spacer 5’ Amino group (IDT Technologies) and incubated in a 1:1 molar ratio with a cyanur-PEG₂₀₀₀-DSPE (Avanti Polar Lipids) in 0.1M boric acid (pH 8.8) for 24 hours. Unconjugated aptamer was separated by dialysis (MWCO 13 000) in 0.01M HEPES buffer (pH 7.4). The concentration of DNA in the samples was measured at 260/280 nm using a nanodrop spectrophotometer. The coupling yield of aptamers to cyanur-PEG₂₀₀₀-DSPE was 80%.
4.2.6. Liposome preparation

The liposomes were prepared as previously described (Zheng et al., 2006). Briefly, DPPC, cholesterol, and DSPE-PEG$_{2000}$ were dissolved in ethanol (250mM) at 70°C at a percent molar ratio of 55:40:5 DPPC:CH:DSPE-PEG$_{2000}$. Omnipaque® (300mg/ml of Iodine, GE Healthcare, Mississauga, ON) was added to yield a final lipid concentration of 100 mM after ethanol removal and the mixture was kept at 70°C for 6 h with intermittent vortexing. The resulting multilamellar vesicles were then extruded at 70°C through a 10-mL Lipex Extruder (Northern Lipids Inc, Vancouver, British Columbia, Canada) with five passages through two stacked 200 nm pore size polycarbonate filters and five passages through two stacked 80 nm polycarbonate filters (Nucleopore; Track-Etch Membrane, Whatman Inc., Clifton, NJ). Unencapsulated contrast agent was removed by dialysis overnight (MWCO 8000) against HEPES buffered saline (HBS, pH 7.4). The liposome formulation was then concentrated to a final iodine concentration of 40mg/mL using a MidJet membrane separation system with a 750,000 NMWC MidGee cross flow cartridge (GE Healthcare, Mississauga, ON). Targeted and untargeted liposomes were generated by adding a specific aptamer-PEG$_{2000}$-DSPE conjugate (~2.5mg/250mM of lipid resulting in ~50 aptamers per liposome assuming a homogenous size distribution) or a control PEG$_{2000}$-DSPE conjugate to a liposome suspension and each mixture was stirred at 37°C for 5 hours following by stirring at room temperature for 18 hours.
4.2.7. Efficiency of aptamer-DSPE-PEG insertion into liposomes

Aptamers were synthesized with a 5’ Cy5 fluorophore and a 3’ amino group (IDT Technologies) and conjugated to cyanur-PEG\textsubscript{2000}-DPSE as previously described. The fluorescence signal of Cy5-labeled aptamer-PEG\textsubscript{2000}-DPSE solutions (excitation 625 nm; emission 670 nm) was measured prior to adding Cy5-aptamer-PEG\textsubscript{2000}-DPSE (from 30 µg to 2mg) to 25 mM of lipids for 30 minutes at 37°C then overnight at room temperature (Ishida et al., 1999; Moreira et al., 2002). Unincorporated Cy5-aptamer-PEG\textsubscript{2000}-DPSE conjugates were removed by dialysis (MWCO 30,000) in 0.01M HEPES buffer (pH 7.4). The fluorescence signal of aliquots from each liposome formulation was then measured and compared to our standard curve. Close to 95% of each evaluated Cy5-aptamer-PEG\textsubscript{2000}-DPSE conjugate was incorporated into liposomes. Assuming that a 100 nm diameter \textit{d} liposome contains ~80,000 lipids, the total number of lipids \( N_{\text{tot}} \) in solution was calculated as follows \( N_{\text{tot}} = 17.69 \times [ (d/2)^2 + (d/2 - 5)^2 ] \) (Pidgeon and Hunt, 1981) with the number of liposomes \( N_{\text{lipo}} \) estimated to be equal to \( (M_{\text{lipid}} \times N_A) / (N_{\text{tot}} \times 1000) \) wherein \( M_{\text{lipid}} \) is the molar concentration of lipid and \( N_A \) is Avogadro’s number (6.02×10\textsuperscript{23} mol\textsuperscript{-1}). The estimated average number of aptamers per liposome was then calculated by dividing the total number of aptamers by the total number of liposomes assuming a homogeneous distribution of the aptamers on the liposomes and the near complete integration of the Cy5-aptamer-PEG\textsubscript{2000}-DPSE conjugate added (as discussed above).

4.2.8. Aptamer-liposome characterization \textit{in vitro}

The human breast cancer cell lines MCF-7 and MDA-MB-231 as well as the human cervical adenocarcinoma cell line HeLa, murine fibroblast cell line L929 and rat
gliosarcoma cell line 9L were cultured at 37°C, 5.0% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and dihydrostreptomycin (100 µg/mL). MDA-MB-231 or HeLa cells at a final density of 10⁷ cells/ml in 100 µl of DMEM were treated to a final concentration of 20 µM of either Tn antigen-directed, CEA-directed, cApt-directed aptamer-liposomes or non-targeted liposomes for 1 hour at 4°C to reduce internalization. The cells were then washed three times with cold PBS and analyzed by flow cytometry. For aptamer-liposome concentration studies, MDA-MB-231 and HeLa cells at a final density of 10⁷ cells/ml in 100 µl of DMEM, were exposed to increasing two-fold concentrations (from 5-80 µM) of aptamer-liposomes displaying ~50 aptamers per liposome or to non-targeted liposomes for 1 hour at 4°C.

For internalization studies, MDA-MB-231 or HeLa cells [10⁷ cells/ml in 100 µl DMEM] were incubated with 20 µM aptamer-liposomes (~50 aptamers/liposome) or non-targeted liposomes at 4°C or 37°C, for 0.5, 1, 2, 4, 8 and 24 hours. All studies were repeated in triplicate and averages are represented as mean fluorescence intensity ± S.E.M.

4.2.9. Physio-chemical characterization of aptamer-liposome formulations

The hydrodynamic particle diameter and zeta potential of aptamer-liposome formulations as well as non-targeted liposomes were determined by dynamic light scattering (DLS) using a Zetasizer nano ZS (Malvern Instruments Ltd.).
4.2.10. Aptamer-liposome pharmacokinetic profile

The liposome preparations (0.35g/kg iodine and 1.8g/kg lipid) were injected into athymic female CD-1 mice via the tail vein. The animals were subsequently anaesthetized using 2% isoflurane and full body, 16-second anatomical micro-CT scans (GE Locus Ultra micro-CT, GE Healthcare, Waunakee, WI) were recorded prior to liposome administration and at 5 min, 2h, 4h, 8h, 24h, 48h and 72 hours post-injection intervals. CT images were acquired at 80kVp and 70mA with a voxel size of 0.15mm x 0.15mm x 0.15mm and a field of view of 0.57cm x 0.57cm x 10.5cm.

CT images were analyzed using MicroView v2.2 (GE Healthcare, Waunakee, WI). Briefly, the mean iodine concentrations in selected organs were determined through contouring the desired region on multiple 2D slices to generate a 3D volume and calculating the mean voxel intensity (expressed in CT attenuation Hounsfield Units, or HU) for each volume. Iodine concentrations in blood were determined by contouring the aorta.

4.2.11. Statistical methods and data analysis

Data sets derived from individual groups of mice were compared using student’s t-test and grouped data sets were analyzed by ANOVA. Statistical analyses and graphs were assembled using GraphPad PRISM (version 5.01, GraphPad software for Science, San Diego, CA). P values ≤ 0.05 were considered significant unless otherwise indicated. Flow cytometry statistics were analyzed using WinMDI (version 2.8, Windows multiple document interface for flow cytometry, Scripps Research Institute).
4.3. Results

4.3.1. Identification and characterization of MUC1-VR1, a MUC1-binding aptamer

The target used to identify MUC1-binding DNA aptamers generated using a SELEX approach, was a recombinant MUC1 peptide corresponding to five consecutive 20-residue long tandem repeats of the mucin MUC1 protein extracellular domain. This peptide was enzymatically modified with the human glycosyltransferase ppGalNAc-T1 to harbor up to 15 GalNAc sugars known as Tn antigens (Brokx et al. 2006). Forty recovered oligonucleotide inserts from the SELEX search were sequenced yielding 37 DNA aptamer sequences that could be regrouped into 4 unique sequences (Figure 4.2A). In particular, MUC1-VR1 (40-base long variable region) and MUC1-FL1 (primers and the same 40-base long variable region) accounted for 86% of identified aptamer sequences. All 4 DNA aptamer sequences were then synthesized with a 5’ biotin group to confirm their binding to the MUC1 Tn antigen using a modified ELISA protocol (Appendix 7) (Orava et al., 2012). The ELISA binding assay confirmed that of the 4 identified sequences, 3 of the 40-base aptamers specifically bound to the Tn antigen MUC1 peptide, namely MUC1-VR1, MUC1-VR4 and MUC1-7.

The ability and specificity of these aptamers to bind to Tn antigen-containing mucin MUC1 on the surface of cancer cells was subsequently assessed using FITC-labeled MUC1 aptamers as well as the FITC-labeled CEA-binding aptamer N54 (5’ GAC GAT AGC GGT GAC GGC ACA GAC GTC CCG CAT CCT CCG CCG TGC CGA CCC GTA TGC CGC TTC CGT CCG TCG CTC 3’; Orava et al. 2013). The MUC1-VR1, -VR4 and -VR7 aptamers all bound to the CEA+/MUC1+ cell lines MDA MB 231 (Gilbey et al., 2004; McGuckin et al., 1995) and MCF-7 while none of these fluorescent aptamers...
recognized CEA\(^{-}/MUC1\(^{-}\) cell lines 9L, L929 and HeLa (Appendix 8 and 9). MUC1 expression on cells was confirmed using the anti-MUC1 M23 antibody (Linsley et al., 1988) and a FITC-labeled mouse anti-human secondary antibody (Appendix 10). Although MUC1-VR1, MUC1-VR4 and MUC1-7 bound to Tn antigen MUC1 peptide using ELISA-based method and flow cytometry showed binding to MUC1\(^{+}\) cells, only MUC1-VR1 and its full length MUC1-FL1 homolog showed binding to the MUC1 peptide and its Tn antigen MUC1 variant by SPR. The binding constant of MUC1-VR and MUC1-FL aptamers were determined by surface plasmon resonance by coupling unglycosylated MUC1 peptide and the Tn antigen-containing MUC1 peptide variant to an amine reactive chip. Of the three aptamers tested, aptamer MUC1-VR1 showed no significant difference in binding affinity between the MUC1 peptide and Tn antigen form (410 ± 130 nM and 380 ± 90 nM). Interestingly, the MUC1-FL1 aptamer was ~7-more avid to the MUC1 peptide relative to the Tn antigen-labeled MUC1 peptide (230 ± 60 nM vs. 1600 ± 400 nM) (Figure 4.2 B). Since there was no significant difference between the binding constants of MUC1-VR1 and MUC1-FL1 to the Tn antigen-labeled MUC1 peptide we chose to continue our studies using the smaller shorter MUC1-VR1 aptamer.
Figure 4.2. Identification of MUC1-specific DNA aptamers and the ability of MUC1+ MDA-MB-231 cells to internalize FITC-labeled aptamer MUC1-VR1. (A) DNA aptamers identified from the SELEX screen against a Tn antigen-modified MUC1 peptide and their frequency of occurrence in the final selection pool. (B) Calculated dissociation constants ($K_D$) for MUC1-FL1 and MUC1-VR1 binding to the glycosylated and unglycosylated form of the MUC1 peptide as determined using surface plasmon resonance. (C) Mean fluorescence intensities of FITC-labeled MUC1-VR1 bound to cells at 4°C after one hour then treated with trypan blue (TB) showing a reduction in fluorescence intensity down to the background fluorescence signal of the control aptamer cApt suggesting that the aptamer MUC1-VR1 is not internalized by cells at 4°C. (D) Mean fluorescence intensities of FITC-labeled MUC1-VR1 bound to cells after one hour at 37°C then treated with trypan blue indicating that this aptamer is rapidly taken up by MDA MB-231 cells at 37°C. Each bar represents the average + SEM (n=3). P-value $>0.05$ using a Welch corrected two-tail unpaired t-test was considered non-significant.
The internalization of FITC-labeled MUC1-VR1 aptamer into cells was measured by FACS analysis at 37°C (surface binding and internalized components) and 4°C (surface binding only), after a 1-hour incubation period. The mean fluorescence intensities (MFI) from aptamer bound to the surface of cells was quenched with trypan blue prior to FACS experiments. Cells treated with trypan blue at 4°C showed a significant decrease in fluorescence signal when compared to MUC1-VR1 with no quenching but no such difference in MFIs was observed for the control FITC-labeled aptamer cApt which does not bind the MDA MB 231. Importantly, cells treated with trypan blue after binding and internalization at 37°C showed no significant decrease in MFI when compared to cells treated with MUC1-VR1 alone suggesting that the aptamer was being internalized into target cells.

4.3.2. Optimization of MUC1-VR1 and N54 aptamer-targeted liposomes in vitro

Cy5-labeled MUC1-VR1, N54 and cApt (control) aptamers were conjugated via their 3’ end amino group to cyanur-PEG2000-DPSE. The resulting conjugates were incorporated into preformed pegylated liposomes. Greater than 95% of the Cy5-labeled aptamer-PEG lipid constructs were inserted into liposomes up to a density of 320 aptamers per liposome (Appendix 11) (Moreira et al., 2002). As well, the binding of Cy-5 labeled aptamer N54 was confirmed by FACS on MCF-7 and MDA-MB-231 cells before incorporation (Appendix 12). At the lowest aptamer concentration tested (10 aptamers per liposome) there was ~2-fold increase in MFIs of MDA-MB-231 cells treated with MUC1-VR1 and N54 as compared to the control aptamer (Figure 4.3A). At a concentration of ~40 aptamers per liposome, MUC1-VR1- and N54-modified liposomes respectively showed a 7-fold and 5-fold increase in cell surface-associated fluorescence.
intensities as compared to cApt liposomes.

The effect of aptamer-liposomes concentration on cell surface association to MDA-MB-231 and HeLa cells at 4°C was monitored by FACS analysis using a loading of 50 Cy5-labeled aptamers per liposome. At the lowest concentration tested of 5 µM, the MUC1-VR1 and N54 liposomes showed ~3-fold and 2.5-fold increase in MFI as compared to the cApt and non-targeted liposomes on CEA+/MUC1+ MDA-MB-231 cells (Figure 4.3C). Interestingly, at concentrations at or above 20 µM, there was a significant increase in MUC1-VR1 liposomes binding (MFI) to CEA+/MUC1+ MDA-MB-231 cells as compared to the N54-tagged liposomes. As expected, no significant difference in MFI was observed between aptamer groups on CEA-/MUC1+ HeLa cells although a small increase in nonspecific binding (MFI) occurred for non-targeted liposomes at concentrations ≥ 40 µM (Figure 4.3D).
Figure 4.3. Liposome internalization into CEA+/MUC1+ MDA-MB-231 cells is dependent on the number of DNA aptamers present on the surface of liposomes. (A) Increasing amounts of Cy5-labeled MUC1-VR1 aptamer (triangles), CEA N54 aptamer (inverted triangles) or control aptamer cApt (squares) were incorporated into pegylated liposomes. Mean fluorescence intensities of liposomes bound to the cell surface of MDA-MB-231 cells after a 1-hour exposure at 4°C were measured by FACS analysis. (B) Lack of binding of targeted and untargeted liposomes to MUC1-/CEA- HeLa cells. (C) Increasing concentrations of aptamer targeted Cy5-liposomes or non-targeted (circles) bound to (C) CEA+/MUC1+ MDA-MB-231 or (D) CEA-/MUC1- HeLa cells after a 1-hour incubation period at 4°C. (E) Physicochemical properties of targeted and non-targeted liposome formulations.
**4.3.3. Aptamer-Liposome internalization in MDA-MB-231 cells**

Cellular uptake and recycling of surface markers takes place at 37°C. The effect of temperature on the binding and cellular uptake of Cy5-labeled MUC1-VR1, N54 or cApt-tagged aptamer-liposomes into MDA-MB-231 and HeLa cells was monitored by FACS analysis at 4°C (surface binding) and 37°C (binding and internalization) using a loading of 50 Cy5-labeled aptamers per liposome. At 4°C, both N54- and MUC1-VR1 liposomes reached a maximum fluorescence signal within the first 2 hours of incubation with MDA-MB-231 cells with no significant difference in MFI being observed after a 24-hour incubation period (Figure 4.4A). The same aptamer liposomes did not significantly bind to MUC1/CEA- HeLa cells even after 24 hours (Figure 4.4C). At 37°C, fluorescence signals associated with both N54- and MUC1-VR1 liposomes increase with time although the uptake of MUC1-VR1 was significantly more rapid reaching a plateau at 4 hours as opposed to the CEA N54-labeled liposome preparation which led to increased MFI values only after 24 hours (Figure 4.4B). Their uptake levels into HeLa cells at 37°C were comparable to that of untargeted liposomes (Figure 4.4D). Although comparable to fluorescence values observed at 4°C, the nonspecific uptake of cApt-labeled or untargeted liposomes by MDA-MB-231 and HeLa cells showed a slight increase in MFI values with time at 37°C (Panels 4.4B and 4.4D).
Figure 4.4. Binding and internalization of targeted liposome formulations in vitro. (A) Mean fluorescence intensities (MFI) of CEA+i/MUC1+ MDA-MB-231 cells maintained at 4°C and treated with either MUC1-VR1-liposomes (triangles), CEA N54-liposomes (inverted triangles), cApt-liposomes (squares) or non-targeted liposomes (circles) reached a plateau after 2 hours (B) MDA-MB-231 cells incubated at 37°C display a pronounced increase in MFI (internalization event) as a function of time when treated with Cy5-labeled MUC1 and CEA liposome preparations when compared to MFI values recorded at 4°C. CEA+i/MUC1− HeLa cells treated with the same liposome formulations at (C) 4°C and (D) 37°C showed no significant increase in MFI.
4.3.4. Aptamer-Liposome physicochemical properties and stability in vivo

Liposome preparations formulated with aptamers were analyzed by light scattering [Zetasizer] to define their hydrodynamic diameter and zeta-potential in relation to non-targeted liposome formulations. The incorporation of MUC1-VR1 and N54 into liposomes resulted in ~10% and ~20% larger liposome diameter as expected from the fact that the CEA aptamer N54 has 35 more bases than the 40-base long MUC1-VR1 aptamer (Figure 4.3E). The incorporation of aptamers decreased the surface potential of the liposomes by ~10% as compared to non-targeted liposomes although these changes were not statistically significant.

To determine whether the incorporation of aptamers MUC1-VR1, N54 and cApt would significantly affect the stability of the liposomes, preparations were injected via the tail vein into CD1 mice and imaged at time intervals of 0, 0.5, 1, 2, 4, 8, 24, 48 and 72 hours. Images were examined at the different time points and the descending aorta was contoured to determine the amount of liposomes in the blood by quantifying the amount of contrast agent. The results showed that there was no significant difference in the elimination half-life of the aptamer-liposomes compared to the non-targeted formulation suggesting that the incorporation of the DNA aptamers did not alter the stability of the liposomes (student’s t-test, p>0.05) (Table 4.1).

Table 0.1. Pharmacokinetics of different Omnipoque encapsulated non-targeted and aptamer targeted liposome preparations in CD1 mice (n=5 per group).

<table>
<thead>
<tr>
<th>Liposome Type</th>
<th>T$_{1/2}$ (h) in blood</th>
<th># Aptamers per liposome</th>
<th>APTAMER SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-targeted Liposome</td>
<td>39.10 ± 6.22</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>cApt Liposome</td>
<td>38.44 ± 4.03</td>
<td>~50±10</td>
<td>40 b (~13 kDa)</td>
</tr>
<tr>
<td>MUC1 Liposome</td>
<td>35.90 ± 2.35</td>
<td>~50±10</td>
<td>40 b (~13 kDa)</td>
</tr>
<tr>
<td>CEA Liposome</td>
<td>37.69 ± 3.12</td>
<td>~50±10</td>
<td>75 b (~24.6kDa)</td>
</tr>
</tbody>
</table>
4.4. Discussion

The targeting of nanoparticles and nanomaterials with aptamers offers the potential to increase their accumulation at tumour sites by binding specifically to unique cancer markers.

For instance, the aptamer TTA1 directed at Tenascin-C, an extracellular matrix protein that is expressed during tissue remodeling and notably tumour growth was radiolabeled with $^{99}$M-Tc and reported to display good tumour penetration and a high tumour-to-blood ratio most likely due to its rapid clearance (Hicke et al., 2006). Aptamers have recently been derived to the mucin MUC1 protein (Ferreira et al., 2009); a well-known tumour marker that is overexpressed and underglycosylated on epithelial cancers. Studies have shown that MUC1-directed aptamers are capable of delivering cytotoxic agents or as targeting agents in the context of PLGA nanoparticles in vitro (Hu et al., 2012; Yu et al., 2011). The biodistribution of a PEGylated MUC1 aptamer as well as their ability to target quantum-dot-doxorubicin conjugates in vivo studies have also been reported (Da Pieve et al., 2012; Savla et al., 2011). To date, no studies have shown the utility of an aptamer recognizing CEA as a targeting agent.

In the present studies, we show that a loading of >80 MUC1-VR1 or CEA N54 aptamers per liposome is not essential for the maximal in vitro uptake of such formulations into the CEA+/MUC1+ human breast cancer MDA-MB-231 cells (Figure 4.3A). This level of aptamer loading is comparable to the density of 34-60 VEGF aptamers per liposome for 50 nm nanoparticles reported in the past (Willis et al., 1998) but less than the loading of 500 aptamers per liposome required to achieve a pronounced targeting effect for an aptamer directed at E-selectin (Mann et al., 2011).

We did observe that the MUC1 aptamer MUC1-VR1 had a greater amount of cell
association as compared to our CEA N54 aptamer (Figure 4.4A). Since the CEA aptamer had a higher reported binding constant than MUC1-VR1 for its target (~45 nM vs. ~400 nM), the lower amount of cell association may reflect a lower level of CEA expression on the cell surface of MDA-MB-231 cells or could be that surface CEA is being shed from cells.

Importantly, the rates of internalization of CEA and mucin MUC1 by MDA-MB-231 cells showed significant differences. At 37°C, the MUC1-VR1 aptamer displays an estimated internalization rate of <1 hour which is consistent with the published recycling rate of MUC1 on cells (Altschuler et al., 2000) (Figure 4.4B). However, MDA-MB-231 cells significantly accumulate CEA N54-tagged liposomes over a period of 24 hours (Figure 4.4B). There was no significant difference in mean fluorescence intensities at 8 hours between 4°C and 37°C exposure of CEA-targeted liposomes to MDA-MB-231 cells, suggesting that the CEA liposomes were being internalized after this time point. This finding is consistent with the metabolic turnover rate of CEA on cells of between ~10 – 17 hours using monoclonal antibodies to CEA (Schmidt et al., 2008). As expected, no significant difference in cell-associated fluorescence was observed for CEA/MUC1-HeLa cells treated with MUC1-VR1- or N54-liposomes at either 4°C or 37°C as compared to the non-targeted and cApt (control) liposome (Figure 4.4C, 4.4D).

These aptamer-targeted pegylated liposomes were loaded with the contrast agent and injected intravenously (tail vein) into mice to establish their circulatory half-life in the blood over a period of 72 hours. CT results confirmed that the incorporation of aptamers into pegylated liposomes did not significantly alter their stability and circulation half-life (Table 4.1). Interestingly, the non-targeted liposomes were considered to possess a very negative zeta potential (~36 ± 4 mV) and it was thought the
incorporation of aptamers would make the formulation significantly more negative. However, it was found that the average zeta potential of these pegylated liposome preparations were not statistically different, being comparable to that of non-targeted pegylated liposomes (Figure 4.3E).

Although tumour accumulation studies have not yet been completed, the affinity difference in these aptamers and their respective targets on cancer cells should allow for an interesting comparative study. Recently, studies using HER2-specific antibodies as targeting agents demonstrated that differing avidities can affect the level of tumour penetration and internalization in solid tumours (Rudnick et al., 2011). This finding would suggest that MUC1-VR1 aptamer liposomes may accumulate to a greater degree in the tumour than untargeted liposome formulations. As well, it was shown in a comparative study that the in vitro internalization and in vivo distribution of fast-internalizing and slow-internalizing receptors showed a significant difference in tumour accumulation patterns (Bryan et al., 2005). This study would suggest that targeting a non- or slow-internalizing receptor such as CEA might yield better tumour accumulation than a rapidly internalizing receptor such as MUC1. As well, characterizing the ability of these aptamers to target this liposome formulation encapsulating a contrast agent for imaging would allow an easy transition to engineering similar liposomes with a variety of chemotherapeutic drugs.

We have shown in vitro that CEA and MUC1 aptamer targeted pegylated liposomes are able to bind and be internalized by CEA⁺/MUC1⁺ MDA-MB-231 cells but not by MUC1⁻/CEA⁻ HeLa cells. The incorporation of these aptamers into pegylated liposomes did not affect their stability in vivo or their circulatory half-life of ~ 36 hours. Studies are currently underway to determine that biodistribution and internalizing rates of
these aptamer-targeted liposomes \textit{in vivo}. 
Chapter 5

Overall Thesis Conclusions and Future Research
5.1. Thesis Conclusions

The main focus of my thesis was to derive functional aptamers to targets that could functionally inhibit their function in vitro and in vivo. The rationale for pursuing such an objective was that therapeutic targets such as TNFα and CEA can have a therapeutic advantage in blocking binding to their cognate receptors as well as their adhesive properties. In chapter 2, I described how one can successfully identify a DNA aptamer able to block TNFα and confirmed this in vitro by monitoring the blocking of the downstream signaling in NFκB (Figure 2.1) as well as blocking TNFα induced cytotoxicity (Figure 2.2). The inhibitory aptamer VR11 and aptamer VR20 both bound to TNFα with low nanomolar affinities and did not bind to the closest known homolog TNFβ (Figure 2.3). Aptamer VR11 was also able to reduce TNFα dependant NO production in macrophages (Figure 2.4). Circular dichroism spectroscopy also suggests that VR11 does not fold into a G-quadruplex structure however there may exist a secondary structure that aids in binding and blocking TNFα (Figure 2.5). Lastly, it was also determined that VR11 does induce an innate immune response via TLR9 in C57BL/6 mice despite harbouring two possible CpG motifs (Figure 2.6).

In Chapter 3, the homotypic binding domain (N domain) of the human carcinoembryonic antigen (CEA) was selected as a target for aptamer searches since cancer cells expressing CEA have been shown to aggregate and promote tumour foci formation. There is a need to develop new concepts and therapies that can halt or control the establishment or expansion of secondary tumour foci. Thus, the identification of aptamers able to block such intercellular interactions could in theory halt the formation of tumour metastasis. CEA represents a logical target for designing new therapies as it is over-expressed on many epithelial cancer tissues and serves key roles in cellular
aggregation processes and attachment to extracellular matrix elements. We initially identified aptamers, specifically N54 and N56, were able to block the homotypic and heterotypic binding of recombinant N and A3B3 domains (Figure 3.1). Subsequently we confirmed the ability of these aptamers to block the adhesion of the CEA expressing murine cell line MC38.CEA to rCEA and r A3B3 immobilized on wells (Figure 3.2). The truncations of aptamer N54 resulted in a loss of anti-adhesive properties while aptamer N56 could be truncated to a 32-base sequence without any loss of its inhibitory properties relative to the full-length sequence (Figure 3.3). Despite a high level of homology between the N domain of CEACAMs (Figure 3.4), Cy5-labelled aptamers N54 and N56 did not bind by FACS to other CEACAMs known to be involved in cellular adhesion processes suggesting these aptamers are specific to CEA (CEACAM5) (Figure 3.5). Most importantly, it was shown that pretreatment of MC38.CEA cells with aptamer N54 and N56 prevented the tumour implantation in an intraperitoneal model (Figure 3.6). This was not due to an induction of apoptosis or an innate immune response caused by the aptamers (Figure 3.7). This study provided the first, direct evidence that aptamer-based, CEA-directed, anti-adhesive strategies can block metastatic foci formation in vivo.

Since DNA aptamers are small molecules displaying short circulating half lives in vivo, I explored in Chapter 4, the design and construction of long-circulating pegylated liposomes decorated on their surface with aptamers. DNA aptamers directed at two well-known tumour markers, namely CEA and the mucin protein MUC1 were derivatized with a pegylated lipid tail and inserted into pegylated liposomes commonly used to deliver imaging agents and therapeutic drugs to tumour xenografts in immunocompromised mice. I explored the design of pegylated liposomes decorated with aptamers. In 2012, approximately 26 percent of all cancer cases in Canadian women
were breast cancer and represents the second leading cause of cancer deaths and third leading cause of death. This year there will be an estimated 22700 more Canadian women diagnosed with breast cancer however breast cancer deaths have decreased nearly 40 percent since its peak in 1986 partially attributed to more advanced screening technology and improved treatments (Jemal et al., 2008). Targeting unique cancer marker such as CEA and MUC1 represents a novel strategy to increase detection of breast cancer tumours by delivering contrast agent encapsulated liposomes. We have shown in vitro that these aptamers can recognize several breast cancer cell lines and appear to have effect on binding to CEA+/MUC− cells. These aptamers also had no effect on the pharmacokinetic profile of the previously reported liposome formulations. Our data suggest that these formulations may have the ability to increase tumour accumulation or internalization into breast cancer cells.

5.2. Future Directions

5.2.1. Modification of aptamer VR11 for in vivo

Single-stranded DNA aptamers themselves cannot be used in in vivo studies that require them to circulate longer without modifications to stabilize them from degradation. Without modification previous studies have shown that the circulation time of these aptamers are only a few minutes (Healy et al., 2004). It has previously been shown that modifications such as conjugation to polyethylene glycol (PEG) groups allow for the prolonged circulation time of aptamers in mice (Boomer et al., 2005). This study demonstrated that DNA aptamers, when conjugated to a 20kDa or 40kDa PEG group were able to achieve therapeutic concentrations at the sites of inflammation. Another
mode of prolonging circulation is by conjugation to liposomes by conjugating the aptamers to PEG-lipid groups and inserting into preformed liposomes (as described in Chapter 4). In the case of the TNFα aptamer VR11, the VR11 conjugated liposomes would significantly increase the circulatory half-life of the aptamer as well as passively target them to the sites of inflammation (Boomer et al., 2005)

5.2.2. Determine the ability of aptamer N54 as a detection and targeting agent

Our study has shown that the aptamer N54 and to a lesser extent aptamer N56 are able to block the adhesive properties of CEA. Our animal data also showed that pretreating colon cancer cells expressing CEA and no other CEACAMs with these aptamers was able to prevent the formation of tumour foci in vivo. This suggested that CEA homotypic binding is an important event in the attachment and establishment of tumour foci. However, CEA has been shown to interact with other CEACAMs and these interactions may contribute to the metastatic potential of cancer cells. As such, a treatment targeting only CEA in a combination therapy could be advantageous in preventing metastasis during chemotherapy. These aptamers may also be used in pathology for detecting CEA-positive tumours in tissue biopsies or as a delivery agent to target therapeutic cargos to CEA-expressing cancers.

5.2.3. Determine the ability of MUC1 and CEA aptamer to target liposomes

A major challenge associated with chemotherapeutic agents remains their toxicity towards normal tissues. This challenge limits their use to suboptimal doses and ultimately leads to disease re-occurrence. Therapeutic cargos linked to antibodies and
proteins are being developed to specifically deliver chemotherapeutic agents to cancer cells (Dillman, 2011). Yet, protein-guided therapies come with major limiting factors including their size, cost and immunogenicity. Accordingly, simpler targeting agents are needed to focus the delivery of useful cargos to cancer cells. Since their inception in 1990, short DNA/RNA aptamers have been developed to recognize therapeutically important molecular targets such as VEGF, thrombin and HIV. More importantly, aptamers can serve as cellular delivery vehicles by targeting cell surface markers that are internalized by cancer cells, allowing for the intracellular localization of therapeutic cargoes. Aptamers can be rapidly developed through SELEX screens, are easily synthesized, are typically non-immunogenic and are readily amenable to modifications leading to increased circulation times and stability. Aptamers directed at internalized surface markers can be conjugated directly to drugs, RNA/DNA, radionuclides, proteins and nanostructures to serve as tumour selective diagnostic and therapeutic agents. Future studies will reveal the potential of CEA and MUC1 aptamers as targeting agents and their ability to increase the accumulation and internalization of drugs in tumours. Biodistribution analysis from imaging studies and examination of tumours by FACS should determine if either of these formulations is able to increase the amount of liposomes internalized into cancer cells. These anticipated results would suggest that aptamers may enhance the therapeutic potential of drug-loaded liposomes such as Doxil®.
Appendix 1. Inhibition of TNFα-induced cytotoxicity in murine fibroblasts with truncations of aptamer VR11. (A) L929 cells were treated with a 10 nM concentration of TNFα alone or in the presence of either the inhibitory anti-TNFα MAb (20 µg/ml) or a 2µM concentration of aptamer cApt (VR), VR20, VR4, VR11 or truncations of aptamer VR11. (B) Table of aptamer sequences used to perform the cytotoxicity assay. Each point represents the average percent survival value ± SEM (n=4). Asterisk denotes statistical significance (P<0.05; student-t-test).
Appendix 2. Displacement of biotinylated VR aptamers bound to immobilized recombinant TNFα by an anti-TNF mAb. Biotinylated VR aptamers (1µg/well) were bound to immobilized recombinant TNFα overnight then dilutions of anti-TNFα MAb was added for 1 hour and the remaining bound aptamer was detected using streptavidin-HRP. Each point represents the average ELISA signal ± SEM (n=4).
Appendix 3. Commercial and recombinantly purified human TNFα are both active.

Cytotoxicity in murine fibroblast L929 cells pretreated with Actinomycin D (1 µg/ml) and increasing concentrations of commercial recombinant human TNFα supplied by Bioclone Inc. (●) or recombinant human TNFα expressed and purified as described in Materials and Methods section (■). Each point represents the average percent survival ± SEM (n=4).
Appendix 4. Representative Cell association curves generated by the Xcelligence system measuring cell index every minute for (A) MC38 cells adhering to wells coated with BSA, (B) MC38 cells adhering to wells coated with rCEA N-domain, (C) MC38.CEA cells adhering to wells coated with BSA and (D) MC38.CEA cells adhering to wells with rCEA N-domain. Wells and cells were treated with either Aptamer N54 (green), N56 (Red), cApt (Turquoise) or BSA (Blue). Association curves for untreated cells are shown in purple. Time points indicate an average of n=5.
Appendix 5. Endogenously expressing CEA cancer lines (A) BxPC3 (human primary pancreatic adenocarcinoma) grown in RPMI-1640 + 10% FBS, (B) HT29 (human colon carcinoma) grown in McCoy’s 5A + 10% FBS and (C) MCF-7 (human breast cancer cells) grown in DMEM + 10% FBS. Cells stained with the COL-1 anti-CEA antibody (Green) and Cy-labeled aptamers N54 (red) and N56 (blue).
Appendix 6. 4-12% SDS PAGE gel confirming the purity of Ni-NTA purified MUC1 peptide and the mass shift confirming the glycosylation of the MUC1 peptide.
Appendix 7. Binding of the 40 base variable region (VR) biotinylated MUC1 aptamers to glycosylated MUC1 immobilized on 96-well ELISA plates. Control aptamer (cApt) gave a signal comparable to streptavidin-HRP treatment alone. Each bar represents the average + SEM (n=4).
Appendix 8. Histograms showing the binding after 1 hour of the MUC1 polyclonal antibody M23 detected with a FITC-labelled secondary antibody and FITC-labelled MUC1-VR aptamers to the MUC1+ and CEA+ cell line MDA-MB-231.
Appendix 9. Histograms showing the binding after 1 hour of FITC-labelled MUC1-VR1 (Green), MUC1-VR4 (Purple), MUC1-VR7 (purple) and CEA aptamer N54 (blue) to MUC1- and CEA- cell lines 9L, L929 and HeLa as well as MUC1+ and CEA+ MCF-7 cells.
Appendix 10. MUC1 antibody M23 and CEA antibody COL-1 was used to confirm there was no expression on CEA-/MUC1- cell lines L929, 9L and HeLa as well as confirm the expression of MUC1 and CEA on CEA+/MUC1+ cells MCF-7 and MDA-MB-231. COL-1 staining was shown in Chapter 3.
Appendix 11. Standard curve of Cy5 fluorescence measured as function of aptamer concentration using a fluorescent plate reader. Each point represents the average + SEM (n=3).
Appendix 12. Histograms showing the binding of cy5-labeled Aptamer N54 (blue) and control aptamer (green) to (left) MCF-7 and (right) MDA MB 231 cells. Aptamer N54 did not bind to HeLa cells (Chapter 3).
References

noninternalizing copper-64 radioimmunoconjugates in cell and animal models of colon cancer. Nucl Med Biol 32, 851-858.


Korotkova, N., Yang, Y., Le Trong, I., Cota, E., Demeler, B., Marchant, J., Thomas, W.E., Stenkamp, R.E., Moseley, S.L., Matthews, S., 2008. Binding of Dr adhesins of
Escherichia coli to carcinoembryonic antigen triggers receptor dissociation. Mol Microbiol 67, 420-434.
aptamer bound to thrombin. RNA 14, 2504-2512.


DNA aptamers targeting internalized surface portals. Biochim Biophys Acta 1798, 2190-2200.


aptamer that selectively inhibits the enzymatic activity of protein tyrosine phosphatase 1B in vitro. Chembiochem 11, 1583-1593.


Woo, J., Chiu, G.N., Karlsson, G, Wasan, E., Ickenstein, L., Edwards, K., Bally, M.B.,