Sialylation of cell surface glycoproteins facilitates formation of 3D multicellular prostate cancer spheroids

by

Sabah Haq

A thesis submitted to the graduate program in Microbes, Immunity, and Inflammation
In the Department of Biomedical and Molecular Sciences in conformity with the requirements
for the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
(June, 2017)

Copyright © Sabah Haq, 2017
Abstract

Prostate cancer spheroid based three-dimensional culture models (referred to as prostaspheres) have provided insight into prostate cancer biology by highlighting the importance of cell–cell interactions and the extracellular matrix in the tumor microenvironment. The prostasphere model have particular advantages over 2D monolayer culture models in terms of 3D structure and having cell-cell and cell-ECM adhesion instead of cell-culture plate adhesion. The cyclo-RGDfK(TPP) peptide-based biochemical method is a simple, reproducible and cost-effective method of spheroid formation. The cyclo-RGDfK(TPP) peptide method of spheroid formation depends on the cell surface glycoproteins, integrins and cadherins. The terminal monosaccharide of these glycoproteins is sialic acid. Using human metastatic androgen independent prostate carcinoma PC3 and DU145 cell lines and their respective gemcitabine resistant (GemR) variants, prostaspheres were generated by using cyclic Arg-Gly-Asp-D-Phe-Lys peptide modified with 4-carboxybutyl-triphenylphosphonium bromide (cyclo-RGDfK(TPP)). By using cyclo-RGDfK(TPP) peptide in a dose- and time-dependent manner, both DU145 and DU145GemR cells formed prostaspheres. In contrast, PC3 and PC3GemR cells formed irregular cell aggregates at all concentrations of cyclo-RGDfK(TPP) peptide. The formation of prostaspheres was not dependent on the level of E-cadherin expression by the prostate cancer cell lines. Using lectin cytochemistry and flow cytometry, it was found that DU145 and PC3 cells and their drug resistant variants expressed different levels of α2,3 sialic acid (SA) and α2,6 SA residues on the cell surface which correlated with the ability to form prostaspheres. Prostasphere volume was dose-dependently reduced following pretreatment with α2,6 SA specific neuraminidase (Vibrio Cholerae). Oseltamivir phosphate (OP), a
neuraminidase1 inhibitor, did not have any effect on the level of sialic acid and therefore in the formation of spheroids. These results suggest that the relative levels of specific sialoglycan structures on the cell surface glycoproteins correlate with the ability of prostate cancer cells to form prostaspheres.
Acknowledgement

The support, assistance and encouragement of my supervisor, friends, parents and husband have made this dissertation possible. I am very grateful to my supervisor Dr. Myron Szewczuk for his persistent guidance and mentorship during my 2 years as a Masters’ trainee. Whenever I needed his advice or opinion regarding any experiments, presentations, papers he readily extended a helping hand. However, he has also taught me to think for myself. His eagerness for the search of knowledge has encouraged me to do the same. He has been very patient with me and helped me overcome the obstacles that I faced. Thank you Dr. Szewczuk for all your help! I would also like to acknowledge the support and advice of my committee members, Dr. Charles Graham and Dr. Madhuri Koti. I really appreciate your suggestions, useful comments and thoughtful discussions, which helped shape my research.

My lab mates and friends Fiona Haxho, Manpreet Sambi and Roman Akasov have always been there for me whenever I needed them. I learned my laboratory techniques, problem solving and technical skills from my lab mates. I would like to give a special shout of thanks to Fiona for making me technologically sound and skilful. I will immensely miss the thoughtful and at times drama-esque life discussions with Manpreet. I am also very thankful for the encouragement and support of my office mates Lori Minassian and Takafumi Ushida during the challenging Masters’ program.

Vanessa Samuel and Bessi Qorri, our two very enthusiastic, lively and helpful undergraduate research students made my time in the Szewczuk lab pleasurable and worthwhile. I enjoyed guiding and teaching Vanessa all about my spheroids.
I would like to thank my mom, dad and sister without whom none of this would have been possible. The support and unconditional love of my parents motivated me to work hard, to believe in myself during challenges and to have confidence in myself. Lastly, I am grateful to my husband, Niaz, who was a wonderful companion and friend during my journey at Queen’s University. The love, friendship, motivation, mentorship and guidance of my supervisor, teachers, friends and family have made my time at Queen’s worthwhile and memorable!
Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgement ........................................................................................................................ iv
List of Publications ...................................................................................................................... ix
List of Figures .................................................................................................................................. x
List of Tables .................................................................................................................................. xii
List of Abbreviations .................................................................................................................. xiii
Chapter 1 Introduction ................................................................................................................ 1
Chapter 2 Literature review ........................................................................................................ 3
  2.1 Prostate cancer .................................................................................................................... 3
  2.2 Multicellular tumor spheroid (MCTS) .............................................................................. 4
    2.2.1 Methods of MCTS formation ..................................................................................... 5
    2.2.2 Biochemical method of MCTS formation .................................................................. 6
  2.3 Integrin and E-cadherin in cancer cell-cell and cell-ECM .................................................. 7
    2.3.1 Integrins ....................................................................................................................... 11
    2.3.2 E-cadherins ................................................................................................................ 12
  2.4 Glycosylation in prostate cancer ....................................................................................... 13
  2.5 Sialic acid .............................................................................................................................. 15
    2.5.1 Role of sialylation in prostate cancer cell-cell and cell-ECM adhesion ......................... 19
  2.6 Neuraminidase inhibitor, oseltamivir phosphate - regulator of sialic acid on epidermal growth factor receptors (EGFR) ................................................................. 21
    2.6.1 G-protein coupled receptor signaling paradigm ......................................................... 22
    2.6.2 Role of oseltamivir phosphate in spheroid formation .................................................. 24
2.7 Significance of the study

2.8 Hypothesis and objectives

Chapter 3 Materials and Methods

3.1 Cell lines

3.2 Reagents

3.3 Antibodies

3.4 Lectin-based cytochemistry

3.5 Water soluble tetrazolium salt -1 (WST-1) cell viability assay

3.6 Flow cytometry analysis

3.7 Live cell sialidase activity assay

3.8 Fluorescence spectrophotometer analysis of EGF-induced sialidase activity

3.9 Phase contrast microscopy and measurement of prostasphere volume

3.10 Agarose-based and cyclo-RGDfK(TPP) peptide-based prostasphere formation

3.11 Immunocytochemistry (ICC)

3.12 Statistical analysis

Chapter 4 Results

4.1 Generation of prostaspheres using cyclo-RGDfK(TPP) peptide

4.2 Relationship between prostasphere formation and E-cadherin expression on surface of prostate cancer cells

4.3 Increased cell surface expression of α2,6 sialic acid on prostate cancer cells promotes prostasphere formation

4.4 Neuraminidase treatment inhibits cell aggregation and prostasphere formation in prostate cancer cells

4.5 Specific sialic acid binding lectins demonstrate no significant effect on prostasphere formation
4.6 Neu1 inhibitor, oseltamivir phosphate (OP), blocks sialidase activity in live EGF-treated prostate cancer cell surface..........................................................59

4.7 Regulatory role of OP in cell aggregation and compaction in prostasphere formation.............................................................................................................67

Chapter 5 Discussion..........................................................................................75

Chapter 6 Conclusion and Future Directions.......................................................80

6.1 Conclusion.....................................................................................................80

6.2 Future Directions.........................................................................................80

Bibliography.........................................................................................................82
List of Publications


   All the figures (except Figure 26, 27 and 28) in the result section of the thesis have been reprinted with permission from Dove Medical Press, *OncoTargets and Therapy*.

   Haq S did all the experiments except for the spheroids/cell aggregates volume measurements; Samuel V measured the volumes of spheroids and cell aggregates in Figures 19, 21A, 23A, 25A and 30A. Leko M and Burov S.V synthesized the cyclo-RGDfK(TPP) peptide.


   **Figure 9 was reprinted with permission from Oncotarget.**

   Haxho F did the fluorescence histochemistry in Figure 9.


   This is a review paper. No data was taken from this paper
List of Figures

Figure 1. Steps of formation of multicellular tumor spheroids by the cyclo-RGDfK(TPP) peptide based biochemical method........................................................................................................8

Figure 2. Summarized schematic of formation of multicellular tumor spheroids.................9

Figure 3. Cyclo-RGDfK(TPP) peptide-based method of spheroid formation.......................10

Figure 4. Integrin and E-cadherin signaling cascade..............................................................14

Figure 5. Cancer associated glycan changes...........................................................................16

Figure 6. The primary forms of sialic acids.............................................................................17

Figure 7. α2,3 and α2,6-sialic acid linkages to the underlying galactose molecule..............18

Figure 8. Inhibition of Neu1 by OP in the G-protein coupled receptor signaling paradigm....23

Figure 9. Fluorescence histochemical detection of α2,3 SA and α2,6 SA expressions in paraffin-embedded tumor tissues archived from xenograft tumors of PANC1 and MDA-MB231 cells growing in RAG2xCγ double mutant mice.................................................................25

Figure 10. Comparison of prostasphere formation with DU145 and DU145GemR cells by the agarose or cyclo-RGDfK(TPP) peptide method.................................................................41

Figure 11. Prostasphere formation with prostate cancer cell lines using different concentrations of cyclo-RGDfK(TPP) peptide.......................................................................43

Figure 12. Expression of E- and N-cadherins on the surface of prostate cancer cells determined by immunocytochemistry..............................................................45

Figure 13. Expression of E- and N-cadherins on the surface of prostate cancer cells determined by flow cytometry..............................................................46

Figure 14. Expression of α2,3 and α2,6 linked sialic acid on the surface of PC3 and PC3GemR cells determined by immunocytochemistry.........................................................48

Figure 15. Expression of α2,3 and α2,6 linked sialic acid on the surface of PC3 and PC3GemR cells determined by flow cytometry.........................................................49

Figure 16. Expression of α2,3 and α2,6 linked sialic acid on the surface of DU145 and DU145GemR cells determined by immunocytochemistry.........................................................51
Figure 17. Expression of α2,3 and α2,6 linked sialic acid on the surface of DU145 and DU145GemR cells determined by flow cytometry ................................................................. 52

Figure 18. Effect of neuraminidase treatment on prostate cancer cell surface sialic acid expression .................................................................................................................. 55

Figure 19. Effect of neuraminidase treatment on prostasphere formation .................. 56

Figure 20. Effect of α2,3 sialic acid binding lectin, MAL-2, on prostasphere formation studied by phase contrast images ................................................................. 60

Figure 21. Effect of α2,3 sialic acid binding lectin, MAL-2, on spheroid/cell aggregate volume and cell viability ................................................................. 61

Figure 22. Effect of α2,6 sialic acid binding lectin, SNA, on prostasphere formation studied by phase contrast images ................................................................. 62

Figure 23. Effect of α2,6 sialic acid binding lectin, SNA, on spheroid/cell aggregate volume and cell viability ................................................................. 63

Figure 24. Effect of β1,3 N-acetylgalactosamine binding lectin, PNA, on prostasphere formation studied by phase contrast images ................................................................. 64

Figure 25. Effect of β1,3 N-acetylgalactosamine binding lectin, PNA, on spheroid/cell aggregate volume and cell viability ................................................................. 65

Figure 26. Effect of OP on sialidase activity of prostate cancer cells assessed by the sialidase assay .................................................................................................................. 68

Figure 27. Effect of OP on sialidase activity of prostate cancer cells assessed by fluorescence spectrophotometry ................................................................. 70

Figure 28. Effect of OP on prostate cancer cell surface sialic acid expression .......... 72

Figure 29. Effect of OP on prostasphere formation studied by phase contrast images .......... 73

Figure 30. Effect of OP on spheroid/cell aggregate volume and cell viability .......... 74
List of Tables

Table 1. Characteristics of prostate cancer cell lines used…………………………………………30

Table 2. List of lectins used and their specificity. Lectins are carbohydrate binding proteins that have been used extensively as probes to study cell surface oligosaccharide structures/sugars……………………………………………………………………………………………………32

Table 3. Effect of Neu and lectin treatment on prostate cancer cell aggregation and prostasphere formation………………………………………………………………………………66
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>4-MUNANA</td>
<td>2-(4-methylumbelliferyl)-(\alpha)-D-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>(\alpha)2,3 SA</td>
<td>(\alpha)2,3 linked sialic acid</td>
</tr>
<tr>
<td>(\alpha)2,6 SA</td>
<td>(\alpha)2,6 linked sialic acid</td>
</tr>
<tr>
<td>(\beta)1,6 GlcNac</td>
<td>(\beta)1,6-N-acetylgalosamine (\beta)1,6 GlcNac</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>AREs</td>
<td>Androgen response elements</td>
</tr>
<tr>
<td>Bkg</td>
<td>Background control sections</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C-1</td>
<td>Carbon-1</td>
</tr>
<tr>
<td>C-2</td>
<td>Carbon-2</td>
</tr>
<tr>
<td>C-3</td>
<td>Carbon-3</td>
</tr>
<tr>
<td>C-5</td>
<td>Carbon-5</td>
</tr>
<tr>
<td>C-6</td>
<td>Carbon-6</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Cellular oncogene myelocytomatosis</td>
</tr>
<tr>
<td>Cyclo-RGDFK</td>
<td>Cyclic Arg-Gly-Asp-D-Phe-Lys peptide</td>
</tr>
<tr>
<td>Cyclo-RGDFK(TPP)</td>
<td>Cyclic Arg-Gly-Asp-D-Phe-Lys peptide conjugated with 4-carboxybutyl-triphenylphosphonium bromide</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DU145GemR</td>
<td>Gemcitabine resistant DU145</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial-cadherin</td>
</tr>
<tr>
<td>EBP</td>
<td>Elastin-binding protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptors</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-Acetylgalactosamine</td>
</tr>
<tr>
<td>Gem</td>
<td>Gemcitabine</td>
</tr>
<tr>
<td>GemR</td>
<td>Gemcitabine resistant</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylgalactosamine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>KDN</td>
<td>2-keto-3-deoxy-D-glycero-D-galactonononic acid</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MAL-2</td>
<td><em>Maackia amurensis</em> lectin 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCTS</td>
<td>Multicellular tumor spheroid MCTS</td>
</tr>
<tr>
<td>MMP1</td>
<td>Matrix metalloproteinase-1</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Neuronal-cadherin</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule N-CAM</td>
</tr>
<tr>
<td>Neu</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>Neu1</td>
<td>Neuraminidase1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OC</td>
<td>Oseltamivir carboxylate</td>
</tr>
<tr>
<td>OP</td>
<td>Oseltamivir phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PC3GemR</td>
<td>Gemcitabine resistant PC3</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
</tr>
<tr>
<td>PPCA</td>
<td>Protective protein cathepsin A</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>r</td>
<td>Average radius</td>
</tr>
<tr>
<td>Ras</td>
<td>RAt sarcoma</td>
</tr>
<tr>
<td>RAG2xCγ</td>
<td>Recombinase activating gene-2 (RAG2) and common cytokine receptor γ chain</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Sarcoma Cc homology 2</td>
</tr>
<tr>
<td>SLe⁺</td>
<td>Sialyl Lewis A</td>
</tr>
<tr>
<td>SLeˣ</td>
<td>Sialyl Lewis X</td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em> lectin</td>
</tr>
<tr>
<td>STn</td>
<td>Sialyl Tn</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMX</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TPP</td>
<td>4-carboxybutyl-triphenylphosphonium bromide</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV (Mouse Mammary Tumor Virus) integration site family</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water soluble tetrazolium salt -1</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Cancer is defined as an abnormal mass of tissue characterized by uncontrolled and uncoordinated proliferation, invasion and destruction of adjacent structures and spread to distant sites.\(^1\) The cellular mechanisms and hallmarks of cancer are complex, making cancer difficult to treat.\(^2\) More than 100 types of cancer have been identified.\(^3\) Among them, prostate cancer is one of the leading causes of cancer death in men.\(^4\) The molecular mechanisms governing prostate cancer initiation, progression and metastasis are not well understood.\(^5\)

Most \textit{in vitro} studies are conducted on two-dimensional (2D) monolayer culture models. An emerging three-dimensional (3D) model to study cancer cell adhesion is the multicellular tumor spheroid (MCTS).\(^6,7\) Prostate cancer MCTS are referred to as prostaspheres.\(^8\) MCTS is a collection of tumor cells or a mixture of tumor and stromal cells that have a 3D spheroidal arrangement.\(^9\) The MCTS model has particular advantages over 2D monolayer culture models in terms of 3D structure and having cell-cell and cell-ECM adhesion instead of cell-culture plate adhesion.\(^6,10\) The cyclo-RGDfK(TPP) peptide-based biochemical method is a simple, reproducible and cost-effective method of MCTS formation as described by Akasov et al.\(^11\) MCTS formation depends on the interaction of the glycoproteins, E-cadherin and integrin with the cyclo-RGDfK(TPP) peptide. The terminal monosaccharides on the E-cadherin glycans are sialic acids (SA).\(^12\) Apart from E-cadherin, many other cell surface receptors and adhesion molecules like growth factor receptors, integrins and other cadherins are also highly sialylated. The role of SA on cancer cell adhesion is controversial. One group reported that SA is required for E-cadherin dependent cell-cell adhesion\(^13\) while other groups found the opposite.\(^14,15\) Akasov et al. have recently reported that α2,3 and α2,6 SA promotes breast and pancreatic cancer cyclo-
RGDfK(PPP) peptide induced spheroid formation by increasing the cell-cell adhesion.\textsuperscript{16} The current research focuses on the effect of cell surface sialylation on cell-cell adhesion and therefore on 3D multicellular prostasphere formation.
Chapter 2

Literature Review

2.1 Prostate cancer

Prostate cancer is the second most commonly diagnosed malignancy among men in Western countries and one of the leading causes of death in men worldwide.\textsuperscript{4,17} Globally, it is the sixth highest cause of cancer related death in men.\textsuperscript{18} The prostate gland is a fibro-musculo-glandular organ located in the lower pelvis below the internal urethral orifice and surrounding the beginning of the urethra.\textsuperscript{19} The primary function of the prostate is to contribute to the formation of the ejaculate by secreting an alkaline fluid, which enhances sperm viability, motility and provides nourishment.\textsuperscript{19,20} Most of the prostate tumors originate in the epithelial cells and are called adenocarcinomas.\textsuperscript{21}

In 2012, there were 21,105 new cases of prostate cancer, which were diagnosed in Canada and accounted for 23.4\% of all cancer cases.\textsuperscript{22} The worldwide incidence of prostate cancer has increased in the last 20 years due to a rise in life expectancy, improved diagnostics, public awareness, and increased implementation of prostate cancer screening programs.\textsuperscript{17,23} The prognosis of prostate cancer is promising if the tumor is organ confined at the time of diagnosis. However, these locally confined tumors can progress to a lethal metastatic castration resistant disease that has a much worse prognosis.\textsuperscript{24} Prostate cancers mainly metastasize to the bones\textsuperscript{24,25} with a median survival of approximately 40 months.\textsuperscript{24}

Growth of prostate cancer cells is regulated by testosterone, the main circulating androgen. Testosterone controls the ratio of proliferating and dying prostate cancer cells. Testosterone is secreted by the testes and is formed by peripheral conversion of adrenal steroids. It circulates as bound and free testosterone in the blood. The free testosterone enters the prostate
cells and is converted to the more potent and active hormone dihydrotestosterone (DHT) by the enzyme 5α-reductase. The DHT binds to the intracellular androgen receptor (AR) and leads to dissociation from the heat-shock protein. This results in AR activation. The activated AR undergoes conformational change and forms a homodimer complex that binds to androgen response elements (AREs) in the promoter region of target genes. Activation of the target genes results in biological responses like growth and survival of prostate cancer cells.26

There are reports suggesting that the development of prostate cancer depends on genetic and environmental factors. Multiple cellular signaling pathways have been reported to be altered in prostate cancer.27 However, the molecular mechanisms governing the initiation, progression and metastasis of prostate cancer are not well understood.5,28

2.2 Multicellular tumor spheroid (MCTS)

One of the ongoing challenges in the advancement of prostate cancer research is the development and application of a physiologically relevant and cost efficient tumor model. Recently, prostate cancer multicellular tumor spheroids (MCTS), or prostaspheres, have emerged as key tools for analyzing the avascular and three-dimensional components of prostate tumor development in vitro.7,29

The spheroid model provides many advantages over monolayer cell culture systems. 3D MCTS have been designed to overcome several deficiencies apparent in monolayer systems. Monolayer cell culture models do not represent tumor cell interactions with its environment, can not develop nutrient, oxygen and catabolites gradients and do not allow for the heterogeneous cell population that exists in solid tumors in vivo.11,30,31 To overcome these deficiencies, 3D MCTS are being designed.9 MCTS closely resemble small avascular tumors with complex cell-
cell and cell-matrix interactions.\textsuperscript{32,33} MCTS can be used as an intermediate between monolayer cultures and \textit{in vivo} studies for the screening of novel anti-cancer drugs.\textsuperscript{32,33} Compared to a 2D monolayer culture system, MCTS express increased resistance to chemotherapy since they mimic the multicellular arrangement and extracellular matrix deposition of avascular tumors, have a realistic drug penetrance gradient, cell cycle distribution, cell-cell contact, varying microenvironment and similar \textit{in vivo} gene expression.\textsuperscript{10} MCTS models reduce 2D monolayer experimental uncertainties and help in better determination of successful drug trials on animal models. This reduces the number of animal studies, time between drug discoveries and clinical trials and ultimately decreases the cost.\textsuperscript{6,10,34} It is also difficult to monitor changes in the cell signaling pathways in animal models.\textsuperscript{35} MCTS is a useful 3D \textit{in vitro} model to study tumor and tumor cell proliferation, differentiation, morphology, cell invasion, metastasis, drug binding and chemoresistance.\textsuperscript{6,36-38} MCTS simulate cell-cell and cell-microenvironment interactions that are necessary for cancer cell processes like epithelial-mesenchymal transition (EMT), chemotherapy resistance, invasion, cell motility and metastasis.\textsuperscript{34,38} MCTS in matrigel or in extracellular matrix (ECM)-based matrixes are good models to study the various cancer cell processes and response to chemotherapeutics \textit{in vitro}.\textsuperscript{34,39} Due to the obvious advantages, the MCTS model has been used in the present research.

\textbf{2.2.1 Methods of MCTS formation}

Spontaneous aggregation, spinner flasks, rotatory cell culture systems, hanging drop, liquid overlay, low binding plates, microencapsulation and cell cultivation in ultra-low attachment round bottom plates are the different techniques of the classical method of MCTS formation. The classical method employs the use of mechanical forces that prevent cells from
attaching to the surface of tissue culture vessels and leads to the formation of cell aggregation, compaction and MCTS formation.\textsuperscript{9,40,41} Ellem et al. have discussed in detail the formation of prostaspheres, which develop into spherical multicellular aggregates within a 3D cell culture system.\textsuperscript{42} These classical methods have drawbacks in terms of rapidity, reproducibility, simplicity and cost-effectiveness.\textsuperscript{43} Due to the drawbacks of the various classical methods, different groups have been working on alternative biochemical methods. One such group reported the augmentation of compaction and MCTS formation with the aggregate forming cell line MDA-MB231 upon addition of a combination of ECM proteins like fibronectin, laminin, collagen type I, IV and reconstituted basement membrane.\textsuperscript{37,44} Akasov et al. has developed a more effective biochemical, one-step, highly reproducible technique of 3D MCTS formation using synthetic cyclic Arg-Gly-Asp-D-Phe-Lys (cyclic RGDfK) peptide.\textsuperscript{11}

### 2.2.2 Biochemical method of MCTS formation

Cyclo-RGDfK mimics the RGD (arginine-glycine-aspartic acid) sequence of natural ECM proteins like fibronectin. Cyclo-RGDfK binds to $\alpha_5\beta_1$ integrin on the cell membrane and promotes self-assembly of the cells by inhibiting cell-plate surface adhesion.\textsuperscript{11} Normally, fibronectin, a component of the fetal calf serum added to Dulbecco’s Modified Eagle’s Medium (DMEM) attaches and spreads over the surface of tissue culture flasks and plates. Cancer cell-tissue culture plate surface adhesion occurs by the interaction of cell surface integrins with the adhered fibronectin. This results in the adhesion of the cells to the surface of the tissue culture flasks.\textsuperscript{45} In the biochemical method of MCTS formation, the cyclo-RGDfK binds to $\beta_1$ integrin on the cell membrane. This prevents the binding of the integrin to the fibronectin of the fetal calf serum and thus inhibits attachment of the cells to the surface of the culture flasks and plates. As
shown in the schematic in Figure 1, the cyclo-RGDfK-\(\alpha_5\beta_1\) integrin interaction is followed by a delay period during which E-cadherins are expressed on the cell membrane. Lastly, cadherin-cadherin interactions between the cells result in cell compaction and MCTS formation.\(^{34,46,47}\) The steps from cell aggregation to spheroid formation have been summarized in Figure 2. The E-cadherin has also been reported to prevent anchorage independent cell death of cancer cells.\(^{47}\) 4-carboxybutyl-triphenylphosphonium bromide cation (TPP) was conjugated to the aspartic acid component of the cyclo-RGDfK to facilitate cell aggregation (Figure 3). Hereafter, the cyclo-RGDfK and TPP complex will be referred to as cyclo-RGDfK(TPP). The mechanism by which the TPP cation facilitates spheroid formation is unknown. Some possible explanations might be that the TPP can stimulate a faster interaction or can change the spatial structure of the cyclo-RGDfK peptide and enhance the interactions between the peptide and integrin. The MCTS had an average diameter of 60–120\(\mu\)m. Similar reports by other groups have proposed that MCTS formation depend on interactions between cell surface glycosylated integrins such as \(\alpha_6\beta_1\), \(\alpha_5\beta_1\), \(\alpha_6\beta_1\), \(\alpha_2\beta_1\) and their ligands fibronectin, laminin, and collagen.\(^{11,46,48,49}\) The cell surface cadherins, integrins and other glycoprotein molecules that may take part in MCTS formation have terminal sialic acids on their N-glycans, which may highly regulate their cell-cell and cell-ECM adhesion function.

### 2.3 Integrin and E-cadherin in cancer cell-cell and cell-ECM adhesion

Integrins and E-cadherins are cell adhesion transmembrane glycoprotein receptors involved in cancer cell proliferation, migration, invasion, apoptosis and survival.\(^{50}\) Cell adhesion receptors facilitate binding to ECM molecules or to receptors on other cells.\(^{51}\) Integrins and E-cadherins play a role in spheroid formation. Since this thesis is on prostate cancer spheroid
Figure 1. Steps of formation of multicellular tumor spheroids by the cyclo-RGDfK(TPP) peptide based biochemical method.
Steps of formation of MCTS by the cyclo-RGDfK(TPP) peptide-based biochemical method. Step 1: formation of loose cell aggregates via $\alpha_5\beta_1$ integrin – cyclo-RGDfK(TPP) peptide binding; step 2: a delay period for E-cadherin expression and accumulation; step 3: formation of compact MCTS through E-cadherin–E-cadherin interactions.
© 2017 Haq et al. This work is published and licensed by Dove Medical Press Limited. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5422540/
Figure 2. Summarized schematic of formation of multicellular tumor spheroids.
Summary of the steps from cell aggregation to spheroid formation. The cells aggregate, come close to each other and ultimately bind to one another to form spheroids.
© 2017 Haq et al.52 This work is published and licensed by Dove Medical Press Limited. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5422540/
Figure 3. Cyclo-RGDfK(TPP) peptide-based method of spheroid formation.\textsuperscript{11}
Conjugation of 4-carboxybutyl-triphenylphosphonium bromide cation (TPP) to the aspartic acid component of the cyclo-RGDfK to form cyclo-RGDfK(TPP) peptide. The cyclo-RGDfK(TPP) peptide was added to the prostate cancer cells on day 0. Spheroids were formed by day 6. Reprinted by permission from Elsevier: International Journal of Pharmaceutics,\textsuperscript{11} copyright, 2016. https://www.ncbi.nlm.nih.gov/pubmed/27107900
formation, the role of integrins and E-cadherins will be discussed in next few sections.

2.3.1 Integrins

Integrins are heterodimeric transmembrane glycoprotein receptors that mediate cell adhesion to the ECM.\textsuperscript{50,51,53} There are 24 distinct integrin heterodimers formed by the non-covalent combination of 18 α-subunits and 8 β-subunits.\textsuperscript{50,54} The integrin receptor has a large extracellular domain, a single membrane-spanning region and a short cytoplasmic domain.\textsuperscript{51} Cytoplasmic proteins like talin and α-actinin anchors the β cytoplasmic domain to actin cytoskeleton.\textsuperscript{51} Integrin mediated adhesion plays a regulatory role in wound healing, embryogenesis, maintenance of tissue architecture and metastasis of cancer cells.\textsuperscript{55} As prostate cancer progresses, the integrin expression is deregulated, specifically, α\textsubscript{3}, α\textsubscript{4}, α\textsubscript{5}, α\textsubscript{7} and α\textsubscript{v} which have been reported to be downregulated where as β\textsubscript{1}, β\textsubscript{3} and β\textsubscript{6} have been found to be upregulated.\textsuperscript{56,57} Among the 24 integrins, α\textsubscript{5}β\textsubscript{1}, α\textsubscript{6}β\textsubscript{1} and α\textsubscript{6}β\textsubscript{4} facilitate prostate cancer cell invasion and motility.\textsuperscript{58-60} We are particularly interested in α\textsubscript{5}β\textsubscript{1} integrin as they play a key role in the biochemical method of spheroid formation, which is an important part of this project. α\textsubscript{5}β\textsubscript{1} binds to the RGD (arginine-glycine-aspartic acid) sequence found in the ECM protein, fibronectin.\textsuperscript{50,51,58,61} More specifically, Mould et al.\textsuperscript{62} showed that the RGD of the fibronectin binds to the β\textsubscript{1} subunit.

Integrin facilitates prostate cancer cell invasion and migration through processes such as providing traction, controlling the localization and activation of matrix degrading enzymes like matrix metalloproteinase-1 (MMP1), promoting anchorage independence, playing an essential role in the homing of myeloid cells and monocytes that secrete cytokines and growth factors for tumor cell migration.\textsuperscript{50,51} Integrin α\textsubscript{5}β\textsubscript{1} has been found to be upregulated as a result of EMT in
prostate cancer cells.\textsuperscript{63} Integrins provide traction for cell adhesion and migration by directing forces from the cytoskeleton onto the ECM.\textsuperscript{64} The first step in cell migration is adhesion of cell to ECM via integrin.\textsuperscript{64} Integrin mediated cell invasion and migration are activated by focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways as illustrated in Figure 4.\textsuperscript{50,51,57} Binding of the RGD sequence of fibronectin to the extracellular domain of $\alpha_5\beta_1$ stimulates integrin clustering and recruitment of multiprotein complexes to FAK. The multiprotein complexes consist of integrins, integrin associated adaptors and signaling proteins, growth factor receptors and their downstream molecules.\textsuperscript{64} FAK becomes tyrosine phosphorylated upon binding of integrins like $\alpha_5\beta_1$ to fibronectin.\textsuperscript{57,65} It then associates with the cytoplasmic kinase Src. It has been reported that the activated FAK binds to the SH2 domain of PI3K, thereby bringing the PI3K to the cell membrane. The PI3K catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3}), which activates protein kinase B and C (PKB and PKC). PKC regulates transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B) that activates MMP1 gene expression for the facilitation of invasion.\textsuperscript{51,63,64} Several groups have reported that integrin mediated cell adhesion leads to the activation of the MAPK cascade, which may be involved in the regulation of contractility and movement of cellular processes.\textsuperscript{51,57}

### 2.3.2 E-cadherins

E-cadherins are transmembrane homophilic, calcium-dependent, cell-cell adhesion glycoproteins on epithelial cells that are linked to the actin cytoskeleton via cytoplasmic catenins ($\alpha$, $\beta$, and $\gamma$).\textsuperscript{66-68} $\beta$-catenin and $\text{p120}^{\text{CTN}}$ are attached to the E-cadherin to anchor it to the actin cytoskeleton and to form the adherens junction, which is essential for cell-cell adhesion and the
maintenance of epithelial tissue homeostatic architecture. The p120-catenin complex prevents endocytosis of E-cadherin and thus stabilizes it at the cell surface. E-cadherin consists of an extracellular domain of about 550 amino acids arranged in 5 subdomains, a single transmembrane domain and a cytoplasmic domain of 150 amino acids. E-cadherins on the cell membrane of adjacent cells interact with one another. E-cadherin signaling recruits Rac, Rho and Cdc42 that are members of the GTPase family. These GTPases regulate the formation of filopodial-like projections (Figure 2). The filopodia helps cells to come close to each other and form cell-cell contacts. E-cadherin has been reported to antagonize the canonical Wingless-type MMTV (Mouse Mammary Tumor Virus) integration site family (Wnt) signaling by keeping the β-catenin anchored to the membrane, and therefore, preventing it from entering the nucleus and transmitting Wnt signals which regulates cell proliferation.

2.4 Glycosylation in prostate cancer

Glycosylation is an enzymatic process that attaches glycans to saccharides, proteins or lipids. Fifty percentage (%) of all proteins are glycosylated. There are five glycan classes: N-linked glycans; O-linked glycans; phosphoglycans; C-linked glycans; and glypiation. Only the N- and O-linked glycans are covalently attached to a polypeptide backbone via a nitrogen or oxygen atom to form glycoproteins. Adhesion proteins like integrins and E-cadherin commonly have N-glycans. N-linked glycans are attached to a nitrogen atom of asparagine or arginine side- chains. In normal cells, N-glycans on the adhesion proteins regulate protein dimer formation and appropriate interaction of the proteins with the ECM. Figure 5 shows that the glycosylation pattern of glycans in tumor cells is significantly altered compared to normal cells. The balance between glycosyltransferases and glycosidases regulates glycosylation in prostate
Figure 4. Integrin and E-cadherin signaling cascade.

Binding of the RGD sequence of fibronectin to the extracellular domain of $\alpha_5\beta_1$ stimulates integrin mediated cell motility, invasion, survival and proliferation activated by focal adhesion kinase (FAK), phosphoinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways. E-cadherin-E-cadherin binding on adjacent cells regulates the formation of filopodial like projections and inhibits Wnt signaling mediated cancer cell proliferation.
cancer.\cite{76,77} Glycosylation plays an important role in cancer pathogenesis.\cite{76,78-81} High expression of some glycosyl epitopes facilitate invasion and metastasis whereas some other glycosyl epitopes inhibit tumor progression leading to higher survival rates.\cite{80} Cancer-associated changes in glycosylation are sialylation, fucosylation, $O$-glycan truncation, and $N$- and $O$-linked glycan branching.\cite{74,76,82} The focus of this project will be on sialylation.

### 2.5 Sialic acid

Sialic acids (neuraminic acid) are a 50 member acidic family of monosaccharides with a nine carbon amino sugar backbone and a carboxylic acid group at carbon-1 (C-1) position.\cite{12} The carboxylate gives sialic acid a net negative charge at physiological pH.\cite{12,83} There are two primary sialic acids (Figure 6): 2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid (Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN).\cite{12} All the other members of the sialic acid family are metabolically derived from the primary sialic acids.\cite{12} Neu5Ac is the most common sialic acid.\cite{12,84} In this project, the focus will be on Neu5Ac, and hereafter, will be referred to as SA. The carbon at position number five (C-5) in sialic acids is considered to be a modification site because distinct functional groups can attach to it. At this position, sialic acids can undergo different modification processes such as acylation, methylation and hydroxylation.\cite{85} The substitution of Neu5Ac at C-5 by a hydroxyl group results in KDN.\cite{12} Sialic acids can also carry one or more additional substitutions at the hydroxyl groups on carbon-4,7,8 and 9 (O-acetyl, O-methyl, O-sulfate, O-lactyl, or phosphate groups).\cite{12} These modifications are essential to the diversity of sialic acids.

Sialic acids are usually the terminal monosaccharide of $N$- or $O$-glycans. Sialic acids have three different types of $\alpha$-glycosidic linkages between carbon-2 (C-2) of sialic acid and
Figure 5. Cancer associated glycan changes.
Cancer cells express glycan structures that are different from their normal counterparts. The cancer associated glycan changes include increased sialyl Lewis A (SLe\(^a\)), sialyl Lewis X (SLe\(^x\)), increased α2,6 SA in truncated O-linked glycans (STn) and in N-linked glycans, formation of polysialic acid, increased β1,6-N-acetylglucosamine (β1,6 GlcNac) branching and the addition of α1,6 fucose to N-glycans. The cancer associated glycan changes have been boxed. The symbolic nomenclature and geometric shape of each monosaccharide used is according to the International Union of Pure and applied Chemistry short code.\(^{74}\) Reprinted by permission from Macmillan Publisher’s Ltd: Nature Reviews Cancer,\(^{74}\) copyright, 2015. [http://www.nature.com/nrc/journal/v15/n9/full/nrc3982.html](http://www.nature.com/nrc/journal/v15/n9/full/nrc3982.html)
There are two primary sialic acids: 2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid (Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN). Neu5Ac is the most common sialic acid.

Figure 7. α2,3 and α2,6-sialic acid linkages to the underlying galactose molecule. Sialic acids are usually the terminal monosaccharide of N- or O-glycans. Sialic acids have three different types of α-glycosidic linkages between carbon-2 (C-2) of sialic acid and underlying sugars. The most common linkages are at the carbon-3 (C-3) or carbon-6 (C-6) positions of galactose residues giving rise to α2,3 and α2,6 linkages respectively.

underlying sugars. The most common linkages are at the carbon-3 (C-3) or carbon-6 (C-6) positions of galactose residues giving rise to α2,3 and α2,6 linkages as shown in Figure 7.12,86

2.5.1 Role of sialylation in prostate cancer cell-cell and cell-ECM adhesion

Tumor developmental patterns, progression, cell invasiveness and metastatic ability are highly influenced by aberrant cell surface specific sialoglycan structures on glycoproteins like integrins and E-cadherin.79,80,82,87-90 The total sialic acid level in prostate tissues has been reported to be increased in prostate cancer patients. The increase was recorded from 13.01µg SA per mg protein in normal prostate tissue to 19.02µg SA per mg protein in prostate tumor tissue.91 Bull et al. indicated that upregulation or altered activity of sialyltransferase involving 20 members of the mammalian sialyltransferase family; increased substrate availability and different expression of endogenous sialidase are the three mechanisms of aberrant sialylation in cancer cells.87,92 The proto-oncogenes Ras and cellular oncogene myelocytomatosis (c-Myc), hypoxia and high hormone levels like androgen-induced transcription of ST6Gal and ST3Gal enzymes are responsible for synthesis of α2,6 and α2,3 sialic acids.74,77,87

The homotypic and heterotypic adhesion of cancer cells is mediated by glycoproteins, E-cadherins and integrins. In normal cells N-glycan is essential for α5β1 and E-cadherin dimerization, cell surface expression and biological function.65 E-cadherin and α5β1 integrin modification by sialylation in cancer cells is instrumental in regulating their function.70,74,89 The role of sialic acids in cancer cell-cell and cell-ECM adhesion is uncertain and a matter of controversy as different research groups has observed dissimilar results.

Slambrouck et al. showed that in LNCaP-derived metastatic C4-2B prostate cancer cells the adhesion between integrin α2β1 and collagen type I requires cell surface α2,3 sialylation of α2 subunits on the integrin receptors.93 α2,6 sialylation of the β1 subunit was reported to be
decreased in the highly metastatic C4-2B prostate cancer cell line. Hypersialylation of integrin β1 in colon cancer cells activates integrin signaling, increasing binding to collagen I and laminin, which leads to enhanced colon cancer cell progression and metastasis. The effect of the altered sialoglycan pattern of α,β3 integrin on the metastatic potential of melanoma cells was examined by Pocheć et al. They observed a reduction of α2,6 linked SA expression and a rise of α2,3 SA of both subunits of α,β3 integrin with the progression of melanoma. α2,6 SA addition to the β1 integrin subunit changes it’s binding activity to its ligands like fibronectin, laminin, and collagen. Lin et al. indicated that increased expression of α2,6 SA on the cell surface of breast cancer cells, MDA-MB-435, reduced E-cadherin mediated cell-cell adhesion resulting in increased invasion and detachment of cancer cells from the primary tumor. Similar observations of reduced E-cadherin mediated cell-cell adhesion with increased sialylation were made by Pinho et al. in the canine mammary carcinoma cell line, CMT-U27. Collectively, these reports agreed that the findings were due to electrostatic repulsion between cells mediated by the terminal location and net negative charge of α2,6 SA on the E-cadherins. In contrast, other groups have indicated the opposite finding of increased cell-cell and cell-ECM adhesion with increased sialylation of E-cadherin or β1 integrin subunit. Sawada et al. observed lower cell surface sialic acid levels in highly metastatic murine colon adenocarcinoma cell lines compared to poorly metastatic ones. Deman et al. showed removal of α2,3 SA from the surface of human MCF-7 cells abolished E-cadherin-dependent cell-cell adhesion in an aggregation assay. The precise mechanisms by which changes in sialic acid expression influence tumorigenesis and/or invasive behavior remain uncertain.

The above studies on elucidating the role of sialic acid in cell-cell and cell-ECM adhesion were conducted on either animal models or on in vitro monolayer cell culture assays. Recently,
prostate cancer multicellular tumor spheroids (MCTS) or prostaspheres have emerged as a promising 3D *in vitro* tumor model to study cancer cell development, cell motility, metastasis and drug resistance as discussed in section 2.2.\(^6,38\) Akasov et al. have shown that specific cell surface sialoglycan structure correlated with the ability of breast and pancreatic cancer cells to form MCTS.\(^{21}\) \(\alpha_{2,3}\) SA promoted spheroid formation in breast and pancreatic cancer while \(\alpha_{2,6}\) SA played a less significant role. These results confirmed that sialylation facilitates breast and pancreatic cancer cells to remain tightly bound in a spheroidal conformation.\(^{16,97}\) To the best of our knowledge, the role of sialic acids in prostasphere formation and its influence on cell-cell adhesion has not been previously studied.

**2.6 Neuraminidase inhibitor, oseltamivir phosphate - regulator of sialic acid on epidermal growth factor receptors (EGFR)**

Oseltamivir phosphate (OP; Tamiflu\textsuperscript{®}) is a sialic acid analogue that inhibits neuraminidase1 (Neu1).\(^{98,99}\) Apart from the known anti-viral effect of OP,\(^{98,99}\) it has recently been reported that OP also has anti-cancer activity.\(^{100-102}\) Also it has been reported that it is in fact the prodrug OP instead of the active metabolite oseltamivir carboxylate (OC), which completely inhibits Neu1 in pheochromocytoma of the rat adrenal medulla (PC-12 cells) and mouse fibroblast cells (NIH/3T3). OP inhibits Neu1 cleavage of \(\alpha_{2,3}\) SA on epidermal growth factor receptors (EGFR), and thereby, preventing dimerization and receptor activation.\(^{103}\) Inhibition of Neu1 activity by OP blocks a novel G-protein coupled receptor signaling platform expressed on growth factor and TOLL-like receptors, which has been implicated in several tumor development processes including chemoresistance, metastasis, tumorigenesis and angiogenesis.\(^{104}\) Details of this receptor signaling paradigm are discussed below.
2.6.1 G-protein coupled receptor signaling paradigm

Epidermal growth factor (EGF) mediates autocrine and paracrine signaling by binding to the upregulated EGFR on prostate cancer cells. The EGFR is part of a subfamily of four closely related tyrosine kinase receptors (RTK): EGFR (or ErbB-1), Her 2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4).

Recently, our lab has reported a novel G-protein-coupled receptor signaling paradigm. This receptor signaling platform is illustrated in Figure 8. Here, a G-protein-coupled receptor (GPCR) forms a complex with mammalian neuraminidase 1 (Neu1) and matrix metalloproteinase-9 (MMP9) tethered to receptor tyrosine kinases (RTK) with EGFR, at the cell surface. In addition, Neu1 forms a tripartite complex with elastin-binding protein (EBP) and protective protein cathepsin A (PPCA). Upon growth factor binding, a conformational change in the structure of receptor occurs, which in turn results in the activation of GPCR. Activated GPCR stimulates intermediate coupling and activation of guanine triphosphate (GTP)-binding G proteins (guanine nucleotide regulatory proteins composed of α, β, and γ subunits). This interactive process results in the dissociation of the GPCR from the G proteins as well as the Gα-GTP subunit from the Gβ and Gγ subunit. The Gα subunit activates MMP9, which leads to EBP dissociation. This process induces Neu1 to cleave α2,3 sialyl residues that are linked to β-galactosides on the RTK, which are distant from the ligand binding site. Cleavage of α2,3 sialic acid from the receptor removes steric hindrance and allows receptor association, dimerization, activation and downstream signal transduction of the MAPK pathway. Thus, by treating prostate cancer cells with OP, the entire RTK signaling cascade will be inhibited. Consequently, after OP treatment of prostate cancer cells, a retention of α2,3 sialyl residues on the cell surface of cancer cells is expected, which may play a role in the formation of prostaspheres.
Figure 8. Inhibition of Neu1 by OP in the G-protein coupled receptor signaling paradigm. OP inhibits Neu1 preventing the cleavage of α2,3 SA from the RTK and thereby inhibits the RTK signaling cascade. Following OP treatment of prostate cancer cells, a retention of α2,3 sialyl residues on the cell surface of cancer cells is expected.

© 2013 Abdulkhalek et al, publisher and licensee Dove Medical Press Ltd. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.
2.6.2 Role of oseltamivir phosphate (OP) in spheroid formation

In a recent report on breast and pancreatic cancer spheroids, it has been shown that OP enhanced cell aggregation and compaction forming spheroids. Breast cancer cells, MCF-7, treated with 400µg/mL OP and 50µM cyclo-RGDfK(TPP) initiated earlier spheroid formation after 1-3 days and an increase in spheroid volume by day 5, compared to OP untreated MCF-7 cells. The pancreatic cancer cells, PANC1 and gemcitabine (Gem) resistant PANC1, produced improved cell compaction of the cell aggregates following exposure to 200µg/mL OP and 50µM cyclo-RGDfK(TPP) for 5 days. This was indicative of tighter cell-cell contacts in the OP treated MCTS compared to the untreated cells. The effect of OP on sialylation of in vivo tumors has been studied in xenograft tumors of PANC1 and MDA-MB231 cancer cells in recombinase activating gene-2 (RAG2) and common cytokine receptor γ chain (RAG2xCγ) double mutant mouse model. The mouse model lacks mature T and B cells, functional natural killer (NK) cells, and is deficient in cytokine signaling, leading to better engraftment of human cells than any other published mouse strain.100,109 Cohorts were treated with 50mg/kg OP for PANC1 tumors or MDA-MB231 tumors, and were analyzed with fluorescence lectin histochemical staining. Tumors from the OP-treated cohort showed higher expression of α2,3 SA compared with the untreated cohort as shown in Figure 9.16

Interestingly, OP has also been shown to increase the expression of E-cadherin in human pancreatic and breast cancers. Pancreatic cancer cell lines, PANC1 and gemcitabine (Gem) and cisplatin resistant PANC1 cells, have been reported to undergo a mesenchymal to epithelial transition following OP treatment at concentration of 500µg/mL for 48 hours and 600µg/mL for 24 hours, respectively. The cells expressed increased levels of E-cadherin and decreased levels of N-cadherin, which were statistically significant.110
Figure 9. Fluorescence histochemical detection of α2,3 SA and α2,6 SA expressions in paraffin-embedded tumor tissues archived from xenograft tumors of PANC1 and MDA-MB231 cells growing in RAG2xCγ double mutant mice.16

Mice were implanted with $1 \times 10^6$ PANC1 or MDA-MB231 cells cutaneously on the rear flank and OP treatment at indicated dosages began at 22–23 days post implantation when tumors reached 100–200mm$^3$. Paraffin-embedded tumor sections (5µm) on glass slides were processed for lectin histochemistry using biotinylated MAL-2 and SNA followed with avidin fluorescein and fluorescence mounting media. Background control sections (Bkg) were prepared without the biotinylated lectins. Tissue sections were visualized and photographed using a Zeiss Imager M2 fluorescence microscope at 200× magnification. Images are representative of at least five fields of view from two tumor sections.

*Figure taken from our published article, Akasov et al. 2016 Oncotarget 7, No.40 66119-66134. Copyright and License Policies. Open-Access License, No Permission Required.*
In vivo studies with OP showed similar findings. PANC1 or MDA-MB231 (breast cancer cell line) cells were subcutaneously implanted in the right back flank of RAG2xCγ double mutant mice. Mice treated with 2mg/kg OP, or 30mg/kg Gem + 5mg/kg OP exhibited greater E-cadherin staining relative to the untreated cohort. E-cadherin and N-cadherin levels of untreated and Gem only treated cohorts were similar.\textsuperscript{110} Haxho et al.\textsuperscript{102} reported reduced tumor growth rate, neovascularization and metastasis to lungs after daily OP treatment of 30mg/kg or 50mg/kg at day 10 post-implantation of MDA-MB231 cells. OP injection at a dose of 50mg/kg resulted in a significant long-term survival at day 180 post-implantation with an overall shrinkage in tumor volume, and no tumor regrowth after 56 days off all drugs. Immunohistochemical staining of paraffin embedded necropsy tumor sections of 30mg/kg OP treated cohort showed high levels of E-cadherin expression. The reduced metastasis of the OP treated cohorts may also be due in part to the increase in E-cadherin expression essentially allowing the tumor cells from disseminating from the primary tumor.\textsuperscript{102}

2.7 Significance of the study

In the present project the role of cell surface sialic acids in prostasphere formation has been studied. The role of sialic acids in cell-cell and cell-ECM adhesion is a matter of controversy as discussed in section 2.5.1. There are also not many studies on prostate cancer cell models. Since cell-cyclo-RGDbK(PPP) peptide and cell-cell adhesion are key steps in the formation of multicellular tumor spheroids (Figure 1), this highly engineered 3D model system was used to study the role of sialic acid in prostate cancer cell adhesion. This study on sialic acid and cell adhesion mediated spheroid formation can play a fundamental step in our understanding of different prostate cell-ECM and cell-cell adhesion mediated processes. Some of these
adhesion dependent processes include local invasion and detachment of metastatic prostate cancer cells from the primary tumor.\textsuperscript{111} Invasion and detachment of prostate cancer cells are caused by the transition from E-cadherin to neuronal (N)-cadherin.\textsuperscript{112,113} N-cadherins mediate cell-ECM binding.\textsuperscript{111,114} This transition is called epithelial-mesenchymal transition (EMT).\textsuperscript{115}

Epithelial morphogenesis is another process that is dependent on cell adhesion. Epithelial morphogenesis is the process by which epithelial tissues acquire its function specific morphological forms. An important aspect of tissue morphogenesis is cell rearrangement controlled by E-cadherin and desmosome mediated cell-cell adhesion and cell motility.\textsuperscript{116,117} Spheroids are good 3D models to study tissue morphogenesis.\textsuperscript{118} α2,8 linked SA of neural cell adhesion molecule (N-CAM) has been shown to be widely expressed in mesodermal and endodermal derivatives during organogenesis.\textsuperscript{119}

\subsection*{2.8 Hypothesis and objectives}

\textbf{Hypothesis:}

Sialic acids of cell surface glycoproteins facilitate cyclo-RGDfK(TPP) peptide formation of prostaspheres by enhancing cell-cell adhesion.

\textbf{Rationale:}

\begin{itemize}
  \item Adhesion glycoproteins like integrins and cadherins are highly sialylated.
  \item Integrins and cadherins mediate the process of spheroid formation.
  \item Sialic acids facilitated the cyclo-RGDfK(TPP) peptide induced formation of breast and pancreatic cancer spheroids.
\end{itemize}
• Using the cyclo-RGDfK(TPP) peptide induced method of 3D spheroid formation, sialic acids of cell surface glycoproteins is predicted to facilitate prostate cancer cell adhesion and thus spheroid formation.

Objectives:

1. To generate prostaspheres with metastatic cell lines DU145 and PC3 and their chemoresistant variants using cyclo-RGDfK(TPP) peptide-based biochemical method.

2. To determine E-cadherin expression levels on the surface of prostate cancer cell lines DU145 and PC3 and their chemoresistant variants.

3. To correlate cell surface α2,3 and α2,6 sialic acid levels and the ability of prostate cancer cell lines to form prostaspheres.

4. To analyze prostasphere formation upon treatment with cell surface sialic acid regulating agents like lectins (SNA, MAL-2) or neuraminidase (Neu) or oseltamivir phosphate (OP).
Chapter 3
Materials and Methods

3.1 Cell lines

PC3 (epithelial-like human prostate carcinoma cells, ATCC®CRL1435™) and DU145 (epithelial-like human prostate carcinoma cells, ATCC®HTB-81™) cell lines obtained from the metastatic site of human bone and brain tissue respectively, were purchased from ATCC (Manassas, VA 20110 USA). PC3 and DU145 cell lines are metastatic, do not express androgen receptors nor prostate-specific antigen (PSA) and exhibit low levels of acid phosphatase and 5α-reductase activity (Table 1). There is loss of expression of the tumor suppressor gene, phosphatase and tensin homolog (PTEN) in PC3 cells whereas DU145 cells express wild-type PTEN. The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), and 5µg/mL plasmocin™ (InvivoGen, San Diego, CA, USA) in a 5% CO₂ incubator at 37°C. At approximately 80% confluence, the cells were passaged at least five times before experimental use. Gemcitabine resistant PC3 (PC3GemR) and DU145 (DU145GemR) cell lines were established as previously described and cultured in media containing 0.01µM gemcitabine for over one year.

3.2 Reagents

The cyclic Arginine-Glycine-Aspartic acid-D-Phenylalanine-Lysine (cyclo-RGDfK) was synthesized and modified with 4-carboxybutyl-triphenylphosphonium bromide to prepare a cyclo-RGDfK(TPP) peptide in the laboratory of Professor Sergey Burov, Saint-Petersburg, Russia, using standard methods of solid phase peptide synthesis.
Table 1. Characteristics of prostate cancer cell lines used.\textsuperscript{120,122}

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology</th>
<th>Source</th>
<th>Androgen sensitivity</th>
<th>Androgen receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>Epithelial</td>
<td>Metastatic site: human brain</td>
<td>Insensitive</td>
<td>Absent</td>
</tr>
<tr>
<td>DU145GemR</td>
<td>Epithelial</td>
<td>Metastatic site: human brain</td>
<td>Insensitive</td>
<td>Absent</td>
</tr>
<tr>
<td>PC3</td>
<td>Epithelial</td>
<td>Metastatic site: human bone</td>
<td>Insensitive</td>
<td>Absent</td>
</tr>
<tr>
<td>PC3GemR</td>
<td>Epithelial</td>
<td>Metastatic site: human bone</td>
<td>Insensitive</td>
<td>Absent</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sensitivity to 0.01µM Gemcitabine</th>
<th>PTEN\textsuperscript{124}</th>
<th>PSA\textsuperscript{125}</th>
<th>Acid phosphatase\textsuperscript{125}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>Sensitive</td>
<td>Present</td>
<td>Absent</td>
<td>Low levels</td>
</tr>
<tr>
<td>DU145GemR</td>
<td>Resistant</td>
<td>Present</td>
<td>Absent</td>
<td>Low levels</td>
</tr>
<tr>
<td>PC3</td>
<td>Sensitive</td>
<td>Absent</td>
<td>Absent</td>
<td>Low levels</td>
</tr>
<tr>
<td>PC3GemR</td>
<td>Resistant</td>
<td>Absent</td>
<td>Absent</td>
<td>Low levels</td>
</tr>
</tbody>
</table>
*Maackia amurensis* lectin 2 (MAL-2), *Sambucus nigra* lectin (SNA) and peanut agglutinin (PNA) are all plant lectins ordered from Vector Laboratories Inc. (Burlingame, CA, USA) (Table 2). MAL-2 binds to terminal α2,3 sialic acid (also called neuraminic acid/Neu5Ac/SA) linked to β-galactose (Gal). SNA binds with high affinity to the terminally linked α2,6 SA, and has a lower affinity for the α2,3 linkage.\(^{126}\) PNA binds to the terminal β1,3-linked N-acetylgalactosamine (GalNAc).\(^{127}\) The plant lectins were used at a concentration of 0.5µg/mL to 50µg/mL for the indicated incubation times. Neuraminidase (sialidase, Neu) from *Vibrio Cholerae* was obtained from GIBCO laboratories (Thermo Fisher Scientific, Waltham, MA, USA). Neuraminidase hydrolyzes terminal α2,6-, α2,3-, or α2,8-linked sialic acids from asparagine (N)- or serine/threonine (O)-linked oligosaccharides, polysaccharides, mucopolysaccharides, glycoproteins and glycolipids (rate: α2,6>α2,3>α2,8). One unit is the enzyme activity that hydrolyzes 1µmol N-acetyl-neuraminosyl-D-lactose within 1 minute at 37°C under the following incubation conditions: 10mM N-acetyl-neuraminosyl-D-lactose, 50mM sodium acetate, 4mM calcium chloride, 100µg/mL bovine serum albumin, pH 5.5. A 20mg/mL stock solution of neuraminidase inhibitor oseltamivir phosphate (OP) was prepared by dissolving 75mg Tamiflu capsule (Hoffmann-La Roche Ltd., Basel, Switzerland) in sterile 1x phosphate buffer saline (PBS) followed by centrifugation at 1000rpm for 10 minutes.\(^{110}\) Working dilutions of OP (50-800µg/mL) were prepared by solvation in cell culture media.

### 3.3 Antibodies

The expression of E- and N-cadherin was determined using antibodies specific for E- and N-cadherin epitopes. E-cadherin or N-cadherin rabbit monoclonal anti-human antibody (Cell Signaling Technology, Danvers, MA, USA) was used for immunocytochemistry and flow cytometry at a dilution of 1:200. These antibodies do not cross-react with related family
Table 2. List of lectins used and their specificity. Lectins are carbohydrate binding proteins that have been used extensively as probes to study cell surface oligosaccharide structures/sugars.12,79,86

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Origin</th>
<th>Target for binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAL-2</td>
<td><em>Maackia amurensis</em></td>
<td>α2,3 linked SA</td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em></td>
<td>α2,6 linked SA</td>
</tr>
<tr>
<td>PNA</td>
<td><em>Arachis hypogaea</em> (peanut agglutinin)</td>
<td>β1,3 linked GalNac</td>
</tr>
</tbody>
</table>
DyLight 594 conjugated goat anti-rabbit secondary IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) was used for immunocytochemistry and flow cytometry to detect E and N-cadherin primary antibodies at a dilution of 1:500.

3.4 Lectin-based cytochemistry

To determine the localization and expression levels of terminally linked α2,3 and α2,6 sialic acids, before and after treatment with different concentration of Neu (0.25-2.5U) or OP (100-400µg/mL) lectin-based cytochemistry was performed. Cells were cultured in a sterile 24-well plate with 12mm glass coverslips for 24 hours in a 37°C, 5% CO₂ incubator. PC3, PC3GemR, DU145, and DU145GemR cells were fixed with 4% paraformaldehyde (PFA) diluted in PBS for 20 minutes at room temperature, followed by blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.4). Cells were incubated with 10µg/mL biotinylated MAL-2 or SNA for 2 hours at room temperature for lectin binding. The cells were then washed with PBS 3X and incubated with AlexaFluor 594-conjugated streptavidin (Vector Laboratories Inc.) for 1 hour at room temperature in a light sensitive chamber. Control wells without lectin were used to normalize for background fluorescence and were only incubated with streptavidin. Cells were again washed with PBS 3X and cover slips were mounted on a glass slide, visualized with Carl Zeiss Imager 2 fluorescence microscopy (20x objective). The density of the cell staining (red fluorescence) was measured using Corel Photo Paint 8.0 software.

3.5 Water soluble tetrazolium salt -1 (WST-1) cell viability assay

Cells were grown to approximately 90% confluence in T25 tissue culture flasks and
seeded in a microWell 96 well plate (10,000 cells/well). After incubation for 3 hours, 50µM cyclo-RGDfK(TPP) peptide was added to every well to form spheroids. Cells were either untreated (control) or treated with different concentrations of MAL-2, SNA, PNA, Neu or OP for 6 days. After 6 days the adhered cells and/or formed prostaspheres were then incubated with 10µL of WST-1 reagent (Roche Diagnostics Division de Hoffman La Roche Limitée, Laval-des-Rapides, QC, Canada) for 2 hours at 37°C. Viable cells cleave WST-1 reagent (insoluble tetrazolium salt) into a soluble formazan product by the mitochondrial dehydrogenase enzyme, the absorbance of which is measured at 450nm and is directly proportional to the number of viable cells in culture.\(^{128}\) Viability of cells or prostaspheres treated with lectin or neuraminidase or OP was presented as a percentage of control (untreated) cells or MCTS, and analyzed using GraphPad Prism software 6.0 (La Jolla, CA, USA). For lectin, neuraminidase or OP treated cells/prostaspheres, cell viability as a percentage of the untreated control was calculated using the following formula:

\[
\frac{\text{(Absorbance of cells in given concentration of treatment)} - \text{(Media absorbance)}}{\text{(Absorbance of cells in 50µM peptide on day 0)} - \text{(Media absorbance)}} \times 100
\]

**3.6 Flow cytometry analysis**

Cells were grown to approximately 90% confluence in T75 tissue culture flasks. For measurement of terminal sialic acid, cells were stained with biotinylated MAL-2 or SNA (10µg/mL) dissolved in PBS-(2%)FBS for 1 hour on ice. The cells were washed 3X with PBS-(2%)FBS. Cells were then stained with DyLight488-conjugated streptavidin (Biolegend Inc., San Diego, CA, USA) for 30 minutes on ice, followed by washing with PBS-(2%)FBS and fixation
with 2% PFA. Control wells without lectin were used to normalize for background fluorescence and were only incubated with DyLight488-conjugated streptavidin. For E- and N-cadherin detection, live cells were stained with anti E- or N-cadherin rabbit monoclonal antibody for 1 hour on ice. The cells were washed 3X with PBS-(2%)FBS, stained with DyLight 488 conjugated goat anti-rabbit IgG for 30 minutes on ice, washed 3X with PBS-(2%)FBS, and finally fixed using 2% PFA. Control wells without primary antibody were used to normalize for background fluorescence and were only stained with secondary DyLight488-conjugated goat anti-rabbit IgG. A total of $5 \times 10^5$ cells were analyzed by Beckman Coulter Cytomics FC500 flow cytometry and CxP software (Beckman Coulter, Brea, CA, USA) in the Queen’s University Biomedical Imaging Center (QUBIC). The median fluorescence for each histogram was represented for 100% gated cells. The relative levels of $\alpha_{2,3}$ and $\alpha_{2,6}$ SA was expressed as a normalized ratio of $\alpha_{2,3}$-SA/ $\alpha_{2,6}$-SA to control cells.

### 3.7 Live cell sialidase activity assay

The live cell sialidase activity assay was performed as previously described.$^{129,130}$ Briefly, cells were incubated overnight on 12mm sterile circular glass slides in conditioned medium in a 24-well plate until they reached a confluency of 75%. Cells were exposed to increasing concentrations of OP (50-400µg/mL) dissolved in 1xDMEM for 24 hours at $37^\circ$C in a humidified incubator. After removing medium, 0.318mM 2-(4-methylumbelliferyl)-$\alpha$-D-N-acetylneuraminic acid (4-MUNANA; Biosynth Intl., Itasca, IL, USA) substrate in Tris-buffered saline (TBS, pH 7.4) was added to cells alone (control), or with receptor ligand i.e. epidermal growth factor (EGF, 100µg/mL) or with EGF in combination with increasing concentrations of the inhibitor OP (50-400µg/mL). The substrate is hydrolyzed by cell surface membrane sialidase
to give free 4-methylumbelliferone that has a fluorescence emission of 450nm (blue color) following an excitation at 365nm. Fluorescent images were taken after 1–2 minutes using epi-fluorescent microscopy (10x objective). Sialidase activity of live cells was denoted by blue fluorescence surrounding the cell periphery. Mean fluorescence surrounding the cells was quantified by selecting 50 random points using Image J (National Institutes of Health, Bethesda, MD, USA).

3.8 Fluorescence spectrophotometer analysis of EGF-induced sialidase activity

Cells were grown to approximately 90% confluence in T25 tissue culture flasks and seeded in black microWell 96 well optical bottom plates at a density of 10,000 cells/well. Cells in the 96 well plates were incubated overnight at 37°C in a humidified incubator. Cells were exposed to increasing concentrations of OP (50-400µg/mL) dissolved in 1xDMEM for 24 hours at 37°C in a humidified incubator. After removing media, 50µL of 1xPBS was added to each of the wells. 0.318mM 4-MUNANA substrate in TBS, pH 7.4, was added to cells alone (control), or with receptor ligand i.e epidermal growth factor (EGF, 1µg/mL) or with EGF in combination with increasing concentrations of the inhibitor OP (50-800µg/mL). The substrate is hydrolyzed by cell surface membrane sialidase to give free 4-methylumbelliferone that had a fluorescence emission of 450nm (blue color) following an excitation at 365nm. The fluorescence intensity readings were immediately taken over 1-2 minutes using the Varioskan Fluorescence Spectrophotometer (Type 3001, Microplate Instrumentation, Thermo Electron Corporation, Vantaa, Finland). GraphPad prism 6.0 was used for analyzing the results.
3.9 Phase contrast microscopy and measurement of prostasphere volume

The morphology of PC3, DU145, PC3GemR and DU145GemR cells were studied before and after the addition of cyclo-RGDfK(TPP) peptide. The cells were plated in a microWell 96-well plate and treated with cyclo-RGDfK(TPP) peptide, or a combination of cyclo-RGDfK(TPP) peptide and lectin, Neu or OP. Any changes in cellular morphology, aggregation, and prostasphere formation was observed. The wells were visualized using inverted phase contrast microscopy and images were acquired using a scope-mounted camera (Fisher Scientific) at 40x and 100x magnification over 6 days. The diameter of the prostaspheres were calculated using the scale bar in the phase contrast images. The scale bar was used to measure two diameters from each individual spheroid, which were then averaged and divided to calculate the average radius. 20 spheroids were measured per bar. The determinant criteria for what was considered a prostasphere included: compact rounded sphere; distinct border; a diameter of ≥60µm; cells within the prostasphere indistinguishable from one another.\textsuperscript{11} When these criteria were not met the term cell aggregates was used. The following formulae were used to calculate prostasphere volume:\textsuperscript{16,30}

\textbf{10x objective: } V = \frac{4}{3} \pi r^3; \quad \textbf{4x objective: } V = (2.5)\left(\frac{4}{3}\right) \pi r^3; \quad r = \text{average radius (µm)}

For the 4x objective images, the formula includes 2.5 to normalize values to the 10x objective images.

3.10 Agarose-based and cyclo-RGDfK(TPP) peptide-based prostasphere formation

MCTS or prostasphere formation using the classical (agarose-based) method was performed as previously described.\textsuperscript{37} Briefly, 1.5% weight of agarose in 1x DMEM was heated
in a water bath for 15 minutes. 50µL of liquid agar was then pipetted into each well of a flat bottom microWell 96-well plate. Cooling at room temperature for 15-20 minutes solidified the agar and formed agarose-coated plates. Cells were added to the agarose-coated plates at a concentration of 10,000 cells/well and incubated at 37°C for up to 1 week. Alternatively, cyclo-RGDfK(TPP) peptide-induced intercellular assembly and prosastphere formation was performed as previously described. Using this method, cells were grown in a T25 tissue culture flask to 90% confluence, and then plated in a flat bottom microWell 96-well plate at a density of 10,000 cells/well (100µL/well). The cells were incubated for 2-3 hours at 37°C to settle to the bottom of the wells. Next, the medium was replaced with 100µL of cyclo-RGDfK(TPP) peptide at concentrations ranging from 6-100µM, diluted in DMEM. The cells were incubated for 6 days at 37°C in a humidified incubator.

3.11 Immunocytochemistry (ICC)

The localization and expression levels of E- and N-cadherin on PC3, DU145, PC3GemR and DU145GemR cells were determined using ICC analysis. Cells were plated on 12mm glass coverslips in a sterile 24-well plate and incubated for 24 hours at 37°C. The cells were fixed with 4% PFA for 30 minutes on ice, followed by washing with PBS and a protein block with 1% BSA in PBS for 1 hour. Cells were then incubated with primary E- or N-cadherin rabbit monoclonal antibodies for 2 hours at room temperature. The cells were then washed 3X with PBS, stained with DyLight 594 conjugated goat anti-rabbit IgG for 2 hours at room temperature. Background fluorescence was normalized by controlling for cells incubated with secondary antibody alone. Fluorescent images were captured using a Zeiss M2 fluorescence microscope (Carl Zeiss AG, Germany) at 200x and 400x magnification, and quantified using Corel Photo Paint 8.0 software.
3.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0. Results were compared by a one-way ANOVA at 95% or 90% confidence interval using Fisher’s LSD (Least Significant Difference) test or Bonferroni’s test.
Chapter 4

Results

4.1 Generation of prostaspheres using cyclo-RGDfK(TPP) peptide

Here, we compared two methods of \textit{in vitro} prostasphere formation: the classical agarose-based method versus the biochemical cyclo-RGDfK(TPP) peptide-based method. Androgen independent metastatic parental DU145 and PC3 cells and gemcitabine resistant DU145GemR and PC3GemR prostate cancer cells were used to form prostaspheres using the classical\textsuperscript{9} and cyclo-RGDfK(TPP) peptide based methods.\textsuperscript{11} The purpose of using four cell lines was to correlate the ability of prostasphere formation with the different sialylation levels at the cell surface. As mentioned in section 3.9 the determinant criteria for what was considered a prostasphere included: compact rounded sphere; distinct border; a diameter of ≥60µm; cells within the prostasphere indistinguishable from one another.\textsuperscript{11} When these criteria were not met the term cell aggregates was used. Using the classical technique, both DU145 and DU145GemR cells formed loose and irregular cell aggregates but did not form prostaspheres (Figure 10A and 10B). Using the cyclo-RGDfK(TPP) peptide based method, both DU145 and DU145GemR formed small prostaspheres. It was found that DU145 cells formed tight compact prostaspheres with an average diameter of ≥60µm. These results are consistent with another report that has shown that DU145 cells form tight prostaspheres by the 3D matrigel culture method.\textsuperscript{7}

Figure 11 illustrates the formation of prostaspheres by the cyclo-RGDfK(TPP) peptide based method. Prostasphere formation using the four prostate cancer cell lines was analyzed with various cyclo-RGDfK(TPP) peptide concentrations. DU145 cells formed tightly compact prostaspheres with increasing incubation time (6 days) and concentration of cyclo-RGDfK(TPP) peptide, ranging from 12.5-100µM (Figure 11A). DU145GemR cells started to
Figure 10. Comparison of prostasphere formation with DU145 and DU145GemR cells by the agarose or cyclo-RGDfK(TPP) peptide method.

Prostasphere formation of (A) DU145 and (B) DU145GemR cells by the classical agarose and cyclo-RGDfK(TPP) peptide methods in a time-dependent manner. The images were taken with a digital camera mounted on a phase contrast inverse light microscope at 100x magnification. 10,000 cells/well were plated on 1.5% weight of agarose coated 96-well plates for 7 days in the classical agarose method. For the cyclo-RGDfK(TPP) peptide method, 10,000 cells were added to each well in a 96-well plate. Cyclo-RGDfK(TPP) peptide was added at a concentration of 50µM for both DU145 and DU145GemR cells (A, B). The images are a representation of one out of two independent experiments showing similar results. The scale bar in the top left image of panel A represents 508µm. The scale bars of the all the other images in panel A and B have the same measurements. (C) Enlarged image of top right image of panel A. The scale bar represents 508µm. The scale bars of the all the enlarged images have the same measurements. (D) Enlarged image of bottom right image of panel A. (E) Enlarged image of top right image of panel B. (F) Enlarged image of bottom right image of panel B. The term TPP method refers to the cyclo-RGDfK(TPP) method.
aggregate and initiated prostasphere formation at lower concentration of cyclo-RGDfK(TPP) peptide (12.5µM) whereas DU145 cells exposed to the same cyclo-RGDfK(TPP) peptide concentration retained their monolayer morphology.

PC3 and PC3GemR cells were found to form irregular multicellular aggregates at all concentrations of cyclo-RGDfK(TPP) peptide (Figure 11C and 11D), and did not appear to form prostaspheres even after 6 days of incubation. Exposure to 100µM cyclo-RGDfK(TPP) peptide induced PC3GemR cells to form irregular multicellular aggregates, whereas PC3 cells demonstrated a mixture of adhered monolayer cells and irregular multicellular aggregates.

To determine if a longer incubation time had an effect on prostasphere formation and/or size, all the four cell lines were incubated at 37°C for up to 18 days. Notably, there were no significant differences in prostasphere formation and diameter between an incubation interval of 6 days or longer (Data not shown). It was determined that 6 days incubation with 50µM cyclo-RGDfK(TPP) peptide were the optimized conditions for prostasphere formation.

4.2 Relationship between prostasphere formation and E-cadherin expression on surface of prostate cancer cells

ICC and flow cytometry analysis were used to determine if the ability of prostate cancer cells to form prostaspheres was associated with the relative levels of cell surface adhesion molecules. Even though it was found by ICC that DU145 cells expressed higher levels of E-cadherin compared to PC3 cells (Figure 12A and 12B), the results were not significant. Even though N-cadherin expression levels were found to be much lower in DU145 cells than that of PC3 cells the results were also not significant. Flow cytometry analyses (Figure 13A, B, and C) showed no differences between the relative expression levels of E- versus N-cadherin on the
Figure 11. Prostasphere formation with prostate cancer cell lines using different concentrations of cyclo-RGDfK(TPP) peptide.

Cyclo-RGDfK(TPP) method of prostasphere formation using (A) DU145 cells, (B) DU145GemR cells, (C) PC3 cells and (D) PC3GemR cells. 10,000 cells were plated per well in a 96-well plate. Cyclo-RGDfK(TPP) peptide was added in a range of concentrations from 12.5µM to 100µM. The control group was the monolayer cells with no cyclo-RGDfK(TPP) peptide added. The scale bar in the leftmost image of panel A represents 508µm. The scale bars of the all the other images have the same measurements. The images were taken with a digital camera mounted on a phase contrast inverse light microscope at 100x magnification on day 6. Single prostasphere/ cell aggregate has been magnified 7x to illustrate their morphology. The images are a representation of one out of two independent experiments showing similar results. The term TPP refers to cyclo-RGDfK(TPP).
prostate cancer cell surface. This led us to hypothesize that an E-cadherin independent mechanism might be playing a role in prostatosphere formation.

4.3 Increased cell surface expression of α2,6 sialic acid on prostate cancer cells promotes prostatosphere formation

Due to the well-known role of sialoglycan structures in facilitating intercellular interaction and adhesion, the potential link between cell surface sialic acids and prostatosphere formation was investigated. The relative expression levels of α2,3 SA versus α2,6 SA were analyzed in PC3 and PC3GemR (Figure 14 and 15) as well as DU145 and DU145GemR cells (Figure 16 and 17). Using α2,3 SA-binding MAL-2 and α2,6 SA-binding SNA lectins, ICC and flow cytometry analyses were performed. Both DU145 and DU145GemR cells expressed approximately 5-fold greater levels of cell-surface α2,6 SA (Figure 17C) in comparison to α2,3 SA and both cells formed tighter and compact prostatospheres compared to PC3 and PC3GemR cells. In contrast, PC3GemR cells showed only 1.4-fold higher expression of α2,6 SA compared to α2,3 SA. For PC3 cells, the relative expression level of α2,3 SA was approximately 4-fold higher than α2,6 SA (Figure 15C). Among the four cell lines PC3 showed the highest level of α2,3 SA on the cell surface. As mentioned above, PC3 and PC3GemR cells were unable to form prostatosphere using cylo-RGDfK(PPP) peptide.
Figure 12. Expression of E- and N-cadherins on the surface of prostate cancer cells determined by immunocytochemistry.

(A) Immunocytochemistry to determine the expression of E- and N-cadherin on the cell surface of non-permeabilized DU145, DU145GemR, PC3 and PC3GemR cells. DU145, DU145GemR, PC3 and PC3GemR cells were stained with primary antibodies against E- and N-cadherin followed by secondary goat anti-rabbit IgG conjugated with DyLight 594. The background control cells were only stained with the secondary antibody. Images were taken with an epifluorescent microscope using a 20x objective. The bars represent 10µm. Images are representative of three fields of view in two independent experiments.

(B) Quantitative analysis was done by assessing the density of cell staining corrected for background for 5-6 separate image panels using Corel Photo Paint 8.0 software. Each bar in the graphs represents the mean fluorescence corrected density of staining ± S.E. (error bars) for all cells within the respective images. Results were compared by a one-way ANOVA at 95% confidence interval using Fisher’s LSD test and all the results are non significant.
**A**

**PC3 cells**

<table>
<thead>
<tr>
<th></th>
<th>Median Fluorescence</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells unstained</td>
<td>2.71</td>
<td>5.59</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>3.15</td>
<td>8.58</td>
</tr>
<tr>
<td>Anti N-Cadherin rabbit mAb</td>
<td>2.71</td>
<td>8.42</td>
</tr>
<tr>
<td>Anti E-Cadherin rabbit mAb</td>
<td>2.83</td>
<td>8.84</td>
</tr>
</tbody>
</table>

**B**

**PC3GemR cells**

<table>
<thead>
<tr>
<th></th>
<th>Median Fluorescence</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells unstained</td>
<td>123</td>
<td>291</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>145</td>
<td>388</td>
</tr>
<tr>
<td>Anti N-Cadherin rabbit mAb</td>
<td>146</td>
<td>351</td>
</tr>
<tr>
<td>Anti E-Cadherin rabbit mAb</td>
<td>125</td>
<td>384</td>
</tr>
</tbody>
</table>
Figure 13. Expression of E- and N-cadherins on the surface of prostate cancer cells determined by flow cytometry.

(A) PC3, (B) PC3GemR and (C) DU145GemR cells were stained with primary antibodies against E and N-cadherin, followed by secondary goat anti-rabbit IgG conjugated with DyLight 488. Control cells were stained with only secondary antibodies. Cells were analyzed by Beckman Coulter Cytomics FC500 flow cytometry and CxP software (Beckman Coulter). Overlay histograms are displayed. The median fluorescence for each histogram is for 5x10^5 acquired cells (100% gated). The data are a representation of one out of two experiments showing similar results.
Figure 14. Expression of α2,3 and α2,6 linked sialic acid on the surface of PC3 and PC3GemR cells determined by immunocytochemistry.

(A, C) Immunocytochemistry to determine the expression of α2,3 and α2,6 linked sialic acid (SA) on the surface of non-permeabilized PC3 and PC3GemR cells. PC3 and PC3GemR cells were stained with biotinylated lectins, MAL-2 specific for α2,3 and SNA specific for α2,6 SA. Stained cells were then treated with DyLight 594 streptavidin. The background control were cells with only DyLight 594 streptavidin staining. Images were taken with an epifluorescent microscope using a 20x objective. The bars represent 10µm. Images are representative of three fields of view in three separate experiments.

(B, D) Quantitative analysis was done as described in Figure 12B. Results were compared by a one-way ANOVA at 90% confidence interval using Fisher’s LSD test.
A

PC3 cells

Gate: 100%

<table>
<thead>
<tr>
<th></th>
<th>Cells unstained</th>
<th>DyLight 488 Strept-avidin</th>
<th>Biotinyalted SNA+DyLight 488-Streptavidin</th>
<th>Biotinyalted MAL-2+DyLight 488-Streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Fluorescence</td>
<td>0.608</td>
<td>0.988</td>
<td>1.55</td>
<td>4.99</td>
</tr>
<tr>
<td>Mean Fluorescence</td>
<td>0.778</td>
<td>1.69</td>
<td>3.17</td>
<td>6.63</td>
</tr>
</tbody>
</table>

DyLight 488 fluorescence (525 ± 20 nm)

B

PC3GemR cells

Gate: 100%

<table>
<thead>
<tr>
<th></th>
<th>Cells unstained</th>
<th>DyLight 488 Strept-avidin</th>
<th>Biotinyalted SNA+DyLight 488-Streptavidin</th>
<th>Biotinyalted MAL-2+DyLight 488-Streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Fluorescence</td>
<td>0.398</td>
<td>0.592</td>
<td>6.59</td>
<td>5.31</td>
</tr>
<tr>
<td>Mean Fluorescence</td>
<td>0.449</td>
<td>1.03</td>
<td>7.19</td>
<td>5.88</td>
</tr>
</tbody>
</table>

DyLight 488 fluorescence (525 ± 20 nm)
Figure 15. Expression of α2,3 and α2,6 linked sialic acid on the surface of PC3 and PC3GemR cells determined by flow cytometry.

(A, B) Flow cytometry to determine the expression of α2,3 and α2,6 linked SA on the surface of non-permeabilized PC3 and PC3GemR cells. PC3 and PC3GemR cells were stained with biotinylated lectins followed by streptavidin conjugated DyLight 488. Control cells were stained with only DyLight 488 conjugated streptavidin. Cells were analyzed by Beckman Coulter Cytomics FC500 flow cytometry and CxP software (Beckman Coulter). Overlay histograms are displayed. Gray filled histogram represents unstained cells; black-dashed unfilled histogram depicts DyLight 488 conjugated streptavidin treated cells; dotted black line unfilled histogram for biotinylated SNA stained cells plus DyLight 488 conjugated streptavidin; black line unfilled histogram represents biotinylated MAL-2 stained cells plus DyLight 488 conjugated streptavidin. The median fluorescence for each histogram is for 5x10^5 acquired cells (100% gated). The data are a representation of one out of two experiments showing similar results.

(C) Graph of normalized ratio of α2,3 SA/ α2,6 SA to control cells from flow cytometry analyses is displayed.
Figure 16. Expression of α2,3 and α2,6 linked sialic acid on the surface of DU145 and DU145GemR cells determined by immunocytochemistry.

(A, C) Immunocytochemistry to determine the expression of α2,3 and α2,6 linked sialic acid (SA) on the surface of non-permeabilized DU145 and DU145GemR cells by the method described in Figure 14 (A, C).

(B, D) Quantitative analysis was done as described in Figure 12B. Results were compared by a one-way ANOVA at 90% confidence interval using Fisher’s LSD test.
**DU145 cells**

<table>
<thead>
<tr>
<th></th>
<th>Gate: 100%</th>
<th>Median Fluorescence</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells unstained</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DyLight 488 Strept-avidin</td>
<td></td>
<td>56</td>
<td>73</td>
</tr>
<tr>
<td>Biotinylated SNA+DyLight 488-Streptavidin</td>
<td></td>
<td>131</td>
<td>147</td>
</tr>
<tr>
<td>Biotinylated MAL-2+DyLight 488-Streptavidin</td>
<td></td>
<td>121</td>
<td>138</td>
</tr>
</tbody>
</table>

**DU145GemR cells**

<table>
<thead>
<tr>
<th></th>
<th>Gate: 100%</th>
<th>Median Fluorescence</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells unstained</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DyLight 488 Strept-avidin</td>
<td></td>
<td>58</td>
<td>72</td>
</tr>
<tr>
<td>Biotinylated SNA+DyLight 488-Streptavidin</td>
<td></td>
<td>153</td>
<td>167</td>
</tr>
<tr>
<td>Biotinylated MAL-2+DyLight 488-Streptavidin</td>
<td></td>
<td>138</td>
<td>156</td>
</tr>
</tbody>
</table>
Figure 17. Expression of α2,3 and α2,6 linked sialic acid on the surface of DU145 and DU145GemR cells determined by flow cytometry.

(A, B) Flow cytometry to determine the expression of α2,3 and α2,6 linked sialic acid (SA) on the surface of non-permeabilized DU145 and DU145GemR cells by the method described in Figure 16. Overlay histograms are displayed. Red histogram represents unstained cells; grey histogram depicts DyLight 488 conjugated streptavidin treated cells; yellow histogram for biotinylated SNA stained cells plus DyLight 488 conjugated streptavidin; blue histogram represents biotinylated MAL-2 stained cells plus DyLight 488 conjugated streptavidin. The median fluorescence for each histogram is for 5x10^5 acquired cells (100% gated). The data are a representation of one out of two experiments showing similar results.

(C) Graph of normalized ratio of α2,3 SA/α2,6 SA as described in Figure 15G.
4.4 Neuraminidase treatment inhibits cell aggregation and prostasphere formation in prostate cancer cells

DU145 cells were treated with either 0.25U or 2.5U Neuraminidase (Neu, *Vibrio Cholerae*) for 24 hours followed by biotinylated lectin staining. It was determined that 2.5U Neu cleaved >50% of α2,6 SA compared to the untreated control (Figure 18B) However, only about 20% of α2,3 SA was cleaved by 2.5U Neu compared to the untreated control.

When treated with Neu in the presence of 50µM cyclo-RGDfK(PPP), all four prostate cancer cell lines showed a significant inhibition of cell aggregation and prostasphere formation (Figure 19). It was observed that Neu-treated cells better retained their monolayer culture and resisted aggregation, compared to untreated cells. The corresponding prostasphere diameter and volume was then measured and calculated (Figure 19). The graphs show a decrease in prostasphere volume when cells were treated with Neu.

The cytotoxic effect of Neu on the adhered monolayer and suspended prostasphere was analyzed using the WST-1 assay. Cells were treated with 0.025U, 0.25U, 2.5U or 25U Neu in a 96-well plate for 6 days, then WST-1 reagent was added to each well. Cell viability was expressed as the percentage of control ± standard error of the mean of duplicate values. Remarkably, it was found that Neu treatment (0.025U-25U) demonstrated no cytotoxic effects on the viability of prostasphere and multicellular aggregates for any of the four cell lines (Figure 19). This suggests that Neu negatively regulates the formation and size of prostasphere and cell aggregates without affecting cell survival.
Figure 18. Effect of neuraminidase treatment on prostate cancer cell surface sialic acid expression.

(A) Fluorescent microscopy images of DU145 cells stained with biotinylated lectins followed by DyLight 594 streptavidin. The cells were treated with 0.25U or 2.5U neuraminidase (Neu) (*Vibrio Cholerae*) for 24 hours before staining. Images were taken with epifluorescent microscope with 20x objective. The bars represent 36µm.

(B) Quantitative analysis was done as described in Figure 12B.
A

10,000 cells per well, Day 6, 100X

DU145 Spheroids

Volume (µm³)

DU145 Spheroids

Cell Viability (% of Control) ± SEM

B

10,000 cells per well, Day 6, 100X

DU145GemR Spheroids

Volume (µm³)

DU145GemR Spheroids

Cell Viability (% of Control) ± SEM
C

Monolayer  50µM TPP  50µM TPP + 0.025U Neu  50µM TPP + 0.25U Neu  50µM TPP + 2.5U Neu

PC3

10,000 cells per well, Day 6, 100X

PC3 Cell Aggregates

Volume (µm$^3$)

0 0.025 0.25 2.5

Neu, U

*P<0.05

PC3 Cell Aggregates

Cell Viability (% of Control) ± SEM

0 0.025 0.25 2.5

Neu, U

D

Monolayer  50µM TPP  50µM TPP + 0.025U Neu  50µM TPP + 0.25U Neu  50µM TPP + 2.5U Neu

PC3GemR

10,000 cells per well, Day 6, 100X

PC3GemR Cell Aggregates

Volume (µm$^3$)

0 0.025 0.25 2.5

Neu, U

*P<0.05

PC3GemR Cell Aggregates

Cell Viability (% of Control) ± SEM

0 0.025 0.25 2.5

Neu, U
Figure 19. Effect of neuraminidase treatment on prostasphere formation.

Phase-contrast images of (A) DU145, (B) DU145GemR, (C) PC3 and (D) PC3GemR cells treated with a combination of 50µM cyclo-RGDfK(TPP) peptide and Neu at concentrations of 0.025U, 0.25U or 2.5U for 6 days. 10,000 cells were plated per well in a 96-well plate for 6 days. The images are a representative of two fields of view in two independent experiments. The scale bar in the leftmost image of panel A represents 508µm. The scale bars of the all the other images have the same measurements. Prostasphere volume was measured using \( V = \frac{4}{3} \pi r^3 \) where \( \pi = 3.1415 \) and \( r = \) average radius (µm). Radius was measured from the scale bar. Each bar in the graph represents mean prostasphere volume ± S.E. (error bars) for 10 prostasphere within each image (1 image per experiment) from 2 separate experiments (n=20). Results were compared by a one-way ANOVA at 95% confidence using Fisher’s LSD test. Viability of DU145, PC3, DU145GemR and PC3GemR attached cells and prostasphere was determined using WST-1 assay. Cells were plated at a density of 10,000 cells/well in a 96 well plate and incubated at 37°C in a CO\(_2\) incubator for 3 hours. The cells were treated with Neu at the indicated doses with a combination of cyclo-RGDfK(TPP) at a concentration of 50µM for 6 days. 10µL of WST reagent was added to each well on day 7 to get a dilution of 1:10. Cells were incubated for 2 hours at 37°C. Cell viability was expressed as a percent of control SEM of two independent experiments. Statistical analysis was carried out using GraphPad Prism, and the results were compared by a one-way ANOVA at 95% confidence using Fisher’s LSD test. The term TPP refers to cyclo-RGDfK(TPP).
4.5 Specific sialic acid binding lectins demonstrate no significant effect on prostasphere formation

The effect of blocking/masking α2,3 SA and α2,6 SA with specific SA lectins on prostasphere/cell aggregate formation was studied in a lectin inhibition assay. All four prostate cancer cell lines were treated with 0.5µg/mL or 5µg/mL or 50µg/mL of MAL-2 (α2,3 SA) or SNA (α2,6 SA) or peanut agglutinin PNA (β-1,3 GalNac) in combination with 50µM cyclo-RGDfK(TPP). The prostaspheres were observed after 6 days of incubation. Interestingly, we did not observe an inhibition of DU145 and DU145GemR cell aggregation and prostasphere formation with any of the neutralizing lectins (Figure 20, 22 and 24) However, only α2,3 SA specific MAL-2 dose-dependently reduced the volume of PC3 and PC3GemR cell aggregates (Figure 21A). The viability of the prostasphere and cell aggregates formed in the presence of increasing concentrations of each lectin was then measured by the WST-1 assay (Figure 21B, 23B and 25B). DU145 and DU145GemR prostaspheres were viable in the presence of each lectin. However, PC3GemR showed a 30% decrease in cell viability following treatment with 50µg/mL MAL-2, as well as SNA. PC3 cell aggregates also showed a 50% reduction in cell viability after treatment with 50µg/mL MAL-2. The reduction in volume of PC3 and PC3GemR cell aggregates with MAL-2 treatment might be due to decreased cell viability. The effect of Neu and lectins on prostatsphere formation has been summarized in Table 3.

4.6 Neu1 inhibitor, oseltamivir phosphate (OP), blocks sialidase activity in live EGF-treated prostate cancer cell surface

Since Neu1 and MMP-9 cross-talk regulates EGFRs in pancreatic, ovarian and triple-negative breast cancer cells, and EGFRs are overexpressed in prostate cancer cells the
Figure 20. Effect of α2,3 sialic acid binding lectin, MAL-2, on prostasphere formation studied by phase contrast images.

Phase-contrast images of DU145, DU145GemR, PC3 and PC3GemR treated with a combination of 50µM cyclo-RGDfK(TPP) peptide and MAL-2 at concentrations of 0.5µg/mL, 5µg/mL and 50µg/mL for 6 days. 10,000 cells were plated per well in a 96-well plate. The data are a representation of two independent experiments. The scale bar in the top left image represents 508µm. The scale bars of the all the other images have the same measurements. The term TPP refers to cyclo-RGDfK(TPP).
Figure 21. Effect of α2,3 sialic acid binding lectin, MAL-2, on spheroid/cell aggregate volume and cell viability.
(A) Spheroid/cell aggregate volume was measured using the method described in Figure 19.
(B) Viability of attached cells and prostasphere was determined using WST-1 assay as described in Figure 19.
Figure 22. Effect of α2,6 sialic acid binding lectin, SNA, on prostasphere formation studied by phase contrast images.
Phase-contrast images of DU145, DU145GemR, PC3 and PC3GemR treated with a combination of 50µM cyclo-RGDfK(TPP) peptide and SNA at concentrations of 0.5µg/mL, 5µg/mL and 50µg/mL for 6 days. 10,000 cells were plated per well in a 96-well plate. The data are a representation of two independent experiments. The scale bar in the top left image represents 508µm. The scale bars of the all the other images have the same measurements. The term TPP refers to cyclo-RGDfK(TPP).
Figure 23. Effect of α2,6 sialic acid binding lectin, SNA, on spheroid/cell aggregate volume and cell viability.
(A) Spheroid/cell aggregate volume was measured using the method described in Figure 19.
(B) Viability of attached cells and prostasphere was determined using WST-1 assay as described in Figure 19.
Figure 24. Effect of β1,3 N-acetylgalactosamine binding lectin, PNA, on prostasphere formation studied by phase contrast images.
Phase-contrast images of DU145, DU145GemR, PC3 and PC3GemR treated with a combination of 50µM cyclo-RGDfK(TPP) peptide and PNA at concentrations of 0.5µg/mL, 5µg/mL and 50µg/mL for 6 days. 10,000 cells were plated per well in a 96-well plate. The data are a representation of two independent experiments. The scale bar in the top left image represents 508µm. The scale bars of the all the other images have the same measurements. The term TPP refers to cyclo-RGDfK(TPP).
Figure 25. Effect of β1,3 N-acetylgalactosamine binding lectin, PNA, on spheroid/cell aggregate volume and cell viability.

(A) Spheroid/cell aggregate volume was measured using the method described in Figure 19.

(B) Viability of attached cells and prostaticspher was determined using WST-1 assay as described in Figure 19.
Table 3. Effect of Neu and lectin treatment on prostate cancer cell aggregation and prostasphere formation.

<table>
<thead>
<tr>
<th>Compound+cyclo-RGDfK(TPP) peptide</th>
<th>Specificity</th>
<th>Effectiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>α2,6&gt;α2,3&gt;α2,8 linked SA</td>
<td>Inhibits cell aggregation and prostasphere formation</td>
</tr>
<tr>
<td>MAL-2</td>
<td>α2,3 linked SA</td>
<td>No effect on prostasphere formation</td>
</tr>
<tr>
<td>SNA</td>
<td>α2,6 linked SA</td>
<td>No effect on prostasphere formation</td>
</tr>
<tr>
<td>PNA</td>
<td>β1,3 linked GalNac</td>
<td>No effect on prostasphere formation</td>
</tr>
</tbody>
</table>
molecular targeting potential of the Neu1-MMP-9 signaling platform was investigated in DU145, PC3 and in their gemcitabine resistant variants. A live-cell sialidase assay was performed as previously established and optimized.\textsuperscript{129,131,132} Cell surface sialidase activity was detected in the periphery of cells due to the lysis of Neu1-specific fluorogenic substrate, 4-MUNANA, into 4-methylumbelliferone, with an emission wavelength of 450nm. This fluorescence was only observed when ligand-receptor interactions occurred, wherein Neu1 was induced. When the ligand EGF was added to the cells, it bound to EGFR, which is overexpressed on prostate cancer cell surface.\textsuperscript{105-107} The EGFR went through a conformational change that activated Neu1, cleaved 4-MUNANA and produced blue fluorescence as seen in Figure 26. The neuraminidase inhibitor OP dose-dependently reduced Neu1 sialidase activity associated with EGF treated live DU145 (Figure 26A), PC3 (Figure 26B), DU145GemR (Figure 26C) and PC3GemR (Figure 26D) cells comparable to the levels of no EGF treated controls represented by the dark blue images and decreased mean fluorescence density.

OP induced reduction of prostate cancer cell sialidase activity was also tested by the fluorescence spectrophotometry method. As seen in Figure 27, OP reduced sialidase activity of all four cell lines. At OP concentrations $\geq 400\mu\text{g/mL}$, the reduction in sialidase activity was significant.

4.7 Regulatory role of OP in cell aggregation and compaction in prostasphere formation

Since OP inhibits Neu1, it blocks the cleaving of $\alpha_2,3$ sialyl residues on EGFRs\textsuperscript{104} resulting in enhanced $\alpha_2,3$ sialyl residues expression on the cell surface of cancer cells. Here, the role of $\alpha_2,3$ SA expression levels on the efficacy of cyclo-RGDfK(PPP)-induced MCTS formation in parental and gemcitabine resistant DU145 and PC3 cells was investigated.
A

DU145

<table>
<thead>
<tr>
<th></th>
<th>EGF 100µg/mL</th>
<th>OP 50µg/mL+ EGF</th>
<th>OP 100µg/mL EGF</th>
<th>OP 200µg/mL EGF</th>
<th>OP 400µg/mL EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
</tr>
</tbody>
</table>

B

PC3

<table>
<thead>
<tr>
<th></th>
<th>EGF 100µg/mL</th>
<th>OP 50µg/mL+ EGF</th>
<th>OP 100µg/mL EGF</th>
<th>OP 200µg/mL EGF</th>
<th>OP 400µg/mL EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
</tr>
</tbody>
</table>

C

DU145GemR

<table>
<thead>
<tr>
<th></th>
<th>EGF 100µg/mL</th>
<th>OP 50µg/mL+ EGF</th>
<th>OP 100µg/mL EGF</th>
<th>OP 200µg/mL EGF</th>
<th>OP 400µg/mL EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
</tr>
</tbody>
</table>

D

PC3GemR

<table>
<thead>
<tr>
<th></th>
<th>EGF 100µg/mL</th>
<th>OP 50µg/mL+ EGF</th>
<th>OP 100µg/mL EGF</th>
<th>OP 200µg/mL EGF</th>
<th>OP 400µg/mL EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
<td>&lt;Image&gt;</td>
</tr>
</tbody>
</table>
Figure 26. Effect of OP on sialidase activity of prostate cancer cells assessed by the sialidase assay.
Sialidase activity in live DU145 (A), PC3 (B), DU145GemR (C) and PC3GemR (D) cells. Cells were allowed to adhere on 12mm circular glass slides in media containing 10% fetal calf serum for 24 hours. Cells were exposed to increasing concentrations of OP dissolved in 1xDMEM for 24 hours. After removing media, 0.318mM 4-MUNANA substrate in Tris buffered saline pH 7.4 was added to live cells alone (control) or with EGF at 100µg/mL or with EGF and increasing concentrations of OP (50µg/mL-800µg/mL). The substrate was hydrolyzed by sialidase enzymes to give free 4-methylumbelliferone, which had a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at 2 minutes after adding substrate using epifluorescent microscopy (10x objective). The mean fluorescence of 50 multi-point replicates surrounding the cells was quantified using the Image J software. Results were compared by one-way ANOVA at 95% confidence interval using Bonferroni test. Data are a representation of one out of two independent experiments showing similar results.
Figure 27. Effect of OP on sialidase activity of prostate cancer cells assessed by fluorescence spectrophotometry.
DU145, DU145GemR, PC3 and PC3GemR cells were allowed to adhere on a black 96 well optical bottom plate for 24 hours. Cells were exposed to increasing concentrations of OP dissolved in 1xDMEM for 24 hours. After removing media, 50µL of PBS was added to the cells. 0.318mM 4-MUNANA substrate in Tris buffered saline pH 7.4 was added to cells alone (control) or with EGF at 1µg/mL or with EGF and increasing concentrations of OP (50µg/mL-800µg/mL). The fluorescence intensity readings were immediately taken over 1-2 minutes by the Varioskan Fluorescence Spectrophotometer using fluorescence excitation at 365nm and emission at 450nm. Results were compared by one-way ANOVA at 95% confidence interval using Fisher’s LSD test. Data are a representation of one out of two independent experiments showing similar results.
DU145GemR cells were treated with either 100µg/mL or 200µg/mL or 400µg/mL of OP for 24 hours followed by biotinylated lectin staining. OP did not have any significant effects on the expression of α2,3 or α2,6 SA levels (Figure 28A, B). The prostate cancer spheroids were treated with increasing concentrations of OP (50µg/mL to 200µg/mL) in the presence of 50µM cyclo-RGDfK(PP) peptide for 6 days. Interestingly, the volume of resultant MCTS/cell aggregates from all cell lines except PC3 decreased as the concentration of OP was increased from 50-200µg/mL (Figure 30A). The volume of PC3 cell aggregates remained unchanged. However, none of the decreases except for DU145 MCTS volume were significant. A WST-1 cell viability assay was performed to determine if the decrease in MCTS/cell aggregates volume was the result of a cytotoxic effect of OP and that the MCTS/cell aggregates being measured were not mostly rounded dead cells. It was observed that ≥200µg/mL OP significantly reduced the cell viability of DU145, DU145GemR, PC3 and PC3GemR MCTS and cell aggregates (Figure 30B). These results indicate that high concentrations of OP were cytotoxic to the prostate cancer spheroids but did not have any effect on the formation of the spheroids.
Figure 28. Effect of OP on prostate cancer cell surface sialic acid expression.

(A) Fluorescent microscopy images of DU145GemR cells stained with biotinylated lectins followed by DyLight 594 streptavidin. The cells were treated with 100µg/mL or 200µg/mL or 400µg/mL OP for 24 hours before staining. Images were taken with epifluorescent microscope with 20x objective. The bars represent 36µm.

(B) Quantitative analysis was done as described in Figure 12B.
Figure 29. Effect of OP on prostatesphere formation studied by phase contrast images.
Phase-contrast images of DU145, PC3, DU145GemR and PC3GemR cells at 10x objective treated with a combination of 50µM cyclo-RGDfK(TPP) and OP or without OP at concentrations of 50µg/mL, 100µg/mL or 200µg/mL for 6 days. 10,000 cells were plated per well in a 96-well plate for 6 days. The images are a representative of two fields of view in two independent experiments. The scale bar in the top left image represents 508µm. The scale bars of the all the other images have the same measurements. The term TPP refers to cyclo-RGDfK(TPP).
Figure 30. Effect of OP on spheroid/cell aggregate volume and cell viability.
(A) Spheroid/cell aggregate volume was measured using the method described in Figure 19.
(B) Viability of DU145 and PC3 attached cells and prostasphere was determined using WST-1 assay as described in Figure 19.
Chapter 5
Discussion

The classical agarose-coated well technique and the biochemical synthetic cyclo-RGDfK(TPP) peptide based method were used to generate matrix free prostaspheres with the prostate cancer cell lines. Both DU145 and DU145GemR cells formed loose and irregular cell aggregates in the agarose-coated well technique and did not form prostaspheres. However, the same cell lines formed tight compact prostaspheres with a diameter of \( \geq 60 \mu M \) when the cyclo-RGDfK(TPP) peptide based method was applied.\(^{11}\) This finding is consistent with another report where DU145 cells formed tight prostaspheres using the 3D matrigel culture method.\(^{7}\) DU145GemR cells started to aggregate and formed prostaspheres at a lower concentration of cyclo-RGDfK(TPP) peptide compared to DU145 cells that retained their monolayer culture at the same concentration. Parental and resistant PC3 cells were found to form irregular multicellular aggregates. However, at 100\( \mu M \) cyclo-RGDfK(TPP) peptide PC3GemR cells formed only multicellular aggregates with minimal adhered monolayer cells; whereas the PC3 cells were a mixture of adhered monolayer cells and multicellular aggregates after 6 days of incubation. Akasov et al. has previously reported irregular semi-spheroidal aggregates for malignant melanoma A-375 and human glioblastoma A-172 cell lines using the cyclo-RGDfK(TPP) peptide method.\(^{11}\) Hedlund et al. and Vinci et al. both reported consistent formation of PC3 cell aggregates using the classical agarose-coated technique\(^{133}\) and ultra-low attachment plate technique.\(^{41}\) In contrast, Harma et al.\(^{7}\) showed that tightly compact spheroids with PC3 cells could be formed using the 3D matrigel culture system. These differences might be explained by differences in culture conditions, such as cellular density at the moment of seeding, medium composition and the absence of standardized morphological criteria.\(^{134}\) The cyclo-
RGDfK(TPP) peptide based method uses the biochemical properties of the cell surface to form prostaspheres as opposed to the mechanical forces used in the classical method. Cyclo-RGDfK(TPP) peptide mimics the ability of natural ECM protein to induce cell aggregation through binding to \(\alpha_5\beta_1\)-integrin. The 3D spheroid characterization showed unimodal structures, ranging from 60-120\(\mu\)m in diameter, and varying between cell lines and medium serum concentration. The ability of different prostate cancer cell lines to form prostaspheres by the biochemical method might be explained by their expression levels of \(\alpha2,3\) SA and \(\alpha2,6\) SA.

The present study shows that sialic acid content of parental and resistant DU145 and PC3 prostate cancer cell lines influences a cell line’s ability to form prostaspheres. Both the parental and resistant DU145 cells expressed approximately 5-fold greater cell surface \(\alpha2,6\) SA than \(\alpha2,3\) SA. PC3GemR cells showed only about 1.4 times higher expression of cell surface \(\alpha2,6\) SA compared to \(\alpha2,3\) SA. However, only for PC3 cells the relative level of \(\alpha2,3\) SA was 4-fold higher than \(\alpha2,6\) SA. PC3 and PC3GemR cells were unable to form prostaspheres using cyclo-RGDfK(TPP) peptide at all concentrations and time points that were tested. \(\alpha2,6\) SA may play a greater role in cell-cell adhesion and prostasphere formation than \(\alpha2,3\) SA. This is in accordance with reports from other groups that have proposed that the characteristic and biologic outcomes of tumor cells is more influenced by \(\alpha2,6\) sialylation compared to \(\alpha2,3\) sialylation due to its more favorable conformation.\(^{92}\) Takano et al. also reported that the MDAY-D2 murine lymphoma cell line mutant overexpressing \(\alpha2,6\) SA showed 3-10-fold fewer metastases and 60% slower tumor growth. The mutant cells had more \(\alpha2-6\) SA than \(\alpha2,3\) SA on the cell surface.\(^{135}\)

On pretreatment with neuraminidase (\textit{Vibrio Cholerae}) in the presence of 50\(\mu\)M cyclo-RGDfK(TPP) peptide, all four prostate cancer cell lines showed a significant inhibition of cell aggregation and prostasphere formation without having any cytotoxic effects. These findings are
in agreement with our previous reports. These findings suggest that cell surface sialylation may facilitate cell-cyclo-RGDfK(TPP) peptide and cell-cell adhesion and aggregation in prostasphere formation. Our results showing that sialylation plays a role in prostasphere formation is very similar to the reports of Terao et al. who demonstrated that MCTS forming cells and CD24\textsuperscript{high}/CD44\textsuperscript{high} cancer stem-like cells of pancreatic PANC1 cells express higher levels of fucosylated glycans than the monolayer cells. Results in the present study are supported by our previous report on breast (MCF-7) and pancreatic (PANC1) cancer cells lines. MCF-7 and PANC1 cells, and their drug-resistant cancer cell lines (MCF-7 TMX and PANC1-GemR) expressed different SA content, which influenced their ability to form spheroids by the cyclo-RGDfK(TPP) peptide-based biochemical method. Removal or blockage of SA inhibited cell aggregation and spheroid formation with MCF7 and MCF-7 TMX cells under cyclo-RGDfK(TPP) peptide treatment.

E- and N-cadherin expression on the cell surface of prostate cancer cells was analyzed by ICC and flow cytometry to elucidate whether there was a relationship between E-cadherin expression level and the capacity to form prostaspheres. DU145 cells that formed tightly compact prostaspheres had the highest level of cell surface E-cadherins. N-cadherin expression levels were found to be much lower in DU145 cells than that of PC3 cells. However, none of the ICC results were significant. As illustrated by the flow cytometry findings, the expression of both E- and N-cadherin was very weak for all four cell lines. This might be due to the fact that prostate cancer cells DU145 and PC3 retain some stem cell characteristics and their ability to alter its cadherin expression during the process of invasion. Another reason for this unexpected finding may be due to the fact that both E-cadherin positive and negative cell populations naturally co-exist in the DU145 cell lines. However, whether the same can be said
about the PC3 cell line is not known. These unexpected results indicate that spheroids and therefore cell-cell adhesion may be mediated by an unknown E-cadherin independent mechanism.

Glycosylation regulates different aspects of cancer cell behaviour. It effects cell survival, evasion of apoptosis, invasion, metastasis, cell-cell and cell-ECM adhesion. Branched N-glycans influence cell-cell and cell-ECM adhesion mediated through molecules like E-cadherin and integrins. Sialic acid, the terminal monosaccharide on the N-glycan plays a controversial role in cell-cell adhesion as discussed in section 2.5.1. We have previously shown that cell surface sialylation facilitates cell-cell adhesion and thus MCTS formation with human breast MCF-7 and pancreatic PANC1 cell lines using the cyclo-RGDfK(PP) peptide method. The results with prostate cancer MCTS further validate these findings. As stated by Christie et al. increased sialylation of the β1 integrin can result in alteration in the conformation of the integrin receptors, which ultimately affects its interaction with other glycolipid and glyproteins on the cell membrane. Sialic acids regulate conformation and ligand binding site access of cell membrane receptors and adhesion proteins and also alter lateral association between membrane associated proteins. Sialic acids control the bonds and orientation between the N-linked oligosaccharide and the underlying protein. This controls the exposure of the ligand binding site of the adhesion molecules. Lateral association between membrane proteins like growth factor receptors can control intracellular signal transduction pathways. Sialic acid on the surface of one cell might bind to an unknown sialic acid binding site on adjacent cells, pulling the cells close together.

Neu1 has been demonstrated to have a major role in multistep tumorigenesis. Cell surface Neu1 has also been documented to desialylate integrin β4 as evaluated by PNA- and
MAL-2 lectin blotting of immunoprecipitates with anti-integrin β4 antibody. The Neu1 inhibitor OP targets Neu1 and significantly inhibits the survival of cancer cells. It is proposed that this process is initiated by the desialylation of receptor tyrosine kinase. OP has been shown to inhibit Neu1 sialidase activity of LPS-stimulated BMC2 macrophage cells, EGF-stimulated PANC1 pancreatic cancer cells, EGF-stimulated A2780 ovarian carcinoma cells and EGF-stimulated triple negative breast MDA-MB231 cells. Live cell sialidase assay showed that OP has similar Neu1 inhibitory activity on EGF-treated prostate cancer cell lines. The results of the sialidase assay were further confirmed with fluorescence spectrophotometry findings.

Since OP inhibits Neu1, the study hypothesized an increased expression of α2,3 sialyl residues on cell surface glycoproteins of the prostate cancer cell lines. Cell viability of prostaspheres/cell aggregates decreased at ≥200µg/mL OP concentrations. It was also observed that the volume of the prostaspheres decreased with increasing concentration of OP. The decrease in volume could be either due to inhibition of MMP9 mediated cell motility or cytotoxic effect. It was reported that αvβ3 integrin cooperates with MMP9 in breast cancer cell migration. The results of the WST-1 assay showed that the decrease in volume was due to reduced cell viability. Thus, OP did not have any effect on spheroid formation. This can be explained by the finding that OP had no effect on the level of prostate cancer cell surface sialic acid.
Chapter 6
Conclusion and Future Directions

6.1 Conclusion

As stated in section 2.8, this research had four primary objectives: generation of prostaspheres using the cyclo-RGDfK(TPP) based biochemical method, determination of E-cadherin on prostate cancer cells, determination of correlation between ability of metastatic prostate cancer cells to form prostaspheres and sialic acid expression, and analysis of prostasphere formation upon treatment with cell surface sialic acid regulating agents. From these objectives, it was observed that cell surface α2,6 sialic acid may facilitate prostasphere formation and that spheroids may be formed by an E-cadherin independent mechanism. The α2,6 sialic acid may facilitate cyclo-RGDfK(TPP) induced cell aggregation, cell compaction and ultimately tight spheroid formation. Expression of α2,6 sialic acid on cell surface glycoproteins may play a fundamental role in cancer cell-cell adhesion with important implications in the detachment stage of the metastatic cascade and epithelial morphogenesis.

Altered sialylation of tumor cell surface glycoproteins has been described to be highly associated with the metastatic phenotype of cancer. The significance of these data identify the key sialyl residues in the formation of 3D multicellular prostate cancer spheroids mimicking small avascular tumors with complex cell-cell and cell-matrix interactions in culture and help in understanding the mechanism and processes involved in prostate cancer biology.

6.2 Future Directions

This project was focused on the role of prostate cancer cell surface sialic acid on prostasphere formation. The effect of reducing sialic acid on the cell surface was investigated in
this project. It will be interesting to study how the upregulation of sialyltransferase and therefore the increased expression of α2,6 and α2,3 sialic acids influences spheroid formation. This will further our understanding of cell-cell adhesion. The α2,6 sialic acid linkage can be further characterized to determine its role in spheroid formation, metastasis and epithelial morphogenesis. Exploring the different effects of sialylation on spread of cancer cells will help us develop alternate and novel methods of targeting cancer cell glycobiology. It has been reported that the neuraminidase1 inhibitor OP has anti-cancer activity against pancreatic, ovarian and triple-negative breast cancer cell types. In the future, preclinical in vivo anti-cancer activity of OP should be investigated in an animal model of human androgen independent prostate cancer.


