A LOCALIZED CONTROLLED DELIVERY SYSTEM UTILIZING A BLOCK COPOLYMER TO IMPROVE THE CURRENT PARADIGM OF CHEMOTHERAPY

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A LOCALIZED CONTROLLED DELIVERY SYSTEM UTILIZING A BLOCK COPOLYMER TO IMPROVE THE CURRENT PARADIGM OF CHEMOTHERAPY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctorate of Philosophy
Bioengineering

by
Jason Olbrich
August 2013

Accepted by:
Dr. Karen Burg, Committee Chair
Dr. Frank Alexis
Dr. Joel Corbett
Dr. Waleed Shalaby
Dr. Kenneth Webb
ABSTRACT

The goal of the field of Bioengineering has always been to bring about a better standard of care for patients. As a field encompassing a broad array of researchers and talents, such improvements often are brought about through the integration of different fields. This work advances the integration of controlled delivery and chemotherapy. The use of cytotoxic chemotherapy has become and will continue to be a powerful tool for the treatment of solid malignancies. Conventional dose scheduling of cytotoxic agents and newer biological response modifiers are investigated under a clinical paradigm initially designed to identify a drug dose that elicits acceptable levels of toxicity before the rigorous testing of efficacy. Newly developed regimens involving altering of the conventional scheduling are always compared to the current “gold standard” of chemotherapy. This process of evaluation becomes quite cost prohibitive through the number of clinical trials required, and often the “clinically meaningful endpoint” is no longer overall survival, but some more specific event, e.g. the targeted death of a specific type of cell. The traditional method for evaluating new anti-cancer drugs is appropriately intended to minimize patient toxicity and optimize safety. However, the inherent flaw in this approach is that the effective dose and the toxic dose are often not concurrently considered (e.g. the therapeutic index); rather, the approach is geared toward maximizing death of a particular cell type. That is, the traditional approach is to provide the maximum tolerated dose at defined time intervals; the intervals allow recovery of the normal tissue but they also provide time for tumor cell regrowth and potential chemo-resistance. Another obstacle is the conventional (intravenous) routes of administration which often
expose the tumor to relatively short durations of drug, based on half-lives at excessively high systemic levels; toxicity is cumulative and associated with high systemic levels of drug. The theoretical benefits of controlled drug delivery can minimize significant fluctuations in systemic toxicity while optimizing efficacy. Such controlled therapy requires a defined therapeutic index and a delivery platform that can be tailored to meet the desired clinical endpoint. As understanding of cancer has grown, novel strategies have been developed to selectively administer conventional chemotherapy to manage advanced cases of solid malignancies that historically have a poor prognosis. The largest obstacle to the use of such approaches is the dearth of technology for delivering a chemotherapeutic in a repeatable controlled manner. To date, there is a paucity of existing biomaterials whose properties allow (1) in situ gelation kinetics that can be modulated to produce a predictable and sustainable drug delivery depot, (2) the ability to control in situ gelation kinetics to maximize vascular and micro-vascular access to the tumor, (3) predictable controlled release kinetics and duration of release in concert with the total drug load to minimize dose dumping and maximize release, (4) and modulated absorption kinetics to minimize collateral damage to healthy tissue and facilitate repeat administration based on tumor response or treatment requirements.

At Poly-Med Incorporated, the OC Polymer system was specifically designed to combat current issues with chemotherapy. These polymers, when combined in a novel delivery system, allow localized controlled delivery. The system advanced in this work is comprised of the OC polymer, solubilized in a low molecular weight polyethylene glycol,
and further absorbable polymers to help modulate release. This system is injectable through a standard Leur-Lok needle and syringe system and forms a stable depot after injection into an aqueous environment. The primary objective of the work was to test the OC delivery system in an *in vitro* and an *in vivo* setting, laying the foundation for investigation of this system in multiple chemotherapeutic treatments. The specific aims of the project were to (1) determine the potential efficacy of the system through the performance in *in vitro* studies to determine release characteristics with a model drug; (2) ensure the relevancy of the results to a chemotherapy-based system through further *in vitro* testing with the platinum-based chemotherapeutic carboplatin; and (3) exhibit the clinical potential of the product through *in vivo* testing of a promising OC system in two administration methods using a mouse flank model.

The OC polymer system was examined for release *in vitro* and was shown capable of release of a water-soluble model drug from 3 days to greater than 45 days. Also, the OC system was shown capable of release of the chemotherapeutic carboplatin in a relatively linear manner over a period of 3 weeks, the course of a typical chemotherapeutic regimen. Finally, the OC polymer system was examined in an *in vivo* model and tolerance of the non-drug loaded system was exhibited. Also, mice were shown to tolerate higher levels of total carboplatin exposure than the literature suggested LD$_{50}$ of 150 mg/kg. The next steps in preparing such a system involve further *in vitro* studies and *in vivo* testing to validate the efficacy of such a system prior to clinical trials.
DEDICATION

I would like to dedicate this manuscript to my loving and caring wife Katie, without her support this endeavor would not have been possible.

I would also like to dedicate this manuscript in the memory of Dr. Shalaby Wahba Shalaby, who touched many lives through his body of work as well as his personal mentoring of students such as myself. I hope to honor his memory with my contributions to the field.
ACKNOWLEDGMENTS

I would like to thank Poly-Med Incorporated and the Shalaby family for their support throughout this process. I would also like to thank the members of my committee, Dr. Frank Alexis, Dr. Joel Corbett, Dr. Waleed Shalaby, Dr. Kenneth Webb and especially my committee chair Dr. Karen Burg, for their time and mentorship at multiple instances throughout my scholastic career thus far. Further, I thank those such as Dr. Patilee Tate and the staff at Godley-Snell Research Center who took the time to help teach me patiently.

Lastly, I would like to thank my parents who sacrificed much to provide me the best education and opportunity to succeed.
This dissertation describes the development of a drug delivery system through the early stages of design, \textit{in vitro} testing and \textit{in vivo} screening. The chapters each represent a different stage of that process, and are outlined in the Figure provided. Chapter 1 provides a general overview of cancer and chemotherapy. Specifically, many different chemotherapy agents are reviewed for clinical significance. Chapter 2 further elucidates the background of the field through a review of oncological methods. New and novel methods and technologies are reviewed. Chapter 3 provides the background on the OC system itself. The polymers involved are discussed and characterized, and a representative polymer is advanced. In Chapter 4, release is explored from the OC system, with the model drug doxycycline. Release is studied from many different configurations of the system, showing the modularity of the platform as a whole. Chapter 5 further illustrates the modularity of the system with the use of many different pharmacuetics in regards to release. These studies were designed to show the many different indications for which the system can be used. Finally, Chapter 6 details the \textit{in vivo} work performed. Preliminary studies, assay exploration and a maximum tolerated dose study are all recorded to lay the ground work.
for a future full-scale animal study. Portions of many of these chapters have been published in the following:


# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>PREFACE</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. CANCER AND CHEMOTHERAPY</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Types of Cancer</td>
<td>4</td>
</tr>
<tr>
<td>Chemotherapeutic Agents</td>
<td>12</td>
</tr>
<tr>
<td>Tumor Microenvironment and Growth</td>
<td>26</td>
</tr>
<tr>
<td>II. TUMOR TREATMENT IN MODERN MEDICINE</td>
<td>33</td>
</tr>
<tr>
<td>Tumor Treatment and Administration Methods</td>
<td>33</td>
</tr>
<tr>
<td>Extension – What Next?</td>
<td>44</td>
</tr>
<tr>
<td>III. THE OC SYSTEM: A METHOD FOR LOCALIZED SUSTAINED DELIVERY</td>
<td>48</td>
</tr>
<tr>
<td>Overview</td>
<td>48</td>
</tr>
<tr>
<td>Polymers</td>
<td>50</td>
</tr>
<tr>
<td>Diluent</td>
<td>57</td>
</tr>
<tr>
<td>Additives</td>
<td>59</td>
</tr>
<tr>
<td>Injectability Checks</td>
<td>60</td>
</tr>
<tr>
<td>Strengths of Such a System</td>
<td>62</td>
</tr>
</tbody>
</table>
Table of Contents (Continued)

IV. DELIVERY OF MODEL HYDROPHILIC DRUG AND EVALUATION OF VARIABLES THEREIN ................................................................. 64

- Introduction ............................................................................. 64
- Liquid OC Comparison ......................................................... 68
- Solid OC Release Profiles ..................................................... 73
- Additive Use – A6 ................................................................. 74
- SW Polymer Additives .......................................................... 80
- BTO3 Additive ...................................................................... 84
- Diluent Effect on Release ....................................................... 85
- Blending of OCs and Effect on Release ................................. 89
- Conclusion ........................................................................... 93

V. RELEASE OF OTHER DRUGS FROM THE OC SYSTEM; USE OF REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY .......................................................... 94

- HPLC ................................................................................ 95
- HPLC System and Parameters .............................................. 97
- Method Development: A General Outline ........................... 97
- Antibiotics, the OC System, and Clinical Need .................... 101
- Cefuroxime ....................................................................... 101
- Clindamycin ...................................................................... 105
- Dicloxacillin ...................................................................... 108
- Metronidazole .................................................................... 111
- Paclitaxel .......................................................................... 115
- Tobramycin ........................................................................ 119
- Carboplatin ........................................................................ 123
- Conclusion .......................................................................... 126

VI. THE USE OF THE OC SYSTEM TO DELIVER CARBOPLATIN IN AN ATHYMIC NUDE MOUSE MODEL ........................................... 128

- The Murine Model and the Relation to the OC System .......... 128
- Preliminary Work ................................................................. 131
- Cell Culturing and Testing of Efficacy with Carboplatin Eluents ...... 145
- Full Pilot Study ................................................................. 146
- Discussion ........................................................................... 156

VII. CONCLUSIONS .................................................................... 164
<table>
<thead>
<tr>
<th>Table of Contents (Continued)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII. RECOMMENDATIONS FOR FUTURE WORK</td>
<td>166</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>168</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>The OC Polymerization: A General Procedure</td>
</tr>
<tr>
<td>3.2</td>
<td>Pertinent Properties of OC9 Lot 1 Purified</td>
</tr>
<tr>
<td>3.3</td>
<td>Injectability of Multiple Polymers in Differing Diluent Percentages</td>
</tr>
<tr>
<td>4.1</td>
<td>Optimized Gradient HPLC Method for Detection of Doxycycline</td>
</tr>
<tr>
<td>4.2</td>
<td>Partial Mass Balance Evaluation of Drug Release from the Liquid OC Systems</td>
</tr>
<tr>
<td>5.1</td>
<td>Percent Inhibition of S. Aureus by Cefuroxime Eluents</td>
</tr>
<tr>
<td>5.2</td>
<td>Percent Inhibition of S. Aureus by Clindamycin Eluents</td>
</tr>
<tr>
<td>5.3</td>
<td>Percent Inhibition of S. Aureus by Dicloxacillin Eluents</td>
</tr>
<tr>
<td>5.4</td>
<td>Percent Inhibition of V. parvula by Metronidazole Eluents</td>
</tr>
<tr>
<td>6.1</td>
<td>Results of Preliminary Animal Studies</td>
</tr>
<tr>
<td>6.2</td>
<td>Groups and Subjects for the Pilot Study</td>
</tr>
<tr>
<td>6.3</td>
<td>Subject Weights during Course of Study</td>
</tr>
<tr>
<td>6.4</td>
<td>Results of GPC Data from Mice Within The Study</td>
</tr>
<tr>
<td>6.5</td>
<td>Selected Results of CBC Testing (Cell Count)</td>
</tr>
<tr>
<td>6.6</td>
<td>Selected Results of CBC Testing (White Blood Cell Makeup)</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Methotrexate, the original chemotherapeutic (Source <a href="http://www.chemicalbook.com">www.chemicalbook.com</a>)</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Carboplatin, the second generation member of the platinum drug family (Source: <a href="http://www.chemistry.about.com">www.chemistry.about.com</a>)</td>
<td>14</td>
</tr>
<tr>
<td>2.1</td>
<td>Example configuration of a liposome (Source: <a href="http://www.thescientist.com">www.thescientist.com</a>)</td>
<td>39</td>
</tr>
<tr>
<td>2.2</td>
<td>Example of a thermogelling system</td>
<td>43</td>
</tr>
<tr>
<td>3.1</td>
<td>Theoretical mechanistic diagram of OC system introduction into an aqueous environment</td>
<td>49</td>
</tr>
<tr>
<td>3.2</td>
<td>Complex viscosity over time for gelation of OC polymer and organic solvent</td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td>Theoretical demonstration of mechanism of large molecular weight additive interaction with depot formation</td>
<td>59</td>
</tr>
<tr>
<td>4.1</td>
<td>Example chromatogram of doxycycline HPLC run</td>
<td>66</td>
</tr>
<tr>
<td>4.2</td>
<td>Example of doxycycline standard curves utilized throughout study</td>
<td>67</td>
</tr>
<tr>
<td>4.3</td>
<td>Doxycycline release from the liquid OC polymers</td>
<td>69</td>
</tr>
<tr>
<td>4.4</td>
<td>Release profiles of the solid OC polymers</td>
<td>73</td>
</tr>
<tr>
<td>4.5</td>
<td>Effect of doxycycline release from OC2 system and the effect of A6 inclusion</td>
<td>75</td>
</tr>
<tr>
<td>4.6</td>
<td>Effect of doxycycline release from OC4 system and the effect of A6 inclusion</td>
<td>76</td>
</tr>
<tr>
<td>4.7</td>
<td>Effect of doxycycline release from OC6 system and the effect of A6 inclusion</td>
<td>77</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>78</td>
</tr>
<tr>
<td>4.8</td>
<td>Effect of doxycycline release from OC4 system and the effect of A6 inclusion</td>
</tr>
<tr>
<td>4.9</td>
<td>81</td>
</tr>
<tr>
<td>4.9</td>
<td>The effect of the SW family of additives on release from the OC9 delivery system</td>
</tr>
<tr>
<td>4.10</td>
<td>82</td>
</tr>
<tr>
<td>4.10</td>
<td>Comparison of A6 and SW2: effect on DOX release from OC9</td>
</tr>
<tr>
<td>4.11</td>
<td>83</td>
</tr>
<tr>
<td>4.11</td>
<td>Release of DOX from OC9/SW2 system over 42 days with line of best fit</td>
</tr>
<tr>
<td>4.12</td>
<td>84</td>
</tr>
<tr>
<td>4.12</td>
<td>Effect of doxycycline release from OC4 system and release difference between BTO3 and BTO3S</td>
</tr>
<tr>
<td>4.13</td>
<td>86</td>
</tr>
<tr>
<td>4.13</td>
<td>Comparison of DOX release from OC9 system between PEG and G4A diluents</td>
</tr>
<tr>
<td>4.14</td>
<td>87</td>
</tr>
<tr>
<td>4.14</td>
<td>Effect of doxycycline release from OC4 systems with varied diluents</td>
</tr>
<tr>
<td>4.15</td>
<td>88</td>
</tr>
<tr>
<td>4.15</td>
<td>Comparison of DOX release from OC9 system between a 67/33 and 50/50 system of G4A</td>
</tr>
<tr>
<td>4.16</td>
<td>90</td>
</tr>
<tr>
<td>4.16</td>
<td>Comparison of DOX release from blended OC systems</td>
</tr>
<tr>
<td>4.17</td>
<td>91</td>
</tr>
<tr>
<td>4.17</td>
<td>Comparison of DOX Release from OC Blends</td>
</tr>
<tr>
<td>4.18</td>
<td>92</td>
</tr>
<tr>
<td>4.18</td>
<td>Comparison of DOX release from OC blends of differing diluent composition</td>
</tr>
<tr>
<td>5.1</td>
<td>98</td>
</tr>
<tr>
<td>5.1</td>
<td>A 5-95% run of cefuroxime on HPLC</td>
</tr>
<tr>
<td>5.2</td>
<td>99</td>
</tr>
<tr>
<td>5.2</td>
<td>A first attempt at a “15% method” for cefuroxime</td>
</tr>
<tr>
<td>5.3</td>
<td>100</td>
</tr>
<tr>
<td>5.3</td>
<td>Acceptable method for cefuroxime analysis on HPLC</td>
</tr>
<tr>
<td>5.4</td>
<td>102</td>
</tr>
<tr>
<td>5.4</td>
<td>Cefuroxime standard curve at 275 nm</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>Release of cefuroxime from OC4 gel systems</td>
<td>103</td>
</tr>
<tr>
<td>5.6</td>
<td>Clindamycin standard curve at 210 nm</td>
<td>106</td>
</tr>
<tr>
<td>5.7</td>
<td>Release of clindamycin from OC4 gel systems</td>
<td>107</td>
</tr>
<tr>
<td>5.8</td>
<td>Dicloxacillin standard curve at 225 nm</td>
<td>109</td>
</tr>
<tr>
<td>5.9</td>
<td>Release of dicloxacillin from OC4 gel systems</td>
<td>110</td>
</tr>
<tr>
<td>5.10</td>
<td>Metronidazole standard curve at 275 nm</td>
<td>112</td>
</tr>
<tr>
<td>5.11</td>
<td>Release of metronidazole from OC4 gel systems</td>
<td>113</td>
</tr>
<tr>
<td>5.12</td>
<td>Paclitaxel standard curve at 230 nm</td>
<td>117</td>
</tr>
<tr>
<td>5.13</td>
<td>Paclitaxel extraction efficiency study</td>
<td>118</td>
</tr>
<tr>
<td>5.14</td>
<td>Tobramycin standard curve at 254 nm</td>
<td>120</td>
</tr>
<tr>
<td>5.15</td>
<td>Tobramycin time complex stability study</td>
<td>121</td>
</tr>
<tr>
<td>5.16</td>
<td>Release of tobramycin from OC4 gel systems</td>
<td>122</td>
</tr>
<tr>
<td>5.17</td>
<td>Carboplatin standard curve at 240 nm</td>
<td>124</td>
</tr>
<tr>
<td>5.18</td>
<td>Release of carboplatin from OC9 gel systems</td>
<td>125</td>
</tr>
<tr>
<td>5.19</td>
<td>Release of carboplatin from an OC9/PEG/SW2 gel system</td>
<td>126</td>
</tr>
<tr>
<td>6.1</td>
<td>Image of depot deposited in Mouse A (24 hours post injection)</td>
<td>134</td>
</tr>
<tr>
<td>6.2</td>
<td>Liver from Mouse A swollen and visibly diseased</td>
<td>135</td>
</tr>
<tr>
<td>6.3</td>
<td>Hind flank placement of polymer depot (Mouse B 6 days post implantation)</td>
<td>137</td>
</tr>
<tr>
<td>6.4</td>
<td>Mouse B following euthanasia (21 days post implantation)</td>
<td>138</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>Mouse C at sacrifice (6 days post implantation)</td>
<td>139</td>
</tr>
<tr>
<td>6.6</td>
<td>In clockwise order from top-left (1) Mouse D kidney at 40x (2) Mouse D kidney at 10x (3) Mouse D liver at 10x (4) Mouse D liver at 40x</td>
<td>140</td>
</tr>
<tr>
<td>6.7</td>
<td>GPC results of OC system following mixing but prior to injection</td>
<td>141</td>
</tr>
<tr>
<td>6.8</td>
<td>GPC results of OC system extracted from murine model</td>
<td>142</td>
</tr>
<tr>
<td>6.9</td>
<td>GPC results of neat OC9 polymer</td>
<td>142</td>
</tr>
<tr>
<td>6.10</td>
<td>GPC results of neat PEG 400</td>
<td>143</td>
</tr>
<tr>
<td>6.11</td>
<td>GPC Results of neat SW2 (no peak present)</td>
<td>143</td>
</tr>
<tr>
<td>6.12</td>
<td>Liver histology images (H&amp;E staining) of mice 1, 5, 9, 16, and 20. Images are displayed clockwise in numeric order with mouse 1 in the top-left corner. (10x objective magnification)</td>
<td>154</td>
</tr>
<tr>
<td>6.13</td>
<td>Kidney histology images (H&amp;E staining) of mice 1, 5, 9, 16, and 20. Images are displayed clockwise in numeric order with mouse 1 in the top-left corner. (10x objective magnification)</td>
<td>155</td>
</tr>
</tbody>
</table>
CHAPTER ONE

CANCER AND CHEMOTHERAPY

Introduction

One malady has hung over society more than any other in the history of modern medicine. Cancer has seen a steady increase over the years; one out of every two children born today will be diagnosed with the disease at some point in their lives\(^1\). Malignant tumors can manifest in almost any system in the human body and, untreated, will metastasize to other organs and tissues leading inevitably to death of the host. The mortality of the disease and the lack of a “cure” lead to the vital necessity of effective treatments. Treatments will vary slightly depending on the area of affliction; however they commonly consist of surgery, radiation and chemotherapy\(^2\). Surgery is the first line of defense against most operable tumors and is usually at least partially effective against localized, relatively early stage cancers\(^3,4\). However, in patients with recurrent tumors or tumors in inoperable areas, chemotherapy is indicated or possibly the only option\(^3,5\).

Chemotherapy has evolved over the years, and is generally considered to originate in the period surrounding World War II (1939-1945). After observing rapid tissue growth brought on by unchecked cell proliferation, Louis Goodman and his group at Yale University investigated means to slow this phenomenon\(^6\). The effect of a toxic agent used in the war, mustard gas, was studied and found to retard the growth. Concurrently, a group at Harvard University examined means with which to synthesize antagonists for tumor growth. Their work culminated with the development of one of the first
synthesized folic acid antagonists, aminopterin. This discovery lead to significant success in treating lymphomas and pediatric acute leukemia.

Despite the evidence presented in Farber’s publication, skepticism remained prevalent in the field toward the effectiveness of aminopterin and the next generation amethopterin. It took fully a decade more before solid evidence came forward describing the effectiveness of these antagonists, specifically amethopterin (modern methotrexate) in combating unrestricted cellular growth. More significant than the proof of folic acid antagonists was the fact that researchers at the National Cancer Institute were able to force a solid tumor into remission for the first time using chemotherapeutic agents. Methotrexate (Figure 1.1) is still used to this day in combating choriocarcinoma.

Chemotherapeutics became an area of intense interest for research, both public and private. The first alkaloid chemotherapeutic, Vinblastine, was independently developed in 1958 at both Eli Lilly and the University of Western Ontario. The alkaloid group of drugs was significant because their mechanism of action differed from that of methotrexate in that they interfered with tubulin. This in turn disrupted microtubule production during mitosis, and hence retarded cellular reproduction. This development gave

![Figure 1.1: Methotrexate, the original chemotherapeutic (Source www.chemicalbook.com)](image-url)
rise to a revolution in the field of chemotherapeutics, leading to drugs designed to interfere with the mitotic process rather than design of simple antagonists such as methotrexate.

The next key breakthrough in the development of chemotherapy was the concept of combination therapy, or the use of multiple drugs against a single tumor. Combination therapy relies on the principle of “multiple pathways”, meaning that, should an individual tumor develop a resistance or immunity to a certain type of chemotherapeutic, the treatment paradigm also contains a complimentary drug that acts through a differing mechanism. In this way the tumor is theoretically always subjected to an effective chemotherapeutic drug.

However, even with combination therapy, large volume or advanced stage disease would be refractive to treatment. Time and time again it has been proven that chemotherapy is more effective against smaller groups of cells, perhaps because most chemotherapeutic agents are more effective against mitotic cells than cells in the resting phase\textsuperscript{11} and larger, more developed tumors will have a smaller percentage of cells undergoing mitosis at any one time. Due to the relative ineffectiveness of chemotherapeutics in affecting medium and advanced stage tumors, chemotherapy is often combined with other methods\textsuperscript{12}. This combination is referred to as adjuvant therapy, which infers any combination of surgery, chemotherapy, radiotherapy, or hormonal therapy to combat cancerous growths.
Adjuvant therapy is the modern primary care paradigm for most operable tumors such as melanoma, colon cancer and prostate cancer, amongst others\textsuperscript{11,12,13}.

Modern chemotherapy revolves around adjuvant therapies with multiple different drugs. Over the years, different families of chemotherapeutic drugs have been developed. Some of the more commonly used among these are the taxanes, anthracyclines, and platinum-based drugs. Upon diagnosis, attending physicians design the best course of treatment for each individual and choose the components of adjuvant therapy and the drugs used based on previous clinical data.

\textit{Types of Cancer}

Proper and prompt diagnosis of cancer is the most vital step in combating the disease. Local cancers have been documented as appearing in almost every organ in the human body. Of all cancers diagnosed in the United States, at least one out of every two is defined as lung, female breast, prostate, or colorectal\textsuperscript{14}. These cancers are colloquially referred to as the “Big 4” cancers. Appropriately, a significant portion of research and development funding is directed at each of these diseases.

Lung cancer affects nearly 7\% of the American population during their lifetime according to 2011 National Cancer Institute statistics\textsuperscript{1}. Lung cancer is divided into Small Cell Lung Carcinoma (SCLC) and Non-Small Cell Lung Carcinoma (NSCLC). NSCLC accounts for 70-80\% of diagnosed cases and is usually relatively advanced at the time of
Due to the nature of the disease, specifically the presentation of a tangible tumor, surgery is usually the primary mode of treatment. However, this treatment only results in a 20 to 30% survival rate. Early detection and adjuvant therapy including radio- or chemotherapy can improve this rate, but NSCLC mortality rates remain high even with such treatment. Chemotherapeutic agents used in conjunction with surgery include taxane, pyrimidines, camptothecin analogues, vinorelbine, and platinum combinations thereof. SCLC is more difficult to operate upon and responds better to primary chemotherapy than NSCLC, with upwards of a 45% survival rate. Surgery can be used as an adjuvant therapy in cases of SCLC, but is not common.

While lung cancer is the leading cause of death by cancer in males, breast cancer claims more females every year than any other form of cancer. Breast cancer usually manifests itself within the mammary areas of the breast. As recently as 1980, the main method for treatment was mastectomy, in which the cancerous portion of the breast was removed as well as a majority of the surrounding tissue, the latter as a prophylactic measure. Due to the high public profile of the disease, a large amount of funding and research targets this area. Through research and the advent of screening technologies, multiple new avenues are available for treatment, and mastectomy has been restricted to preventative measures and “last resort” cases. Simple radiotherapy or primary chemotherapy is capable of inducing tumor remission or at least requiring only a partial mastectomy, leaving a cosmetically acceptable breast. Early screening procedures have provided the largest opportunity to combat breast cancer non-operatively, by allowing...
chemotherapeutics to be used in the early stages of the disease\textsuperscript{21}. Indeed, the paradigm for treating breast cancer calls for early detection and primary treatment with drugs such as doxorubicin or paclitaxel\textsuperscript{22}. If the primary treatment is not entirely successful, surgery can be used to augment chemotherapy; this approach is called neoadjuvant therapy\textsuperscript{23}. Other less traditional treatment mechanisms include hormonal therapy, oral chemotherapy, and anti-angiogenesis therapy\textsuperscript{24, 25}. Each of these treatments, while originating in breast cancer treatment, is beginning to gain traction in other areas of cancer research.

The third “Big 4” cancer is cancer of the prostate. Affecting mainly the male organ used in reproduction, this disease will affect 16\% of men at some point during their lives\textsuperscript{1}. Myriad prostate cancer treatments have been developed over the years, but few have been proven repeatedly effective\textsuperscript{26}. The most common primary treatment is surgery\textsuperscript{27}. The procedure, radical prostatectomy, is intended to remove or reduce the cancerous tissue\textsuperscript{28}. Other options range from hormonal therapy, to radio therapy, to active non-action (“Watchful Waiting”)\textsuperscript{29}. Chemotherapy has been largely ineffective in the realm of prostate cancer, but recent studies with the taxanes have shown promise\textsuperscript{30}. Because of the absence of effective chemotherapy, the leading adjunctive therapy is hormonal. Hormonal therapies, such as those including antiandrogens, are as effective as castration in arresting prostate cancer\textsuperscript{31}. The quality of life advantages of this treatment far exceed castration, in at least the obvious matter of retaining sexual function. However, the largest recent contribution to the battle against this disease has come in the form of
enhanced screening and early detection. Prostate-specific antigen (PSA) is a protein, the level of which can be monitored to allow extremely early detection of prostate problems. Over half of prostate issues detected by this mechanism can be readily treated. Cancer allowed to progress past this stage, often to hormone refractory prostate cancer which cannot be treated by castration or hormone therapy, is often terminal. In the absence of a highly effective treatment, early detection is by far the best available practice for combating this disease.

The final, and perhaps least publicized, of the Big 4 cancers is colorectal cancer. In this instance, carcinomas develop from polyps within the intestinal tract. While not all polyps will develop into carcinomas, the most effective way to treat colorectal cancer is to identify and swiftly deal with those polyps which have the potential to proliferate in a malignant manner. When early detection fails, colorectal cancer often leads to a poor prognosis as the highly vascularized nature of the area allows quick and widespread metastization. Many individuals are reluctant to undergo screening; this failure to seek medical screening largely contributes to colorectal cancer’s moniker “the silent killer”. When the disease is allowed to progress, surgery is the frontline treatment prescribed in the majority of cases. Prior to metastasis, when tumors are relatively small, surgical treatment for colorectal cancer has a relatively high success rate. This success rate can be enhanced by radiologic imaging techniques which include barium enemas prior to positron emission tomography (PET).
are particularly useful in the colorectal arena due to the non-uniform nature of the lower intestinal tract.

Secondary treatment, where surgery alone is not successful, usually focuses on adjuvant therapy using chemo- or radiotherapy, with physicians often favoring anti-neoplastic drugs\textsuperscript{37}. The current paradigm of chemotherapy for colorectal cancer is seen in the form of the drug 5-fluorouracil, a fluoropyrimidine which is capable of oral administration\textsuperscript{39, 40}. This drug, which disrupts deoxyribonucleic acid (DNA) replication and thus cell division, has been shown to combat localized forms of colorectal cancer as well as metastasized forms of the disease\textsuperscript{40}. Accordingly, oral fluorouracil is often employed in conjunction with surgery in frontline treatment for colorectal cancer, which is detected only at a late, aggressive stage. Other chemotherapeutics have been developed\textsuperscript{39}, mostly within the fluoropyrimidine family, but 5-fluorouracil remains the chemotherapy of choice for colorectal cancer.

Aside from the aforementioned “Big 4” group of cancers, carcinomas can manifest themselves in almost any organ of the body. Perhaps the deadliest area for cancer to develop is within the cranial cavity. Rarely developing spontaneously, brain cancer often is the result of metastasis of another cancer, such as that of the breast or lung\textsuperscript{41, 42, 43, 44}. Unlike other cancers, surgery is only a viable option in about 25\% of cases\textsuperscript{45}. Lack of suitability for surgical treatment\textsuperscript{49} means that chemotherapy\textsuperscript{43, 45} and, to a lesser extent, radiotherapy\textsuperscript{42}, are the frontline treatments for brain cancer. Indeed, chemotherapy is
indicated for at least a portion of the primary therapy in up to 72% of brain cancer cases\textsuperscript{46}.

While the necessity of chemotherapy in cases of brain cancer is obvious, the methodology is not. The lipophilic nature of the blood-brain barrier (BBB) is seen as a deterring factor in the delivery of almost any water-soluble anti-neoplastic\textsuperscript{43} and, as such, systemic delivery of lipophilic drugs will often preferentially deliver to sites other than the brain\textsuperscript{47}. To this end, direct injection and implantation of delivery vectors such as drug loaded “wafers” have been investigated\textsuperscript{43, 47}. Regrettably, even with these theoretically more efficient methods, drug delivery to the brain remains difficult\textsuperscript{48}. Great advances will be needed in the coming decade to improve the poor mortality rates (around 80%) associated with brain cancer\textsuperscript{50}.

Brain cancer can occasionally be caused by the metastasis of ovarian cancer\textsuperscript{51}. Ovarian cancer is an important malady as it is the highest gynecological cause of death among women\textsuperscript{52}. While the origins of this disease are not fully understood, carcinomas in the ovary and surrounding tissue are thought to stem from hormonal imbalances/inductions\textsuperscript{53}. Further complicating the issue is the high rate of metastasis and secondary cancers, thought to be brought about by cellular transport through the lymphatic drainage system, the latter which is prevalent in the gynecologic region\textsuperscript{54}.
Ovarian cancer treatments often use chemotherapy as a frontline option, due to the high rate of metastasis and difficulty of surgery\textsuperscript{55}. The current paradigm relies on the use of a taxane, usually paclitaxel, in conjunction with a platinum drug\textsuperscript{56, 57, 58, 59}. Other drugs, such as doxorubicin, can be used as long as their toxicity levels are held in check\textsuperscript{60}. One such method for reducing toxicity that has been studied effectively in ovarian cancer is dose-dense therapy\textsuperscript{61, 62, 63}. This therapy relies on the shortening of the treatment window and the use of lower doses of drug. Other treatments have been attempted, such as administration of vascular endothelial growth factor (VEGF) or retinoid analogs in order to circumvent traditional chemotherapeutic toxicity\textsuperscript{64, 65}. However, these therapies have a toxic effect, as is expected from almost any anti-angiogenic treatment\textsuperscript{66}. Further work is obviously needed as the prognosis for this disease is still poor throughout the world\textsuperscript{67}.

Any discussion of cancers would be incomplete without inclusion of skin cancer, the most common cancer in man\textsuperscript{68}. While this disease is largely treatable, with lower than average mortality rates among cancers, it will afflict large proportions of the population during their lives\textsuperscript{69}. While skin cancer is largely initiated by overexposure to ultraviolet radiation in the form of sunrays, it can still afflict humans with higher proportions of pigmentation in their skin\textsuperscript{69, 70}. Also, some sections of the population will be more susceptible to developing the more malignant forms of this cancer based on genetic disposition\textsuperscript{71}. The deadliest form of skin cancer is a malignant melanoma.
Malignant melanomas will account for less than 2% of cancers annually\textsuperscript{72}, but if the disease is not caught in the early stages, the 5-year survival rate shrivels to less than 10\%\textsuperscript{73}. If caught early, surgery and palliative treatment can often be enough to check the growth of the tumor; however, as the disease progresses metastasis occurs\textsuperscript{72}. Malignant melanomas are among the most aggressive of all cancers in terms of metastasis, hence the need for early detection and treatment\textsuperscript{73}. Following this metastasis, there is still no clear path for treatment as most therapies in use are poorly tolerated or produce unacceptably low rates of success\textsuperscript{74}. Therapies such as retinoid chemotherapy\textsuperscript{75} and molecular/gene therapy\textsuperscript{76,77} have been used to attempt to combat melanoma metastasis. However, the current benchmark for success uses systemic distribution of platinum or nitrosourea drugs to attempt to halt and force recession of the aggressive cancer\textsuperscript{74}. Due to the generally poor prognosis, current detection is more important in this case than in almost any other cancerous case, due to the dramatic difference between early and late detection survival rates. Future work in the battle against metastasized melanomas will focus on more effective administration of systemic chemotherapeutic/gene therapy.

For the past hundred years cancer has predominantly been treated with surgical resection\textsuperscript{78}. However, a shift towards non-surgical techniques is occurring, with promising results. More and more commonly, chemotherapy is being used to treat cancer. Increases in lifespan and elimination of malignant tissue have been realized in all age groups, even in pediatric groups\textsuperscript{79}. Chemotherapy is administered in myriad ways, including orally, intravenously, subcutaneously and intralesionally\textsuperscript{78,79,80}. The treatment
can be used independently in the frontline, adjuvantly (following surgery), concurrently with surgery or radiotherapy, or as a method of last resort (salvage therapy)\textsuperscript{78, 79}. The delivery pathway and usage are determined through the cancer presented and the patient’s disposition.

\textit{Chemotherapeutic Agents}

Chemotherapy offers advantages over traditional surgical, and even radiological techniques\textsuperscript{81}. These benefits largely stem from the mechanism of action, as chemotherapy targets cells mainly during the proliferative stage. Thus, by interfering with the reproduction of cells, the growth of large, fast-growing populations of cells can be checked or the cells may even be forced to recede. One of the distinguishing elements of tumor cells is that they reproduce more quickly than normal human cells. Therefore, by administering an agent which disrupts cells during this phase of cell life, cancer cells can be targeted preferentially over normal human cells. Because of this induction of cell death, chemotherapeutics are often referred to as “cytotoxic drugs”\textsuperscript{78, 82}.

It is important to understand the types of agents used to undertake this treatment, as they all differ in properties and mechanism of action. While most act to disrupt cell division in some way, different groups of drugs act in different manners. Similar to selecting an antibiotic, choosing the correct drug often makes the difference between success and failure of a given treatment. There are nine major families of cytotoxic drugs.
Alkylating agents are among the most widely used chemotherapeutics, due to their broad spectrum of action\textsuperscript{83, 84}. This class of drugs, comprising mainly cyclophosphamide and ifosfamide, acts mainly by covalently binding to exposed DNA, hence halting or disrupting the replication of cells. These drugs are regarded for their large therapeutic window\textsuperscript{85,86} which allows lessening of virulent side-effects during use. Another interesting attribute of these drugs is that their mechanism of action has been shown to be catalyzed through an increase in heat; as increased temperature (hyperthermia) is often a condition surrounding malignant tumors, this characteristic serves to increase the alkylating agent’s effectiveness\textsuperscript{87}. Further use of the alkylating agents, cyclophosphamide especially, is in metronomic dosing. This dosing schedule entails the use of a more frequent, less intense treatment. Cyclophosphamide has exhibited anti-angiogenic properties which add even further to the effectiveness of the drug\textsuperscript{88}. This class of drugs is not without drawbacks, among which are low selectivity and targeting ability for cancerous cells and the quick inducement of tumor resistance\textsuperscript{83}. However, numerous studies have shown one alkylating agent to be effective against tumor growth once the tumor has become resistant to the other\textsuperscript{89}. Thus these agents are indicated as first and secondary treatments against a variety of cancers, including head and neck\textsuperscript{84}, non-small cell lung\textsuperscript{84}, breast\textsuperscript{84,90}, cervical\textsuperscript{94}, soft-tissue\textsuperscript{91}, bone\textsuperscript{93}, uterine\textsuperscript{84} and ovarian cancer\textsuperscript{84,92}. These compounds are often used in conjunction with other cytotoxic drugs in combination therapies\textsuperscript{91,93}. 
Another family of chemotherapeutics, often used in combination with the alkylating agents\textsuperscript{92}, is the platinum family of drugs. This family consists of three generations of drugs. The first, cisplatin, is among the most active agents used in the field\textsuperscript{95}. The second generation drug, carboplatin (Figure 2) has fast gained notoriety as being much more stable and as having fewer and milder side effects than cisplatin\textsuperscript{96}. The third generation, and most cytotoxic, platinum drug is oxaliplatin\textsuperscript{97, 98}. While the mechanism through which these drugs prove effective is not fully understood, it is theorized to involve the binding of the eponymous platinum group of the drugs to DNA\textsuperscript{99, 100}. The bound drug then prevents translation and transcription, effectively halting mitosis. The drawback of this family lies in its higher than normal systemic toxicity\textsuperscript{95} and the fact that platinum resistance is inherent in many cancer lines\textsuperscript{95, 96}. This platinum resistance is normally presented by the detoxification of the drug by the tripeptide glutathione, which is normally tasked with mitigating metals\textsuperscript{101}. However, oxaliplatin has been shown to be efficacious where cis- carboplatin are found ineffective\textsuperscript{98}, and all of the platinum drugs can be combined with glutathione inhibitors to increase their effect\textsuperscript{101}. These drugs are administered through a wide range of routes, including intravenous, intraperitoneal, and subcutaneous\textsuperscript{102}. Because of the wide range of delivery options and a broad spectrum of
action, platinum drugs are used in combination and single agent therapies for a wide range of cancers, including but not limited to oral, ovarian, lung, biliary and colorectal.

Another group of cytotoxic drugs developed out of everyday antibiotics. The anti-tumor antibiotics work through DNA disruption, similar to the previously described groups, but do so in non-covalent binding manners. Cultivated from natural means, hence the antibiotic distinction, these are among the oldest anti-neoplastic drugs. The drugs included in the category include actinomycin-D, doxorubicin, and bleomycin. While distinct for their wide range of efficacy, these drugs are also noted for their exceptional systemic toxicity issues, even among chemotherapeutics. This toxicity usually manifests in the areas of the heart and lung and poses a significant side effect when used in systemic chemotherapy. Because of these toxicity issues and because of the potential of the drugs against a wide range of cancers, much work has gone into attempting to control this toxicity. The toxicity can be manipulated through methods which center on specific targeting, such as intra-tumoral injection and polymer conjugation. The most successful of these manipulations, however, involve the liposomal encapsulation of doxorubicin, both pegylated (Caelix®) and unpegylated (Doxil®). This encapsulation not only limits the toxicity but also can increase drug penetration into the tumor. Regardless of the toxicity issues, this group of drugs is used to combat almost any cancer known to man.
The group of anti-neoplastics, known as the anti-metabolites, contain some of the oldest and the newest drugs in the battle against cancer. Methotrexate, in use since 1956\textsuperscript{9}, spans many decades of drugs, from 5-fluorocil to recent purine analogs and conjugates. Despite the vast time frame, these drugs all act to disrupt the protein cascade involved in mitosis and thus prevent cell reproduction\textsuperscript{121,122,123}. The disruption of the normal cell cycle has allowed remission rates as high as 90%, in the case of methotrexate treatment against some cancers\textsuperscript{124}. The predominant issue with methotrexate lies in the associated virulent side effects\textsuperscript{124}, an issue that the use of 5-fluorocil was meant to address\textsuperscript{123}. The even newer, more effective and less toxic pemetrexed has already become a consistent choice for malignant pleural mesothelioma, a carcinoma of the lung tissue caused by asbestos\textsuperscript{125}.

Aside from the normal toxicity issues with chemotherapeutics, the anti-metabolites also suffer from short exposure to the tumor microenvironment due to low retention and high renal clearance\textsuperscript{126}. Thus, these drugs require metabolic rates above a minimum threshold to allow their mechanism of action to be effective, e.g. to halt the metabolic pathway\textsuperscript{127}. To this end, much thought has been placed into entrenching the individual drugs into the target cells so they are not cleared from the environment\textsuperscript{123}. Interestingly, methotrexate and 5-fluorocil have a synergistic effect on each other’s effectiveness, i.e. increasing retention and penetration, and are thus often used sequentially\textsuperscript{128, 129}. The anti-metabolites are also often used in conjunction with other chemotherapeutics in combination therapy\textsuperscript{130}. Platinum-based drugs pair nicely with the anti-metabolites, as
their mechanisms of action are different enough so as to not conflict\textsuperscript{131, 132}. Also, folinic acid has worked well with anti-metabolites, alone and in combination therapy\textsuperscript{132, 133}. This success is theorized to be due to the catalyzing effect that folinic acid has on metabolism, thus enlarging the anti-metabolites window for action\textsuperscript{133}.

Newer methods for enhancing the effectiveness of the anti-metabolites rely on encapsulation and the purine analogs. Liposomal encapsulation and nanoparticle conjugation are means through which renal clearance can be lessened and targeting can be strengthened\textsuperscript{126, 134}. These methods hold much promise for this group, as their major issues lie in these areas. Purine analogs attached to polymeric carriers also hold promise, as they can exhibit anti-metabolic activity, thus theoretically turning a polymer into a cancer fighting agent\textsuperscript{135}. These analogs attach to polymers, as well as other chemotherapeutics such as platinum-based drugs\textsuperscript{136}, opening the door of the anti-metabolites to many other modes of action.

The platinum drugs also work in conjunction with another group of anti-neoplastics, the microtubule inhibitors. The microtubule inhibitors are a group of cytotoxic drugs, named for their mechanism of action, which inhibit mitosis through interference with the normal production/reduction of microtubules during metaphase\textsuperscript{137, 138}. The two main divisions of drugs in this group are the taxanes and the vinca alkaloids. The commercially available taxanes are paclitaxel and docetaxel, which are among the most active chemotherapeutics against solid tumors\textsuperscript{139}. The taxanes were originally identified from portions of yew
trees, *Taxus brevifolia* and *Taxus baccata* respectively, but today most of the supply of drug is synthetic\textsuperscript{140, 141}. As the mechanism of action for the taxanes is rather unique amongst chemotherapeutics\textsuperscript{142}, they are ideal for adjuvant therapy as the taxanes allows effective therapy against cells in a larger range of the cell cycle\textsuperscript{139,143,144}.

The taxanes are not without drawbacks, however. Taxanes have several systemic toxicity issues, including neural toxicity and hypertension\textsuperscript{145}. Also, paclitaxel has an extremely limited solubility in water\textsuperscript{146}, making conventional release studies difficult\textsuperscript{147}. Also, due to the low water solubility, oil and emulsion vehicles are required for intravenous taxane administration to the body\textsuperscript{148}. The formulation for paclitaxel is traditionally Cremophor EL\textsuperscript{®}, while the solubulizer for doxetaxol is usually polysorbate 80\textsuperscript{141}, while other solvents and agents have been investigated\textsuperscript{148}. With these thoughts in mind, any type of localized delivery must be designed around these properties. Devices such as coated stents, microspheres, surgical pastes, biodegradable implants, and liposomes have been investigated\textsuperscript{149, 150}. Prodrugs, such as isotaxel, have been investigated as well to boost water solubility and allow easier administration and targeting\textsuperscript{151}.

The other, currently less popular, division of the microtubule inhibitors incorporates the vinca alkaloids. Derived from the periwinkle plant *Cantharanthus roseus*, the first of these microtubule inhibitors was discovered in the 1950s\textsuperscript{138, 152}. This compound, vinblastine, was the first drug which used the principle of interfering with tubulin, symbolizing a shift in chemotherapy paradigm. The release of vinblastine was followed
closely by the development of vincristine and, subsequently, vinorelbine and vinflunine\textsuperscript{138}. With each iteration, systemic toxicity was reduced while anti-tumor efficacy was increased\textsuperscript{153}. Vinflunine, developed in the early 2000s, is now used as a single agent therapy as well as in combination therapies against a wide range of solid and metastasized cancers\textsuperscript{10}. Chemically, the vinca alkaloids all share a common chemical structure with only slightly differing side chains. This difference has led to much research into slight chemical modifications aimed at creating analogs of the alkaloids to further reduce toxicity and boost efficacy\textsuperscript{152}.

Research into drug modification has paid dividends in the past, as is exhibited by the development of the topoisomerase inhibitors. This family’s mechanism of action revolves around the inhibition of the enzymes DNA topoisomerase I and II\textsuperscript{154, 155,156}. Through this inhibition, mitosis is disrupted, and tumor growth is retarded. These antineoplastics are derived from the drug podophyllotoxin\textsuperscript{157} which has been used for hundreds of years as a folk medicine but for which the anti-mitotic properties were discovered within the past century\textsuperscript{154}. While podophyllotoxin proved to create unacceptable toxic side effects, development work led to the discovery of the semi-synthetic derivative etoposide in the 1950s.

Etoposide is still known to be a highly toxic drug, causing myelosuppression and mucositis\textsuperscript{158}. Due to the high efficacy and the long period of history of development, much work has gone into quantifying the therapeutic window of etoposide\textsuperscript{159}. Data
indicates that antitumor activity begins as low as 0.5 ul/mL, and treatment can be tolerated in a dose as high as 10 ug/ml\textsuperscript{160}. This, still imprecise, quantification of the therapeutic window is rare to find amongst chemotherapeutics and could eventually lead to more effective treatment options for patients as more work is continuing on the topic.

This therapeutic window work has also led to further quantification of etoposide properties which allow more unique usages of etoposide. For example, thermal characterization has been executed on etoposide and many of its derivatives\textsuperscript{161}, allowing further processing of the drug. Also, much microbiological analysis has proven capable of identifying protein binding sites and methodology\textsuperscript{162}. The ability to process the drug at temperatures up to 300°C, along with the advanced elucidation of metabolic pathways, allows novel formulation and administration such as phosphate prodrug forms\textsuperscript{163}, but perhaps more importantly as an oral form\textsuperscript{164}. The oral form allows the use of outpatient chemotherapy, which has been shown to be on par in terms of tumor response with in hospital intravenous administration.

While etoposide works through inhibition of DNA topoisomerase II, two other drugs, topotecan and irinotecan, work through inhibition of DNA topoisomerase I\textsuperscript{155,156}. These drugs were developed as analogs of camptothecin, in a similar manner as etoposide was an analog developed from podophyllotoxin. Topotecan is administered as a single agent and in combination therapies quite effectively due to the synergistic activity it exhibits with other antineoplastics such as the platinum drugs\textsuperscript{165}. Also, the lipophilic nature of the
drug allows it to cross the blood brain barrier easily, aiding in treating more dangerous cancers such as that of the brain and spine. It is not, however, immune to the side effects that plague etoposide, being quite toxic to the nervous system. Attempts to limit this toxicity and increase the water solubility of the drug center around nano-preparations involving lipid and PEG preparations.

The other type II inhibitor, irinotecan, has become a frontline, single agent treatment for colorectal cancer. This water soluble topoisomerase inhibitor follows a very complex metabolic pathway to the form of its active metabolite SN-38. However, this metabolite is more toxic in some patients than are the other topoisomerase inhibitors. The reasons for this difference between patient groups have yet to be fully investigated, however data collected is beginning to reveal clues. Further investigation will allow more accurate use of this effective drug.

Another drug of which not much is known is altretamine. The mechanism of action for this drug has yet to be fully understood, however chemically it resembles members of the alkylating agents. This antineoplastic requires metabolic activation for its cytotoxic effects to be activated. Because of this requirement, altretamine is administered orally so as to take greatest advantage of its properties. Altretamine does not have high success rates (10-20%) and so is often administered as a salvage therapy drug, a “drug of last resort”, in malignant metastasized cancers such as ovarian which have proven resistant to other more traditional treatments. The side-effects associated with
altretamine target the digestive system, due to the metabolic requirement, and include nausea and vomiting\textsuperscript{180}.

Non-cytotoxic drugs have also found their way into the fight against cancer. Certain cancers, such as that of the breast, are resistant to cytotoxic chemotherapy but respond to other types of regimens. One such regimen involves the deprivation of certain hormones, such as estrogen or androgens, in order to “starve” hormone-resistant tumors. The majority of work in alternate regimens of this type has manifested itself in breast cancer research, where up to approximately two-thirds of tumors are hormone-resistant\textsuperscript{181}. This research has produced two types of drugs, aromatase inhibitors (AI) and selective estrogen receptor modulators (SERM), which are designed to slow growth of such cancers. Both of these drugs have applications, such as ovulation induction and endometriosis treatment, outside tumor treatments, \textsuperscript{182,183,184}. SERMs such as raloxifene have been investigated in tumor regression, but tend to be overshadowed by the aromatase inhibitors.

The aromatase inhibitors, for the quarter century prior to 2000, were dominated by tamoxifen\textsuperscript{181}. Tamoxifen was developed quickly on the heels of the first AI, aminoglutethimide, and greatly increased the efficacy of the class of drug\textsuperscript{185}. Tamoxifen and other AIs inhibit the enzyme aromatase, which is the key enzyme in estrogen biosynthesis\textsuperscript{186}. Breast cancer is highly dependent on estrogen for sustenance and replication. Thus, with use of AIs such as tamoxifen, breast cancer can be forced into
remission in a wide range of cases\textsuperscript{187}. In the past decade, two new AIs have come to light. Anastrozole and letrozole, the third generation of AIs, have been shown in multiple studies to be more effective than tamoxifen in combating growth of breast tumors\textsuperscript{188-191}. One such study even shows that letrozole can inhibit more than 99\% production of estrogen\textsuperscript{192}. The ability to administer anastrozole orally makes the drug even more attractive\textsuperscript{193}. While shown to be effective in treatment of male breast cancer\textsuperscript{194}, hormone treatment has been shown to be potentially damaging to quality of life of premenopausal women post treatment. For this reason, AIs are usually used in postmenopausal women and men. The potential of drugs such as these should not be overlooked, as the ability to treat cancer without cytotoxic drugs is one way of limiting the well-documented and generally distasteful side effects of chemotherapy.

Continuing with the theme of non-cytotoxic drugs for the treatment of cancer leads to the anti-angiogenesis agents. These drugs attempt to combat tumor growth through an indirect route, by depriving them of the vasculature necessary to grow, rather than by inducing apoptosis. This inhibition is possible because cancers have been observed to be incapable of growing beyond two millimeters in size without the production of a stable vasculature\textsuperscript{195}. With this in mind, bevacizumab was developed and first approved by the United States Food and Drug Administration (US FDA) in 2004\textsuperscript{196}. Bevacizumab is a human monoclonal antibody which targets vascular endothelial growth factor (VEGF), a factor which is largely responsible for the production of vascularization in the human body\textsuperscript{197}. VEGF is often over expressed in cancerous growths, as the goal of such growths
is to replicate as quickly as possible; thus, combating the expression of this growth factor can slow tumor progression\textsuperscript{198}. Preventing the angiogenic shift from unvascularized 2-3 mm tumors to vascularized, metastasizing cancer can be critical to combating such a growth, but action to stop such a progression must be taken early\textsuperscript{199}. As early detection abilities improve and increase the number of tumors which can be made visible, drugs which can retard angiogenic growth and maintain tumors at manageable sizes will become more and more valuable. Further, the various other effects of bevacizumab, such as regeneration of normal vasculature and tumor oxygenation, occur in concert with the effects of cytotoxic drugs, thus making bevacizumab ideal for adjuvant/combination therapies\textsuperscript{200, 201}. Furthermore, bevacizumab is well tolerated\textsuperscript{202, 203} and associated with only mild or treatable side effects. The drug is even inexpensive as compared to other chemotherapies\textsuperscript{204}. However, despite all these positive attributes, bevacizumab is still only truly effective in combating very early stage tumors. Anti-angiogenic research does hold promise for future success in the clinic.

The other big name in the field of anti-angiogenesis is an old one, soiled through a textbook example of poor pre-market testing. Thalidomide is a glutamic acid derivative which was first synthesized in 1954\textsuperscript{205}. The drug was immediately noted for being so non-toxic that a lethal dose causing death of half an animal test group (LD\textsubscript{50}) could not be established and the fast acting relief of myriad symptoms, including morning sickness\textsuperscript{206}. The drug was marketed over the next decade as a non-barbituate sedative, especially popularly prescribed to pregnant women. However, thalidomide proved to be tetragenic
and over 10,000 babies were born with severe debilitating birth defects prior to the discontinuation of the drug’s usage. From this negative story comes a silver lining of good news, as thalidomide was not completely abandoned from research laboratories. Over the next fifty years, a range of uses for the drug have been developed. Most importantly to the oncologic community, it has been shown to be anti-angiogenic in nature\textsuperscript{207}. This characteristic, coupled with low toxicity, has led to experimental use against a wide range of solid tumors\textsuperscript{208}, and even FDA approval for cancers such as multiple myeloma\textsuperscript{209}. While being administered exclusively through oral tablets\textsuperscript{210}, the mechanism of action for thalidomide has yet to be elucidated fully, even after decades of study\textsuperscript{211}. What is known is that thalidomide is composed of a racemic mixture of two enantio-isomers, which racemize rapidly under physiological conditions\textsuperscript{212}. This attributes to the speedy effect of the drug, as well as the masked tetragenic effects, as only one of the isomers is tetragenic and only in the racemized form. Also, the drug is rapidly broken down in the body, with a half-life of only 4-5 hours. This point explains the almost negligible toxicity of the drug, as chemical build-up in tissues and organs is almost impossible on this short timeframe. All of these properties make thalidomide an attractive choice for non-cytotoxic chemotherapy. However, concerns raised by the thalidomide failure half a century ago, must still be addressed before this drug will become widely accepted.
After reviewing the major players in the field of chemotherapy today, the next logical step is to examine the target for the drugs, i.e. the tumor and associated metastasis. Clinically, much work has been performed, using tumor size reduction and associated progression-free survival rates as the metrics by which cancer treatment “success” is determined. While these metrics are the quantitative measurements with which treatment should be concerned, research must keep in mind the reasons for this success as this line of inquiry will inform new approaches and directions. Description and visualization of the tumor microenvironment has taken a back seat to drug development and mechanism elucidation in the past twenty years. Luckily however, researchers are beginning to direct more attention to the how and why of cancer development, angiogenesis and growth. This knowledge will allow more successful treatment of carcinomas in the long run, ultimately boosting the most important metric, survival.

The first and most important item of note when examining the tumor microenvironment is the diversity of the area. Human neoplasms contain a plethora of differentiating cells in terms of function\textsuperscript{213}. From this variation comes perhaps the most important facet of knowledge related to the tumor microenvironment, that tumors exhibit heterogeneity in terms of cell type\textsuperscript{214}. While one school of thought advances that tumors originate from a single, malformed cell, tumor cell differentiation occurs early in the growth process. This process leads to a series of concurrent factors which either inhibit or encourage growth\textsuperscript{215}. The carcinoma grows outward, through stromal cells on the leading edge of the tumor.
These cells in turn interact with the surrounding extracellular matrix (ECM). The complicated interactions between these fronts of cells are often the largest indicator of either growth or stagnation of the tumor. Dr. Stephen Paget was the first to put forward the “seed and soil” analogy\(^ {216} \), in which he compared a neoplasm to that of a seed and the surrounding ECM to the soil. By controlling the soil he postulated, one could retard the growth of a seed. As any farmer knows, it is easier to salt the ground than explant a series of seeds.

This analogy leads easily to the question of how the cancerous “soil” differs from normal tissue. Studies have been performed to answer this question and have identified numerous differences between cancerous and non-cancerous cells\(^ {216} \). Cancerous tissue often exhibits low levels of pH as well as glucose, which is attributed to the rapid replication of cells, indicative of a tumor. Also, hypoxic conditions are almost universally present at tumor sites. The hypoxia is due to the lack of a comprehensive vasculature; the lack of oxygen contributes to the large number of mutations seen in malignant tumors due to the disrupted replication of DNA. Also, tumor tissue exhibits high levels of necrosis as cells struggle to survive in the hazardous conditions described. Ironically, necrosis of cells at the core of solid tumors has been postulated\(^ {216} \) to actually catalyze outward growth of the tumor as macromolecules and nutrients are released within the core of the growth and become available to the proliferating cells on the leading edge.
This process explains why the core of the tumor differs greatly from the outer palisade cells\textsuperscript{217}. This point is also highlighted by the difference in interstitial fluid pressure (IFP) in the tumor. Due to the poorly vascularized nature of the core of the tumor, caused by the vascular irregularities inherent in tumors, IFP rises. The rise in IFP, coupled with the generally porous or “leaky” nature of tumor vasculature\textsuperscript{218}, makes drug delivery via normal routes e.g. blood pathways, very difficult. Also, the cells in the outer region of the tumor, close to the blood vessels have a higher rate of proliferation than those found in the necrotic core\textsuperscript{219}. The increased cell density in the outer region makes penetration deep into the tumor very difficult. Finally, the last major difference between healthy and cancerous tissue can be seen in the difference in temperature. Temperatures in cancerous tissue can rise as high as 113\textdegree F (45\textdegree C)\textsuperscript{219}. This excess energy can be used to catalyze intracellular processes and thus speed processes such as metabolism and mitosis.

These differences in microenvironment amount to a double edged sword. On one hand, properties such as rise in temperature provide the ability to target cancerous tissue specifically using technologies such as temperature-sensitive polymers. Conversely, the tumor microenvironment can present one of the largest obstacles to chemotherapy in the form of drug resistance. Drug resistance can be developed through mutations of cells surviving treatment (occurring rapidly and spontaneously due to the hypoxic conditions) or can reside inherently in the tumor prior to exposure (\textit{de novo} resistance)\textsuperscript{220}. An example of such resistance can be seen in the production of excess glutathione in tumor microenvironments resistant to cyclophosphamide, a cytotoxic drug inhibited by the
presence of glutathione\textsuperscript{214}. Tumor chemoresistance is the leading cause of failure of chemotherapy, because even if a small portion of the heterogenic tumor is resistant to a given drug, that portion has the ability to regenerate to the previous size or larger\textsuperscript{221}.

The investigation and visualization of the tumor microenvironment is an area of ongoing work in the realm of oncology. Probes such as fluorescent nanoparticles and quantum dots have been used to visualize tumor structures and vasculature\textsuperscript{215}. Tissue preparation and immunohistochemical staining allow post-excision analysis. Immunohistochemistry is often used to determine the microvessel density of a tumor, which is directly related to angiogenesis\textsuperscript{222}. This assessment becomes necessary as the quantification of angiogenesis is essential in determining the stage of the tumor and propensity to metastasize\textsuperscript{223}. In general, the more vascularized the tumor the more advanced the growth and hence the more danger to the host\textsuperscript{224}. Thus, visualization of the cells is important and allows further investigation of solid tumors. This heterogeneity of tumors and the surrounding microenvironment, once studied and fully understood, may provide a series of targets for treatments to check the growth of cancers\textsuperscript{225}.

A better understanding of the microenvironment will, in turn, lead to a better understanding of the mechanisms by which tumors grow and better methods through which to model tumor growth. Such models are important in tailoring treatments to patients and predicting efficacy of such treatments. The mainstream of thinking for a long period, prior to the turn of the century, was that every carcinoma was inherently
different and there was little possibility of predicting the growth of such a tumor through a universal mathematical model. However, in 2001, it was shown that tumors throughout nature follow a same general exponential curve\textsuperscript{226}. While simplistic (growth slows as it reaches a limiting factor), this statement describes that cancer cells react as a population, meaning that growth is not completely unchecked. This concept of tumor growth relates directly with the discussion previously on tumor microenvironment, and raises the interesting question of how medicine might force the retardation of growth sooner.

Development of mathematical models not only highlight questions such as how tumor growth can be examined but allow the elucidation of better treatment regimens by identifying key parameters, such as tumor size, vascularity location etc. This is achieved first, much like any other mathematical model, by defining the relevant variables. Items such as cell structure, environmental factors, adhesion between cells and rate of proliferation are often common across different proposed models\textsuperscript{227}. Behind these obvious variables are, however, the complex interactions which one must quantify. For example, as a tumor grows into the surrounding ECM, the ECM structure is damaged and broken. During this destruction, nutrients and growth factors such as VEGF are released\textsuperscript{228} which can then be used by the advancing stromal cells. Therefore, it can be easily argued that this advancement catalyzes itself through a positive feedback loop. The growth, along with the VEGF, will induce angiogenesis which will further speed tumor growth. Hence, mathematical models can be useful in the identification of an
acceleration point for tumor growth and therefore can be useful in the determination of indicated treatment at the present state of the tumor\textsuperscript{229}.

Tumor growth presents a problem to simple modeling because growth is a dual-stage process\textsuperscript{230}. The first stage is that of pre-vascularization. Here, the tumor grows to the size to which it is limited by the limited capacity to provide nutrients to the core via in the absence of a vascular network. In this stage, a tumor can grow no larger than approximately 2 millimeters. However, with angiogenesis and the ability to supply necessary elements to other regions, growth commences again. The second stage progresses until metastasis occurs, at which point modeling becomes unwieldy and less relevant. Thus any model must represent the growth as a dual compartment model\textsuperscript{231}. Therefore, the models can be described in terms of two stages, as outlined by Sachs\textsuperscript{231}; the first stage of tumor growth can be presented as such:

\[
\frac{dN}{dt} = \lambda N
\]

N is the number of clonogenic cells and \(\lambda\) is a constant\textsuperscript{231}. This simple exponential growth equation is adequate for modeling the first stage; however, upon angiogenesis, two equations are needed. The first equation represents the tumor core:

\[
\frac{dN}{dt} = \lambda N \times K
\]

K is a carrying capacity determined by the second compartment volume. The second equation is related to the speed at which vascularization occurs, the depth at which it
occurs and speed of replication resulting from such nutrient availability. These characteristics are represented by the general form:

\[
\frac{dK}{dt} = DS \times (V\rho)_0
\]

D is the depth of penetration, S is the speed of penetration, V is the volume of the tumor at time 0, and \(\rho\) is the density of cells in the tumor at time 0. This simplified model is meant to describe the potential for the tumor to grow to a carrying capacity \(K\), which is in turn defined by the level of angiogenesis, as time progresses. This mathematical description again correlates to the idea of quantification of the stage of cancer relating to that of the level of vascularization of the tumor.

This proposed model can obviously be improved through the inclusion of further variables or the use of further compartments which have been proposed to represent “shells” or areas of differing tumor growth. The example model proposed is intended to provide a preliminary understanding of the complex system that is tumor growth.
CHAPTER TWO
TUMOR TREATMENT IN MODERN MEDICINE

Tumor Treatment and Administration Methods

The information provided to this point describes two of the tools required to treat cancer using chemotherapy. The first tool, the drugs themselves, is specific to a method, whether it be deoxyribonucleic acid (DNA) intercalation, anti-angiogenesis, or any other mechanism. The second tool, knowledge of the physiology of tumors, guides the selection of the drug. The missing tool is the method of drug administration to the tumor.

Modern chemotherapy administration comes in many different forms, the exact mechanism is usually indicated by the location and stage of the cancer.

Intravenous (IV) chemotherapy was first used in 1942 at Yale University\textsuperscript{233}. Following this initial success, IV chemotherapy became the gold standard against which other chemotherapy regimens were judged. IV chemotherapy seems logical at first glance; it is easy to administer, compatible with most hydrophilic chemotherapy agents, poses a low risk of infection, and exhibits a high rate of efficacy. This route of administration relies on the circulatory system to route the drug through the entire body, exposing areas other than the target tumor to the cytotoxic drug. However, recent studies have shown that, for some solid tumors, intraperitoneal (IP) administration is as effective or even more effective than a similar concentration administered through IV\textsuperscript{234, 235}. IP administration has been shown to be more cost effective in terms of cost versus survival-free progression, making it an interesting economic choice\textsuperscript{236}. Other studies have even
examined using the two together, to great effect. The one drawback of IP administration appears to be slight toxicity to the injection site, as one would expect from a higher, localized concentration of drug.

Oral chemotherapy presents the most patient-compliant form of chemotherapy. Patients are often more inclined to take a pill than undergo injections or infusions, and oral chemotherapy does not require a visit to the physician’s office. However, only certain drugs are capable of oral administration while others require modification, such as encapsulation. Even so, oral administration has been found wanting in terms of tumor response when compared to standard IV administration. For this reason, oral chemotherapy is often only used in cases where previous evidence assures success, or when other methods have failed.

The newest iteration of administration is that of intratumoral (IT) injections. This method consists of injecting the chemotherapy directly into the tumor. This has the advantages of completely perfusing the tumor (allowing access of the drug to the poorly vascularized core) and maximizing the drug concentration at the desired site. This method has already been shown to be more effective than IV treatment in some cases, with reduced toxicity allowing treatments of children and the elderly. The reduced toxicity is attributed to the lack of “collateral damage” as the cytotoxic drugs do not penetrate any tissues other than the target and have perhaps very slight diffusion into the surrounding cells. However, the largest obstacles to the acceptance of IT treatment stems from conservative
physicians and the need for a clear, accessible tumor. Surgical removal often does not leave a visible target and so IT treatment is currently regarded as a “pre-operative” modality.

After determining how to administer chemotherapy, one must determine the timing. Traditionally, chemotherapy is administered in high, concentrated doses with weeks in between to allow the body to recover\textsuperscript{243}. This “cool-down” period is necessary when such high levels of drug are introduced because build-up of the cytotoxic drug not only occurs at the tumor site, but also in collateral, healthy tissue. Because of this necessary break in treatment, tumor cells have a chance to replicate and, worse, develop drug resistance mechanisms. Therefore it is imperative to find ways of administering chemotherapy on a more frequent basis. This can be done through simple modification of dosing schemes, such as through metronomic or dose-dense scheduling.

The goal of metronomic scheduling is to expose the tumor to a constant, frequent, low level of drug\textsuperscript{244}. Because of the low levels of drug administered, cytotoxic side effects are seen less frequently, as the body is more capable of clearing the drug not absorbed into the tumor and preventing the build-up of the drug in “healthy” tissue. Also, by constantly exposing the cancerous growth to a given cytotoxic drug, the tumor has no chance to regrow and has much less time in which to develop a drug resistance. Thus, since most cytotoxic drugs attempt to interrupt cell division at a certain stage, it stands to reason that it is essential that all the tumor cells be exposed to the drug while passing
through that stage of the cell cycle. A sound idea in practice, low dose metronomic (LDM) scheduling has been effective in a number of studies involving paclitaxel\textsuperscript{245, 246}, cyclophosphamide\textsuperscript{83}, and anti-angiogenic factors\textsuperscript{247}. Even more interesting, tumors that develop resistance to LDM have been shown to still be sensitive to higher doses of drugs, at least in the case of cyclophosphamide\textsuperscript{83}.

The main difficulty with LDM is a poorly defined lower threshold for the therapeutic window for most chemotherapeutics. That is, it is not clear at what level one can administer the desired drug and still maintain efficacy. Dose-dense (DD) chemotherapy evades that question, and encompasses a more constant schedule. The goal of DD is to maintain a constant, high level of drug at the site of the tumor. This delivery is executed in a similar manner as metronomic scheduling, with higher concentrated doses than LDM but lower doses than traditional means. Thus, by continually replenishing the drug concentration at the tumor site, better clinical results should follow. Success with this treatment process has been seen with multiple paclitaxel/platinum\textsuperscript{62, 63} and other combination regimens\textsuperscript{248}. The greatest problem with dose-dense chemotherapy is the increased toxicity associated with the more frequent treatment administration. However, even this problem has been shown to be controllable through conscientious dose choice\textsuperscript{61}. Thus, DD chemotherapy is becoming a more attractive option in hospitals and clinics around the world.
Biomaterials are increasingly playing a role in aiding the administration of chemotherapeutics, and are being used as *in situ* delivery systems, microparticles, and polymeric conjugates. A chemotherapeutic is often administered in a form other than a free drug in an IV solution. The best example is a diluent such as BASF’s Cremophor EL®. This excipient, which is simply a reaction of ethylene oxide and Castrol oil, is used to solubilize the hydrophobic drug paclitaxel. This allows the administration of paclitaxel through IV means into the circulatory system without massive precipitation of the solute. The problem with such a system lies in the side effects elicited by the inclusion of Cremophor EL, which are highlighted by hypertension. With the already high level of harmful side effects seen in the use of chemotherapeutics, one can understand why any further toxicity caused by additional components would be undesirable.

Not all delivery aids are solubilizers and excipients. Pluronic® copolymers have been shown to interact with tumors, independent of drug presence. The presence of these polymers at the tumor site has been shown to sensitize normally drug-resistant tumors. The mechanism of action for this sensitization is proposed to be the adenosine triphosphate depletion induced by the presence of the polymers, thus weakening the cancerous cells defense mechanisms. Hence the administration of these polymer solutions concurrently with chemotherapeutics has shown promise in overcoming of multi-drug resistance in tumors.
Polymers are valuable to chemotherapy as liquid carriers and polymer drug conjugates are playing an increased role in therapies as well. Because the majority of the drug’s target resides within the cell, usually either DNA or microtubules, transport and uptake to the cytoplasm is a key component of cancer therapy. Initially, viral vectors were attempted to facilitate such transport. However, problems were seen with toxicity and immunogenicity\textsuperscript{250} and so non-viral methods were explored. This led to research involving cationic polymers. Cationic polymers allow the conjugation of many different anti-neoplastic drugs. These conjugates exhibit better biocompatibility, circulation time and drug uptake within cancerous cells, while inflicting lower systemic cytotoxicity\textsuperscript{250}. Cationic polymers rely on the cell substructure to cleave the polymer from the drug, hence catalyzing the prodrug to drug transition \textit{in situ}.

Polymers are also tailored to be environment sensitive to release their drug payload. Polymer drug conjugates have been designed both with pH and temperature sensitive linkages. Polymers conjugated to drug through acid-sensitive linkages are capable of circulating through the body multiple times until the cancer site is reached. Acidic pH is indicative of a tumor site and so the polymer-drug conjugate will preferentially separate at the tumor site\textsuperscript{251}, thus allowing targeted delivery to the tumor microenvironment. Temperature-sensitive polymers are capable of working in the same manner, though the polymer-drug linkage is less likely to be a strict covalent bond and more likely a sol-gel network\textsuperscript{252}. These examples are just a few of the ways that the tumor microenvironment can be targeted by polymeric conjugates.
Through the use of polymeric linkages, delivery mediators can also be attached. Binding the drug to a polymeric scaffold creates other sites for attachment. These sites can be used to bind targeting moieties, such as folic acid. Folic acid constitutes an interesting choice for a targeting element as it is easy to attach, upregulated in many tumors, overexpressed in relation to size and grade of tumors, and normal tissues shield the majority of their folate receptors. Thus the complex will be preferentially incorporated by cancerous cells and will release the drug from the prodrug complex within the desired cells.

Polymeric conjugates are not the only option for facilitated delivery to cancer cells. Liposomes (Figure 2.1) also offer a significant advantage over free drug administration. Liposomes consist of a lipid bilayer surrounding an inner cavity which, through special processes such as water-in-oil-water emulsion preparation, can be made to encapsulate drug. These liposomes then freely circulate the body for extended periods of time, due to their relatively bioinert characteristics, until they settle into tissue. The “leaky” vasculature of cancer tissue allows the preferential deposition of liposomes at the tumor site. However, once in place, the stable liposomes are not inclined to self-degrade and release the encapsulated drug. Many mechanisms, such as phototriggering, have been investigated to allow the in situ release of drug from liposomes.
As their name implies, liposomes are inherently hydrophobic, at least to a slight degree. While useful for applications such as traversing the blood brain barrier, this characteristic is detrimental in general systemic delivery. For this reason, liposomes can be surface modified using a process described as PEGylation\textsuperscript{256}. This process entails the attachment of the biocompatible and hydrophilic polymer polyethylene glycol (PEG) to a given structure to increase the overall hydrophilic and biocompatible nature of the entire complex. The process, first described in the 1970’s, allows the attachment of PEG to a bioactive molecule through various mechanisms such as acylation or methylation, etc.\textsuperscript{257}. This molecule does not have to be a polymeric complex, but can be a protein, peptide or, even for chemotherapeutic applications, a hydrophobic anti-neoplastic drug.

Further work in the field revolves around the use of nanoparticles and microspheres. The aim of such work is similar to that of liposomal delivery, but attempts have been made to use less lipophilic carriers. These carriers generally take the form of nanoparticles or microspheres. These small particles can be prepared from a variety of materials, polymeric and non-polymeric. Nanoparticles prepared from biodegradable polymers such as polylactide-co-glycolide (PLG) are gaining favor currently as PLG has a low risk of a toxicologic or immunogenic response. These items can be prepared for drug delivery and diagnostic uses for cancer therapy\textsuperscript{258}. Nanoparticles will also preferentially seek out the ‘leaky’ vasculature of tumors, much like liposomes. The leaky vasculature provides an almost sieve-like system, leading to the surrounding tumor, in which a properly sized nanoparticle will deposit\textsuperscript{259}. Equally important to the retention of
nanoparticles in the tumor is the poor drainage of the vasculature relative to normal tissue. These two factors combine to allow a majority of the administered nanoparticles to eventually target the tissue. The collection of fluorescent nanoparticles in the tissue can allow non-invasive visualization of neoplasms.

Nanoparticles and microspheres have already begun to show their worth in terms of drug delivery. Studies as early as 1996 showed that microspheres containing methotrexate elicited a better clinical response to treatment than free methotrexate\textsuperscript{260}. Anti-angiogenic delivery is quite popular and effective due to the area of effect\textsuperscript{261}. Nanoparticles have even found use in treating cancers of the central nervous system, where other methods are only effective over a limited area\textsuperscript{262}. Nanoparticles have also been shown to aid in sensitizing tumors which were otherwise resistant to their payload drug, much like the Pluronic\textsuperscript{®} polymers\textsuperscript{263}. Work has also been conducted using microspheres as placeholders for ligands, thus creating actively targeted therapies. These properties, along with the ability to incorporate most drugs used in chemotherapy in a pharmacologically active manner, make nanoparticles and microspheres a key tool in the battle against cancer\textsuperscript{264-266}.

While nanoparticles show promise in targeted therapy, the next step is localized delivery. The advantages of localized delivery over normal systemic treatments include lower systemic toxicity, more efficient usage of the drug, and better treatment outcomes. The difficulty in this field is the ability to deliver a suitable delivery vehicle to the site of
treatment. Medical devices such as implantable chemotherapeutic wafers for brain cancer have been tried with little success. However, devices which do show promise for localized delivery are injectable in situ forming implants.

These implants begin the treatment cycle in a solution (sol) form. This solution is then administered to the desired area of treatment through a syringe or port system. Once in place, or in situ, the implant then converts to the gel form. This transition is often called the sol-gel transition and the mechanism through which this change is effected is one of the distinguishing factors between systems. The majority of injectable in situ-forming systems fall into one of three categories: (1) crosslinking/network formers, (2) thermogelling systems, and (3) self-assembling systems\textsuperscript{267}.

The first group of such systems relies on the difference of the environment at the site to induce cross-linking and thus the development of the desired construct. This difference can either be an inherent physiochemical difference or an induced change. Examples of induced changes include ultraviolet irradiation, temperature and pH modification\textsuperscript{268}. Once cross-linking is induced, networks are formed between chains (usually polymeric). These networks then release their pharmaceutical payload in a controlled manner\textsuperscript{269}. The release of such molecules can be modulated by the level of cross-linking, the polymer scaffold employed, and the method of cross-linking\textsuperscript{270}. The most popular polymer matrices in this area are those of the pH-responsive type, often using the biocompatible natural polymer chitosan\textsuperscript{268}. These in situ implants are capable of being delivered
through physiological pH (7.4), remaining in the sol state, to weakly acidic (pH 6.8) environments where the sol-gel transition occurs\textsuperscript{271}. In this manner, the transition to a “delivery state” can be contained to a pH usually located only in tumor sites. While effective, these types of treatments do have drawbacks. Cancer pH values are not constant across tumors or even within a particular tumor. Also, once formed, hydrogels produced through sol-gel transition tend to be weaker physically then other methods, making stability an issue\textsuperscript{272}. These drawbacks are significant, and much current work is being geared toward overcoming these obstacles.

More popular among researchers are the thermogelling \textit{in situ} forming systems. These systems are activated by elevated temperatures and are characterized by their lower and upper critical solution temperatures (LCST and UCST). The commercially available family of Pluronic\textsuperscript{®} polymers is the best known example of such a system\textsuperscript{273}. These polymers are poly(ethylene oxide) and poly(propylene oxide) block copolymers which allow the thermocontrolled construction of controlled delivery implants by engineering the UCST to be between room temperature (~20ºC) and body temperature (37ºC). When implants from polymers such as those described have been formed \textit{in situ}, controlled release of multiple chemotherapeutics has been exhibited\textsuperscript{274,275}. 

\textbf{Figure 2.2: Example of a thermogelling system}\textsuperscript{280}
Systems engineered using polymers such as elastin-like polypeptides can even be designed to deliver multiple drugs at different temperatures by creating regions with different CSTs\textsuperscript{276}. The flexibility of such systems is attractive to researchers, but the reliance on physiologically fluctuating temperature again poses a hindrance to widespread use.

The final group of \textit{in situ}-forming implants is perhaps the most complicated, yet intriguing, group. This group relies on the self-assembly of implants once introduced to the site. This reliance on mechanisms other than cross-linking, pH, or temperature makes systems such as these more difficult to prepare but more useful once in place. These systems often rely on precipitation and phase-separations to elicit the desired construct. Collagen gels have been shown to be capable of intratumoral delivery using such an approach\textsuperscript{277}. Other materials involved in systems using methods such as these include novel tissue adhesives\textsuperscript{278} and hyperbranched hydrogels\textsuperscript{279} which are designed to be physically more stable and therefore more effective drug delivery systems than those that rely on external stimuli to induce the sol-gel transition.

\textit{Extension – What Next?}

The obvious question for one to ask is “what next”? A significant base of work has been put into understanding chemotherapy. The drugs with which chemotherapy is performed are well documented. Current understanding of the tumor microenvironment and kinetics of growth are becoming better understood at a rapid pace with today’s technology.
Delivery routes other than traditional IV administration are becoming more common and accepted. Chemotherapy has the potential to become a much more prominent player in the war on cancer. Through more effective usage and administration of common chemotherapeutics, chemotherapy can be made into a more acceptable treatment for cancer patients. The goal of such optimization should revolve around limiting side effects and increasing the efficiency of such treatment.

Ovarian cancer is a known chemoresponsive malady. Treatment of this disease commonly focuses around usage of paclitaxel and platinum-based drugs administered intravenously in combination therapy. This treatment regimen has been shown to aid in the regression of tumors following surgical debridement. This treatment is not, however, without drawbacks; systemic IV administration leads to full-body toxicity and a slew of undesired side effects. Also, drug is inefficiently delivered to the tumor, and even less reaches the intracellular environment where anti-neoplastic activity can occur.

Furthermore, this uncontrolled delivery to the tumor site causes the discarding of the traditional clinical tool, the therapeutic window. The therapeutic window is often defined as the concentrations of a bioactive agent above the lowest efficacious dose but below the maximum tolerated dose. Work such as the development of dose dense and metronomic scheduling has contributed greatly to allow controlled drug levels, but still does not account for a truly controllable drug concentration. Also, each time agents are administered, excipients such as Cremophor EL® are required for treatment. These
agents themselves have been shown to elicit harmful side effects when administered systemically. The administration of controlled delivery vehicles also must ensure the biocompatible nature of the device, and the eventual need for clearance or removal from the body.

Treatment must be optimized; this optimization can take the form of an in situ-forming, biocompatible construct. The injectable nature and self assembly of such a device allows localized delivery of chemotherapeutics. Administered intratumorally, such an implant will remove many of the issues resulting from systemic administration of drugs. Also, through the use of a biocompatible, absorbable system, safety can be increased and explantation will never be required.

Lastly, through the controlled diffusive release of encapsulated drugs, such as paclitaxel and carboplatin, treatments can be optimized to provide constant levels of drug. Dose dense and metronomic dosing schemes have already shown that lower levels of drug administered more often can be effective. It therefore stands to reason that a constant delivery of low levels of drug in situ could be even more effective, again limiting systemic toxicity.

With all of these factors considered, an injectable in situ forming implant is therefore proposed. This implant should be biocompatible and capable of controlled release of chemotherapeutics. By using cancer of the ovaries as a model, an effective system could
be developed. Once proven, the developed system could in turn act as a model for development of other treatments for chemosensitive cancers at other sites in the body. Using new polymeric systems, this treatment could open up new, safer, and more efficient pathways for patients stricken with cancer.
CHAPTER THREE

THE OC SYSTEM: A METHOD FOR LOCALIZED SUSTAINED DELIVERY

Overview

As noted in the previous chapter, there is currently a dearth of technology specifically engineered to encompass both local and sustained delivery. If such a candidate could be designed and prepared, a significant step in the war on cancer would be achieved. One such delivery system being developed is the OC system (Poly-Med, Inc., Anderson, SC). This platform technology comprises an injectable in situ forming drug delivery depot that does not rely on fluctuating environmental factors to induce the sol-gel transition. Rather, the mechanism of formation is simply exposure of the formulation to an aqueous environment. The injectable solution is composed of several components. The OC polymer forms the actual depot delivery system. OC polymers are a block copolymer design with both hydrophobic and hydrophilic segments. In order to facilitate the injection of the system, a polymeric diluent is used. If necessary to further modulate release, polymeric additives are introduced which control the structure of the delivery construct and construct drug interactions. The final component of such a system is the bioactive agent, such as carboplatin, which is used for delivery. This system is a homogenous solution prior to introduction into a biological environment. Upon injection into the desired site, a drug delivery depot forms rapidly (Figure 3.1).

This depot formation occurs when the water-miscible polymeric diluent evacuates the solution, allowing the remaining solutes to coalesce. Following this theory, the OC
polymer orients itself into a thermodynamically favorable conformation, resulting in a stable polymeric construct. The uniqueness of this formation in relation to other hydroset gels is exhibited by the hydrophobic and hydrophilic differences throughout the polymer chain. This property difference, inherent in the polymer chain itself, helps drive the orientation of the delivery depot to a repeatable, stable conformation. The inclusion of polymeric additives can be used to further control the structure, and thus the release properties, of the construct. The included bioactive agent is evenly distributed throughout the construct. Delivery of the drug is then controlled through a diffusion-mediated release which is dependent on the surface area of the implant exposed to aqueous environment (blood and living tissue).

Figure 3.1: Theoretical mechanistic diagram of OC system introduction into an aqueous environment. Red indicates the hydrophobic domain of the polymer. Orange indicates the hydrophilic domain of the polymer. Green indicates the hydrophilic diluent. Blue indicates the aqueous environment. Source: “Intravaginal Drug Delivery” in Polymers for Vascular and Urogenital Applications (S.W. Shalaby, K.J.L. Burg, W.S. Shalaby, eds.) J. M. Olbrich, W.S. Shalaby, G.H. Hilas. Taylor & Francis Group, LLC (2012)
Upon exposure, the hydrophobic portions of the copolymer chain orient themselves to an inner core, while the hydrophilic portions form the outer regions of the delivery depot. Following this orientation, a multi-layered construct is developed through which release of the drug payload, homogenously distributed within, is achieved by diffusive release. This diffusion can then be modulated through modifications to either the hydrophobic or hydrophilic segments of the chains.

The novelty of this platform technology lies in the ability to modulate nearly any of the variables. Release speed, degradation period, and even gel formation speed can be tailored to the exact needs of the individual application through manipulation of the described portions of the system. The major portions of such a system are discussed more thoroughly subsequently.

**Polymers**

The OCs are novel polymers capable of sustained controlled release of a pharmaceutical payload in the presence of an aqueous environment\textsuperscript{77,78,79}. These polymers are prepared through a bulk, ring-opening, multi-step polymerization of monomers and short chain polymers including lactide, glycolide, trimethylene carbonate, diisocyanate and polyethylene glycol, depending on the intended application. An example of the OC family is OC9. The polymer is composed of polyethylene glycol (PEG; Mw=400) and DL-lactide/glycolide (80/20) blocks. The initial step in the polymerization of such an agent involves extensive drying of PEG 400, which serves as the initiator, under vacuum.
in a reaction flask. The metallic based catalyst is then added to the flask. To construct the prepolymer, cyclic monomers (DL-Lactide (DLac) and glycolide (Gly)) are added. Ring-opening of the cyclic monomers and chain growth of the polymer occurs. Block structures of PEG and the absorbable monomers, respectively, are prepared. Polymerization is allowed to proceed under elevated temperature to completion. Completion is monitored through gel permeation chromatography (GPC) analysis of molecular weight. Once the prepolymer has been prepared, the second polymerization is initiated. This step is performed by the addition of diisocyanatohexane (diiso) to the reaction flask. The polymer is then catalyzed with heat to allow the linkage of the diiso units to the prepolymer. Following the completion of polymerization, purification is performed on the product to remove impurities and unreacted monomer. Purification is executed through the dissolution of the full contents of the reaction flask in tetrahydrofuran (THF). Once the contents of the flask have been solubilized, a small amount of isopropyl alcohol (IPA) is added to ensure that free diiso groups have been neutralized. The solution is then poured into a chilled aqueous non-solvent to precipitate the desired polymer. Polymer is subsequently dried on Teflon® sheets under low heat and vacuum. This seven step process is outlined in Table 3.1.
Table 3.1: The OC Polymerization: A General Procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Chemical Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diol is dried in a flask under vacuum. This serves as the initiator.</td>
<td><img src="52" alt="Chemical structure of a diol" /></td>
</tr>
<tr>
<td>2</td>
<td>Heavy metal compound is added as the catalyst.</td>
<td><img src="52" alt="Chemical structure of a heavy metal compound" /></td>
</tr>
<tr>
<td>3</td>
<td>Cyclic monomers are added for the pre-polymerization. A ring-opening bulk reaction occurs.</td>
<td><img src="52" alt="Chemical structures of cyclic monomers" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>Chain extending agent is added after the pre-polymerization. Polymerization, chain-extension, occurs.</td>
<td></td>
</tr>
<tr>
<td><strong>5</strong></td>
<td>Organic solvent is added to the polymer to allow purification through precipitation.</td>
<td></td>
</tr>
<tr>
<td><strong>6</strong></td>
<td>Isopropanol is added to neutralize free floating chain extending agents.</td>
<td></td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>The entire solution is then poured into ice water (H₂O). Resulting polymer is collected.</td>
<td></td>
</tr>
</tbody>
</table>
The result of such work is an absorbable block polyether-ester-urethane (PEEU) or polyether-ester-carbonate-urethane (PEECU) copolymer (the latter if trimethylene carbonate is used). These polymer structures are amphiphilic in nature, containing both hydrophobic and hydrophilic segments joined with flexible urethane linkages (contributed by the diisocyanate linkages). Polymeric properties have been manipulated by changing the monomeric composition and order/structure. The amphiphilic state of such polymers allows the unique formation of a polymeric construct when the polymer is exposed to water. The hydrophobic portions, contributed by the lactide and glycolide blocks, of the polymer orient to the inner core of the polymer while the hydrophilic portions, contributed by the PEG blocks, are presented to the aqueous environment. The inclusion of the urethane portions allows the polymer to reach this thermodynamically favorable conformation at an unparalleled speed. This rapid (fully complete in less than 3 minutes) formation allows circumvention of the normal “burst” effect in delivery of a pharmaceutical which usually plagues such technologies. This gelation was quantified using an Anton-Parr Rheometer (Physica MCR 301). The rheological analysis of one such precipitation is depicted in Figure 3.2.
Due to the rapidity of gelation, little (<5%) drug is lost during evacuation of the diluent and bolus formation. Thus, this series of polymers has unique properties not previously explored. These polymers are constructed from generally well characterized and safe monomers used in many materials and devices already on the market which should allow Food and Drug Administration acceptance and eventual marketing. Preliminary cytotoxicity data has been gathered on a majority of the samples, resulting in scores of ‘0’ according to International Organization for Standardization (ISO) 10993-5 testing. This is the best score possible on the ISO scale and analogous to a rubber stopper control test. The properties of the example OC polymer, OC9, can be seen in Table 3.2.
Table 3.2: Pertinent Properties of OC9 Lot 1 Purified

<table>
<thead>
<tr>
<th>Property</th>
<th>OC9 Lot 1 Purified Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td>PEG400 // DL Lactide/Glycolide</td>
</tr>
<tr>
<td>Prepolymer to diiso molar ratio</td>
<td>1 to 0.8</td>
</tr>
<tr>
<td>Number average molar mass (M&lt;sub&gt;n&lt;/sub&gt;)</td>
<td>10,240 Da</td>
</tr>
<tr>
<td>Weight average molar mass (M&lt;sub&gt;w&lt;/sub&gt;)</td>
<td>18,566 Da</td>
</tr>
<tr>
<td>Polydispersity index (PDI)</td>
<td>1.81</td>
</tr>
<tr>
<td>Differential scanning calorimetry (DSC)</td>
<td>No thermal events between 25 and 260 °C</td>
</tr>
<tr>
<td>Mass loss at 6 weeks</td>
<td>17.6 %</td>
</tr>
<tr>
<td>Mass loss at 9 weeks</td>
<td>55.4 %</td>
</tr>
</tbody>
</table>

Composition and pre-polymer ratio were calculated using monomer values. Molecular weights and PDI were gathered from GPC analysis on a Waters GPC system using a four column set-up consisting of Styragel® 7.8 x 300 mm columns using dichloromethane as the mobile phase. DSC was performed on a Perkin Elmer DSC 6 with a heat scheme of 20-300°C and a heating rate of 20°C/minute. Mass loss studies were conducted in vitro.
using laboratory balances, over a period of 4-12 weeks depending on polymer, with storage at 37°C in presence of phosphate buffer (physiological pH 7-7.5).

From this data, many important facets of the OC polymer can be seen. First, the system is degradable \textit{in vitro}, as is evidenced by 55% mass loss after 9 weeks. However, the majority of the construct will still likely be present after 6 weeks, allowing a window of controlled delivery. It is also interesting that the degradation process appears to accelerate, which stands to reason as such a process is often self-catalyzing due to degradation byproducts and the increasing local acidity of the construct. The PDI indicates that a rather wide range of polymer chain lengths are produced, showing the polymer does exhibit some heterogeneity. Furthermore, the lack of thermal events reported by the DSC indicates that the polymer produced is amorphous. This likely aids in the miscibility of the polymer with the diluent.

\textit{Diluent}

The OC gels alone are too viscous for easy application into the body through most normal methods. Accordingly, a method of dilution was developed for these samples that allows delivery through a standard Leur-lok® needle and syringe. This ability entails the dissolution of the OC polymer in a water-miscible polymeric diluent such as PEG. Once dissolved, the polymer is injectable. The water-miscible diluent then evacuates into the surrounding environment, precipitating the other solubilized components and allowing gelation of the desired depot. Low molecular weight PEGs such as 200, 400 and 600 Da
are used for this solubilizing purpose as the PEG itself must be a non-viscous liquid at room temperature. PEG has been shown to be biocompatible and does not elicit a dramatic/malignant immune response upon injection into human tissue.

Experimentation into modification of PEG to aid delivery has also been investigated. The purpose of end-group modification is to make slight changes to the polymer-diluent interaction. The two major modification schemes used in this work are acetylation and succinylation. These processes in general involve the introduction of a functional group compound, succinic anhydride in the case of succinylation, into an organic solvent at a low weight percentage. The solution is then heated and stirred mechanically to dissolve the compound. Once the compound is dissolved, PEG 400 is added to the flask. The temperature is increased and the reaction stirred for an extended period of time. After cooling to room temperature, the contents are poured into centrifuge tubes and the flask is rinsed with organic solvent to collect as much of the polymer as possible. The product is centrifuged and the organic solvent supernatant is decanted. Acetone is added to the tubes and then sonicated to break up the centrifuged pellet. Finally, the polymer is placed under a hood for air drying before being placed in a vacuum oven at room temperature overnight. In the case of PEG, a diol which possesses terminal hydroxyl groups, acetylation was employed to create acetylated PEG 400 (denoted as G4A). This polymer was shown through acid number testing to have converted terminal groups to acetyl groups, while still maintaining the desirable viscosity of stock PEG. G4A was used throughout the project as an experimental alternative to stock PEG 400.
Additives

Polymeric additives are used to control the formation of the construct. These polymers can serve in myriad ways, depending on the given needs of a certain application. They can be used to increase the overall hydrophilicity of a construct and thus slow release. Conversely, they can be large molecular weight or branched structures that allow perfusion of water into the delivery construct, which in turn increases the area for diffusion and thus the overall rate of delivery (Figure 3.3). Beyond this simple additive formation manipulation, charged additives can be used to induce drug/additive interactions which, in effect, produce hybridized drug/polymer salts. Work has gone into determination of additive and polymer pairings which yield desired characteristics. These delivery systems range from 3 day rapid delivery to linear release over a period of weeks capabilities, and can be tailored to specific applications in conjunction with myriad drugs.

Figure 3.3: Theoretical demonstration of mechanism of large molecular weight additive interaction with depot formation. Purple circle indicates additive.
Injectability Checks

The proposed system is intended to be capable of localized injection. The major objective of the diluent portion of the system is to solubilize the OC polymer. The OC polymer itself has been designed to allow superior precipitation mechanics. Diluent has been included in other precipitation systems as mentioned in Chapter 2; however, these systems generally require large amounts of diluent for solubilizing the chosen polymer. Because of this, as the mainly solvent system precipitates from the polymer, it generally carries a large portion of the pharmaceutical payload from the delivery construct. This is a major cause of the well-documented “burst-effect”. To this end, the OC system should be deliverable through an 18 gauge needle with the minimum amount of diluent required for room temperature delivery. This point was tested with a number of OC polymers as well as non-OC polymer controls, with G4A diluent at diluent weight percentages of 40, 33, and 20%. This testing was conducted with BD Precision Glide Needles (size 18G1½”) and 1 milliliter BD Leur-Lok™ syringes. The results of this testing can be seen in Table 3.3 with “Passing” being defined as system being capable of moving through the 18 gauge needle with reasonable force.
Table 3.3: Injectability of Multiple Polymers in Differing Diluent Percentages

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Description</th>
<th>40 wt% G4A</th>
<th>33 wt% G4A</th>
<th>20 wt% G4A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Non-urethane-linked polylactide-co-glycolides</td>
<td>Does Not Pass</td>
<td>Does Not Pass</td>
<td>Does Not Pass</td>
</tr>
<tr>
<td>OC16</td>
<td>Higher Mw OC using a higher Mw PEG prepolymer, slightly crystalline</td>
<td>Passes at Room Temperature</td>
<td>Passes at 50°C</td>
<td>Does Not Pass</td>
</tr>
<tr>
<td>OC12</td>
<td>Similar to OC16 with less PEG content</td>
<td>Passes at 50°C</td>
<td>Does Not Pass</td>
<td>Does Not Pass</td>
</tr>
<tr>
<td>OC9</td>
<td>Base Polymer, amorphous</td>
<td>Passes at Room Temperature</td>
<td>Passes at Room Temperature</td>
<td>Passes at 37°C</td>
</tr>
<tr>
<td>OC4</td>
<td>Similar to OC9, with higher PEG content to encourage better flow</td>
<td>Passes at Room Temperature</td>
<td>Passes at Room Temperature</td>
<td>Passes at 37°C</td>
</tr>
<tr>
<td>OC4-S</td>
<td>Succinylated OC4</td>
<td>Passes at Room Temperature</td>
<td>Passes at Room Temperature</td>
<td>Passes at Room Temperature</td>
</tr>
</tbody>
</table>

From this data, many conclusions can be reached. First, the inclusion of the urethane linkage and PEG segments in the OC polymers does encourage solubility and flow when combined with a PEG based diluent. This can be seen when comparing any of the OC polymers to the non-urethane-linked or PEG containing controls. Secondly, as expected, the amorphous nature of the OC polymers is also a positive influence in regard to this goal. The more amorphous OCs passed more easily when compared to the semi-crystalline OC16 and OC12, which passed more easily in turn than the even more crystalline non-OC polymers. Interestingly, a higher PEG content in the base polymer had effect in one instance (OC4 vs. OC9) while not in the more crystalline instance (OC16 vs. OC12). This can possibly be attributed to a limitation of the experimental set-up as the observations are strictly qualitative and therefore the resolution of differences
between materials is limited by human observation. The final observation of note is the fact that succinylation does not make OC4 more difficult to inject, but rather appears to increase the injectability of the system. This can likely be to the inclusion of extra oxygen groups on the base polymer, increasing the affinity of the base polymer to the heavily oxygenated PEG. With this information in mind it can be concluded that OC9 in a 67/33 weight percent mixture can be injected through an 18 gauge needle in a room temperature setting.

**Strengths of Such a System**

With injectability determined, the described system can be used for intratumoral perfusion of solid tumors, transarterial chemoembolization treatment of eligible cancers, and systemic administration of the desired bioactive agent. The area most likely for use of such disruptive technology is localized chemotherapy delivery. Such a system, assuming controlled delivery, has many advantages over other drug delivery mechanisms such as microspheres and thermogelling polymers with regard to such therapy. These include but are not limited to:

- Reduced systemic toxicity due to the local delivery of such a system
- Higher concentration of the drug at the desired site
- Efficient use of drug through near complete (e.g. ~100%) delivery from constructs and minimal loss in formation
- Lower daily drug requirements due to increased efficiency
• Continuous exposure of drug to target site, reducing the ability of the tumor to regrow or develop resistance
• Perfusion of the tumor and delivery to the entire malignancy
• Negation of the burst effect to allow targeting of a specific therapeutic window
• Independence from oscillating physiological conditions such as temperature of tumor environment for formation and delivery

With these advantages in mind, drug delivery from this system will be investigated to determine the feasibility of using such a system in treatment of solid tumors requiring constant exposure of chemotherapy. The goal of release should be a semi-linear first-order release of drug to allow constant controlled exposure to the tumor of the pharmaceutical payload. The release of this OC system is explored and discussed in detail in Chapter 4.
CHAPTER FOUR
DELIVERY OF MODEL HYDROPHILIC DRUG AND EVALUATION OF VARIABLES THEREIN

Introduction

In continuation of the description and evaluation of the system that began in Chapter 3, release was evaluated with respect to multiple variables within the OC delivery system. In each of the following studies, doxycycline hyclate (DOX) was used as a model drug. DOX is a well characterized and readily available hydrophilic drug used to treat myriad conditions in the clinic at a relatively low cost. Doxycycline’s cost-effective nature allows a large screening process to pinpoint potential studies to undertake with the much more expensive platinum drug carboplatin (CAR). CAR and DOX are both similar in hydrophilic nature and similar in molecular weight, furthering the argument for use of DOX as a model drug. Finally, DOX release will be useful for potential indications where large scale broad spectrum antibiotic use may be of benefit, such as treatment of osteomyelitis or bacterial overgrowth in the gut or female reproductive tract.

DOX is also an excellent model drug as it is easy to assay with reverse phase high-performance liquid chromatography (RP-HPLC). This positive attribute can be attributed to the hydrophilic nature of the drug as well as the presence of the multiple chromophors present in the molecule. The benzene structures impart much of the resonance to allow HPLC Ultraviolet detection; therefore, an HPLC method was developed for detection of doxycycline in phosphate buffer. This analysis was conducted using a Waters (Milford,
Massachusetts, USA) HPLC system. This system is composed of a 717+ Autosampler connected to a 1525 Binary HPLC pump. Analysis was performed with the integrated 2966 photodiode array detector system (see Chapter 5 for more detail on HPLC and the HPLC system). Water’s Empower software and an in-line degasser were used to supplement this arrangement.

The mobile phases for such analysis were ultrapure water and acetonitrile (ACN), both doped with 0.1% trifluoroacetic acid (TFA). A Waters® reverse phase C18 column was used to separate the drug from the mobile phases. The optimum method for doxycycline detection with the described system incorporated a gradient method, as described in Table 4.1. All samples were examined with at least a 10 minute equilibration step prior to analysis to ensure method accuracy. This analysis yielded a well-defined chromatogram, as seen in Figure 4.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Mobile Phase Composition</td>
<td>77% Water/23% ACN</td>
</tr>
<tr>
<td>Final Mobile Phase Composition</td>
<td>62% Water/38% ACN</td>
</tr>
<tr>
<td>Run Time</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 mL/minute</td>
</tr>
<tr>
<td>Approximate Retention Time</td>
<td>9.3 minutes</td>
</tr>
<tr>
<td>Detection Lambda (λ)</td>
<td>350 nm</td>
</tr>
</tbody>
</table>
Accurate and repeatable analysis of doxycycline allowed the preparation of multiple standard curves. Standard curves were developed from HPLC analysis of known concentrations of doxycycline. Standard curves were always found to be linear with an $R^2$ value of greater than 0.995. The linear coefficients (slopes) of the curves were then used to analyze the unknown results from the experimental runs. An example of a standard curve obtained can be seen in Figure 4.2.
The development of robust standard curves allowed the progression of the project to *in vitro* release studies. Standard curves were generated monthly to ensure accurate analysis of data. With the drug described and analytical ability ensured, the following general method was used to evaluate the difference in drug release. After mixing the components for the desired system, the drug-loaded polymer was evenly distributed into three glass 25 ml vials with approximately 1 gram of delivery system. Gel was then allowed to flow out over several hours, to occupy the bottom surface of the vial to allow a reproducible starting surface area for each test. Once the gel evenly coated the bottom of the vial, 10 milliliters of phosphate buffer (pH 7.2) was added to each vial. The samples were then incubated at 37 °C, with gentle gyration, to simulate physiological conditions. At each time period, the buffer was removed from each sample, filtered through an Acrodisc®
0.45 µm filter to remove any insolubles, such as polymer degradation products, and analyzed by HPLC. The results of this analysis were compared with those of the standard curve to allow quantification of drug release from the samples. Using DOX as a model drug, multiple delivery systems were screened for effect on drug release.

**Liquid OC Comparison**

The OC polymers can be divided into two different groups, the liquid and the solid OCs. The distinguishing feature between the two types is the presence or absence of crystalline regions within the polymer orientation. The liquid OCs (OC2 through OC10) are the more amorphous of the two groups, and tend to be more easily injected than the crystalline OC11-OC16. In order to examine the effect on drug delivery from the liquid OCs, drug release from OC2 through OC10 was tested. The results of this testing is presented in Figure 4.3 on the next page.
From this information it can be seen that OC9 and, to a lesser extent, OC2 appear to be superior in regard to sustained release of the model drug doxycycline. Many conclusions can be drawn from these results. These polymers all have similar hydrophilic PEG regions and the associated polyurethane regions. The difference between these polymers lies within the composition of the hydrophobic (e.g. non-PEG) block. First, OC5, 6, 7, and 8 all incorporate the monomer trimethylene carbonate (TMC) in the hydrophobic region. Despite changing percentages and combinations with lactide and glycolide, similar result profiles are seen. From this information, TMC-containing OC polymers do not readily appear promising for the desired release profile.

OC4 and OC3 are similar in chemical structure to OC2, but possess a weight average molecular weight half that of OC2. These polymers consist of a lactide/glycolide
hydrophobic block which exhibits promise in controlled release. As is often hypothesized in the area of controlled release, a higher weight average molar mass (Mw) polymer allows increased retention of the pharmaceutical payload. The increased Mw OC2 exhibits a reduced burst effect as well as closer to zero order release of the subsequent drug. OC10 is also similar in composition to OC3 and OC4, but uses L-lactide rather than DL-lactide. This change in lactide monomer conformation does not have a large effect on drug release from these polymers.

OC9 has the most markedly different release profile within this experimental group. OC9 differs from the other liquid OCs in the base polymer composition percentage of PEG. OC9 has a smaller hydrophilic portion than the other OC polymers. This reduction of hydrophilicity, while allowing the hypothesized phase separation gelation, appears to help controlled delivery. This can be attributed to the concept that DOX, a hydrophilic drug, will preferentially diffuse through the hydrophilic domains of the release construct. By limiting these domains, and thus the surface area of the hydrophilic areas, drug release will be inhibited. The behavior of OC9 lends credence to this idea, i.e. the greatly reduced initial release and greater control as compared to the other OC release profiles.

No polymer system tested exhibited greater drug release than 60% of payload initially, and each had quantifiable release for subsequent days. This result suggests that the system is effectively encapsulating drug in all instances and releasing at least a portion of the administered drug in a controlled manner. Also, it is evident that at day 13, the date
of termination for these studies, there is not 100% release of the hypothesized payload. This payload is calculated based on the drug percentage of the original polymer preparation and the amount of gel administered in each test sample. In order to more fully understand the system, a better accounting of drug would be helpful. To this end, an extraction method was designed.

The extraction method entails the application of 5 milliliters of acetonitrile (ACN) following the completion of the release testing, which in this case is following day 13. The ACN dissolved the polymer matrix and thus releases the remaining captured drug into solution. The polymer/ACN mixture is allowed to shake at room temperature until the polymer solid is no longer apparent in the release vial. This mixture is then combined with 5 milliliters of HPLC grade water to create a bi-phasic mixture. At this point the mixture becomes a cloudy liquid, as the polymer behaves as would be expected and preferentially separates from the aqueous portion of the mixture, theoretically leaving the majority of the hydrophilic drug in the aqueous portion. To further this separation, the mixture is centrifuged at 13000 RPM for 10 minutes. The resultant supernatant is then removed from the centrifuge vials and is thus finally devoid of the higher molecular weight polymer precipitate remaining in the centrifuge vial. This supernatant, clear and theoretically aqueous with solubilized drug, is then filtered and analyzed through HPLC. This method was tested with a known drug value and was evaluated to have a 90% extraction efficiency which was deemed adequate by team decision for evaluation of
these OC systems. Each of the liquid OC release systems was tested using this extraction technique, and the results of this analysis can be seen in Table 4.2.

**Table 4.2: Partial Mass Balance Evaluation of Drug Release from the Liquid OC Systems**

<table>
<thead>
<tr>
<th>OC System</th>
<th>% Drug Released at Day 13</th>
<th>% Drug Found in Extraction</th>
<th>% Drug Unaccounted For</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>82.5</td>
<td>5.5</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>84.5</td>
<td>0</td>
<td>16.5</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>76</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>81</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>78</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>2</td>
<td>18</td>
</tr>
</tbody>
</table>

As can be seen from this table, OC9 is the only polymer with a large amount of drug remaining within the delivery construct following Day 13. In the cases of the other release profiles, the extraction results allow a reasonable degree of certainty that release has reached a completion. Also, the extractions allow a mass balance showing that, in most cases, approximately 80% of the theoretically included drug is accounted for. While not optimal, it is important to note that the extraction methodology is not 100% efficient and that there are many opportunities for drug to be lost throughout the release
study process. Thus, the results of these studies suggest that the OC9 system is the most promising of the liquid OCs in terms of attaining a semi-linear release profile.

**Solid OC Release Profiles**

Following testing of the liquid OCs, polymers from the solid OCs were tested. These polymers have larger hydrophobic block components, have higher molecular weight PEG components in the hydrophilic domains, and have higher Mw values than the liquid OCs. These polymers tend to gel into harder constructs but are difficult to administer through the desired injection system, as described in Chapter 3. These polymers were tested in the described release format and the results of this testing can be seen in Figure 4.4.

![Figure 4.4: Release profiles of the solid OC polymers (n=3)](Error bars omitted for clarity)
From this data it is evident that the solid OC polymers do not have the same aptitude for controlled release that the liquid OC polymers have. The large hydrophobic component of these polymers, combined with the crystalline behavior inherent to such design, likely inhibits the encapsulation/depot formation seen in the liquid OCs. Also, the higher Mw PEG components of the hydrophilic domains also provide less “flexibility” than the lower Mw sub blocks used in the liquid OCs. From this less favorable data, the decision was made to move forward with the liquid OCs for future testing.

Additive Use – A6

Throughout the development of controlled release technology, much effort has been put toward the use of additives to further control the release from a construct. In this regard, four different additive groups were explored in relation to DOX release from the OC system. The first additive investigated was A6. This polymer is low molecular weight acid terminated polyglycolide. This polymer was included in polymer delivery systems at 5 and 10 weight percent loadings. The effect on release of DOX was quantified using OC2, OC4, OC6 and OC9. The results of this testing are shown in Figures 4.5, 4.6, 4.7, and 4.8 on the following pages.
Figure 4.5: Effect of doxycycline release from OC2 system and the effect of A6 inclusion (n=3, error bars indicate 1 standard deviation)
Figure 4.6: Effect of doxycycline release from OC4 system and the effect of A6 inclusion (n=3, error bars indicate 1 standard deviation)
Figure 4.7: Effect of doxycycline release from OC6 system and the effect of A6 inclusion (n=3, error bars indicate 1 standard deviation)
Figure 4.8: Effect of doxycycline release from OC9 system and the effect of A6 inclusion (n=3, error bars indicate 1 standard deviation)
The results from this testing support the idea that polyglycolide (PG) microparticles can impact release. In each instance, better defined in the OC6 and OC9 test batteries, A6 allows slow release from the constructs and encourages a more linear release profile. OC9 shows that A6 is predictably capable of influencing release as the amount of the additive in the composition increases. Greater than 10 weight percent A6 compositions were not tested as these compositions proved difficult to inject and therefore would not be viable clinically.

While these results do show that A6 can be used to control drug release, the mechanism through which this is affected is not perfectly clear. There are two potential theories for the way that A6 causes these changes. The first involves the effect that A6 has on the depot formation. A6 is a large element in comparison to the flexible OC polymers. The presence of these microparticles could cause disruptions in the formation of the typical polymer depot structure, again affecting the hydrophilic delivery domains. Also it stands to reason A6’s surface will protonate in the presence of an aqueous environment, allowing stronger binding to both the positively charged doxycycline and the OC matrix. This is supported by the fact that, as A6 quantities are increased, release is further decreased, indicating that the A6 is potentially limiting this delivery/diffusive surface area further.

A second theory involves a potential drug interaction between A6 and doxycycline hyclate. Doxycycline hyclate is a salt, like most common antibiotics, composed of the
doxycycline freebase drug and the hyclate combination of hydrochloric acid, water, and short chain alcohols. This drug complex has the potential to either interchange some portions of the hyclate with the similar PG/PEG composition of A6 or simply form a larger complex with a drug/polymer conjugate. This conjugate would then, in theory, be slower to diffuse out of the complex. This theory is reinforced by the slowing of the release rate when A6 is used in the delivery systems.

While the exact mechanism of A6 is not fully understood, it is clear that additives can have a great effect on drug release from the OC system. With this knowledge, other additives, and their effect on the system, will be explored.

*SW Polymer Additives*

After the success of A6, the SW family of polymers was developed and synthesized. These polymers are low molecular weight poly-ether-co-glycolide copolymers. These polymers were tested for release, the results of which can be seen in Figure 4.9 on the next page. These additives show even greater promise than the A6 polymers in achieving linear release. The major difference between the A6 and SW polymers is the inclusion of ether segments in the SW polymer. These segments are added through the incorporation of PEG in the copolymer. A comparison of A6 and SW results can be seen in Figure 4.10 on page 77. The decreased hydrophobicity of the construct likely helps maintain the hydrophilic drug. Perhaps most important in terms of SW effect on release, the entire release profile for OC9 is seen in Figure 4.11 on page 78.
Figure 4.9: The effect of the SW family of additives on release from the OC9 delivery system (n=3, error bars indicate 1 standard deviation)
Figure 4.10: Comparison of A6 and SW2: effect on DOX release from OC9 (n=3, error bars indicate 1 standard deviation)
Figure 4.11: Release of DOX from OC9/SW2 system over 42 days with line of best fit (n=3, error bars indicate 1 standard deviation)

From this graph it is apparent that SW additives can have a great effect on the release of DOX from the OC system. A relatively linear release is achieved. As can be seen from the best fit line in Figure 18, a linear fit garners a R² value of 0.9861. This type of release would be highly advantageous for systems requiring strict quantity control due to a limited range of acceptable concentrations between the lowest efficacious dose and the maximum tolerated dose.
BTO3 Additive

The BTO3 additive is a tri-axial ε-caprolactone-based polymer. This polymer was studied to examine the effect of additive structure on release. As a tri-axial polymer, BTO3 often behaves differently than the linear copolymers described so far. Succinylation with BTO3 magnifies the effect of a potentially charged end-group on release; the resulting polymer is denoted BTO3S. DOX release results from BTO3 and BTO3S are compared to A6 in Figure 4.12.

![Comparison of Doxycycline Release from OC4 Polymer With BTO3 Additives Compared to A6](image)

*Figure 4.12: Effect of doxycycline release from OC4 system and release differences between BTO3 and BTO3S (n=3, error bars indicate 1 standard deviation)*

Both BTO3 polymers appear to be superior in relation to A6 (OC4A), demonstrating reduced release, even in the fast releasing OC4 system. This reduction is likely due to the triaxial nature of BTO3 and the hydrophobic nature of the BTO3. However, BTO3 was
not suitable for large scale use in the system, as the caprolactone nature of the polymer caused injection difficulties, imparted a waxy, brittle quality to the final construct, and the mass loss for BTO3 is a wide window. Also, it is interesting that the succinylation (OC4BS) of the BTO3 creates a wide variation in release. This could be attributed to either (1) the lack of efficiency of the succinylation process, or (2) disturbances in the polymer matrix from the increased charge presence. If the succinylation process is not efficient at affecting the BTO3 polymer homogenously, the charges/end group modification will vary from sample to sample, effecting variability as seen in this sample.

_Diluent Effect on Release_

As the OC polymers require a diluent for administration, the effect of this diluent should be examined to completely understand the system. Polyethylene glycol with Mw of 400 (PEG 400) was chosen as the initial diluent for many reasons. PEG 400 is a liquid polymer with a relatively low viscosity, is already used in many medical applications, and is effectively miscible with the OC polymers with the application of heat. The experimental diluent G4A (acetylated PEG 400) was compared to PEG in many instances to determine the effect of end-group chemistry on depot formation and subsequent release. Figure 4.13 illustrates the difference of release on the OC9 system when comparing G4A and PEG 400 as the diluent portion.
Figure 4.13: Comparison of DOX release from OC9 system between PEG and G4A diluents (n=3, error bars indicate 1 standard deviation)

The difference in release between the two profiles, while not profound, is apparent in the initial two week period of release. While potentially helping the system in terms of injectability and shelf-life prior to injection, G4A appears to disrupt the initial release kinetics of the system. This finding was further clarified by studies, see Figure 4.14, conducted in OC4 systems, which are already plagued by quick release as seen earlier, but exacerbated by the use of G4A. Also in this graph can be seen the effect of G4S. G4S is succinylated PEG 400, which was prepared to visualize the effect on release of a different end-group modification.
Figure 4.14: Effect of doxycycline release from OC4 systems with varied diluents (n=1)

As can be seen, neither G4S or G4A appears to be a promising replacement for PEG 400 when striving to attain linear release from the OC delivery systems. These end group modification techniques appear to cause the diluent to carry more drug as they evacuate the depot formation. This is seen through the almost tenfold increase in burst between the PEG and modified diluents. From this data it stands that PEG-based diluents are capable of use with the system, but unmodified PEG should be used to best achieve linear release. Next, it is logical to explore the effect of varying diluent component percentage of the OC system. The effect of a 50/50 polymer/diluent system versus a 67/33 system is seen in Figure 4.15.
Interestingly, these samples show that, while there is little initial burst difference between more or less diluent, there is a significant effect on release in the early days of the system. This leads one to believe that the larger diluent percentage may not be able to fully evacuate the depot initially, but rather, when it is able to escape, carries a large amount of drug with it. The 50/50 composition, while injectable, does not control release as well as the polymer dominant system. Also, due to the reduced polymer presence, the 50/50 mixture does not form as repeatable or as strong depot following injection as the higher polymer percentage compositions.
Blending of OCs and Effect on Release

The OC polymers were also designed to be amenable to blending processes. Polymer blends are often employed to derive properties from multiple polymers into a single polymer. Blends differ from copolymers as the polymeric chains and units are not themselves covalently joined, but rather remain separate while acting as a single unit. In essence the OC delivery system as a whole is a blend of OC polymer, diluent polymer, and polymeric additive. The next logical step of this progression is to examine the effect of blending multiple OC polymers together to examine the effect on release. The blending is simple and achieved through physical mixing of the OC polymers, both together in the same step of the system preparation as the diluent. To first examine the feasibility of blending, a screening study was performed using OC4 and OC9 as test subjects.
Figure 4.16: Comparison of DOX release from blended OC systems (n=3 for OC9 and OC4 release, error bars indicate 1 standard deviation)

From this data it can be seen that blending is indeed possible as all three of the release curves for the blends fall approximately between the release curves for OC4 and OC9. Blending has many benefits to systems such as the OC system. Primarily it allows the development of a more modular platform without the use of complex polymerizations. Also, secondary properties including injectability and depot formation can be tuned to a specific application. The successful blending of the OC polymers opens interesting doors to tailorable delivery systems for individual applications. The ability to use multiple drugs for myriad indications provides an opportunity for personalized medicine. This
idea is discussed in more detail in Chapter 5. Following this success, blending was examined more thoroughly through a series of tests.

![Comparison of Doxycycline Release From OC Blends](image)

*Figure 4.17: Comparison of DOX Release from OC Blends (n=3, error bars indicate 1 standard deviation)*

Figure 4.17 reveals that 1:1 blending of OC4 and OC9 with OC2 produces release curves which contain characteristics of both of the single polymer systems. The OC9 blend still releases at a lower rate than the OC4 blend, but both blends show release similar to that of OC2. Also, both releases still show acceptable repeatability and therefore blending can be expected to combine characteristics of the composite polymers.
Figure 4.18: Comparison of DOX release from OC blends of differing diluent composition \((n=3, \text{ error bars indicate 1 standard deviation})\)

Figure 4.18 shows the effect of diluent change in a blended system is similar to that of the unblended or single system, specifically, the larger the diluent component, the higher the initial release. From the gathered blending data it is reasonable to postulate that blending OC polymers into a single system will result in hybridization of release profiles. These blended results, following initial observation, appear to follow the results one would have expected based on the data gathered from the single OC systems.
Conclusion

Many methods to control a water soluble drug are presented in the context of the OC system. These methods involve the manipulation of assorted variables within the system. Release from the OC system has been shown from a quick 3-day release to an almost linear release over 42 days. Such modularity with the OC system speaks to its potential use as a platform technology. If the OC system can be shown to be useful and controllable with a variety of drugs, such a system could have many uses in a wide range of applications in medicine.
CHAPTER FIVE

RELEASE OF OTHER DRUGS FROM THE OC SYSTEM; USE OF REVERSE
PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Following the examination of doxycycline as a model drug, other drugs were considered for release quantification, in conjunction with the OC system. Various bioactive agents were investigated to show the system’s modularity and potential as a platform technology. In these instances the ability to quantify drug release was vital. Accurate, rapid and repeatable detection of pharmaceutical agents allows comparisons between drug delivery constructs prior to *in vivo* studies as well as identification of therapeutic levels in samples both *in vitro* and *in vivo*. Perhaps the foremost scientific method for monitoring therapeutics is high performance liquid chromatography (HPLC).

HPLC allows clear and simple quantification of therapeutic presence in a sample, with an inherent or induced chromophore, through the detection of bound specimen to the HPLC column. Multiple drugs can be analyzed through this process; methods were developed in this research for the pharmaceutics carboplatin, cefuroxime, clindamycin, dicloxacillin, doxycycline, metronidazole, paclitaxel, and tobramycin. For each drug: (1) a brief background is supplied, (2) a reverse phase HPLC method is described, (3) a standard curve is presented to show the linearity of response for the proposed method within a range of absorbances, and (4) release is examined from the OC4 system. This quick releasing system was chosen both to illustrate the cross-utility of the OC polymers within
the proposed system as well as to illustrate that most antibiotic regimens tend to be shorter in duration than typical chemotherapeutic courses. Analysis of all drugs was performed on a single HPLC apparatus, showing the flexibility of the technology as well as the ability to analyze multiple compounds from one machine, allowing laboratories to save costs on multiple machines and columns.

**HPLC**

HPLC is a widely used method to quantify the presence of a pharmaceutical solute in samples. This analytical method involves the injection of a sample into the chromatographic column, which then retains the desired sample from the mobile phase. The extracted and bound sample can then be analyzed through a variety of methods, which involve the detection of a chromophore of the bound agent. HPLC analysis is superior to other forms of ultraviolet-visible (UV-Vis) quantification due to its ability to clearly quantitate material as well as remove material interaction issues inherent in other processes\(^{281}\). Despite this distinct advantage, HPLC is a very difficult process, with variables such as temperature, column choice, mobile phase composition and mobile phase pH being essential to consider\(^{282}\).

Column choice is a crucial variable, influencing the efficacy of an HPLC method. Columns are offered in a wide variety of types and sizes, but can generally be divided into normal phase and reverse phase columns. These column names relate to the manner in which the desired solute is removed from the mobile phase and drawn to the analytical
column. Reverse phase is by far the more prevalent technology at present and involves hydrophobic columns and aqueous-based mobile phases. Commonly used columns in reverse-phase HPLC (RP-HPLC) include C8 and C18 columns. Column choice parameters have been covered in the literature and, as RP-HPLC is by far the more prevalent, the terms RP-HPLC and HPLC will be used interchangeably in this text as they are in literature.

Following the choice of column type, HPLC methods fall into two groups based on analytical scheme. These two schemes are referred to as isocratic and gradient methods. Isocratic methods refer to the use of a single mobile phase composition for the entire analysis, whereas gradient methods refer to methods which use a changing composition through the process. Isocratic analysis has been considered faster, however gradient analysis allows higher throughput, better resolution, and separation of multiple compounds in a single sample. For these reasons gradient analysis is often employed in analytical settings where applicable. When choosing a gradient method, the mobile phase is of great concern. Mobile phases in RP-HPLC usually involve water, acetonitrile, and/or methanol and sometimes a dopant agent such as triethanolamine or trifluoroacetic acid (TFA). Because of the large amounts of variables present in such systems, method development is quite difficult and results in many methods for analyzing the same drug. For this reason, it is important to select and/or develop the best method for a given application’s indication.
A single HPLC apparatus was used for the detection of all drugs, allowing limited maintenance and ease of work flow interruption between projects. The column used was a Waters Symmetry® C18 5 µm 4.6 x 150 mm column, and the pump was a Waters 1525 binary HPLC pump. The pump and column were coupled with a Waters 2996 Photodiode Array Detector (PDA) for analysis. All mobile phases were first filtered through a Waters four channel in-line degasser prior to injection into the pump housing. Mobile phases used were HPLC grade water with 0.1 % TFA and acetonitrile (ACN) with 0.1% TFA. A Waters 717plus autosampler was employed to allow multiple sample injection. Software for analysis was Waters Empowerer Pro. The flow rate was set at 1 ml/min. Note that the methods are described by percentage of ACN mobile phase, omitting the complimentary percentage of HPLC composition in the mobile phase.

**Method Development: A General Outline**

Methods for each drug were generated specifically for the provided HPLC apparatus. A drug solution of greater than 0.1 mg/ml was prepared in mobile phase solution. If a given drug was not soluble at 0.1 mg/ml, the highest concentration found in lab was used. Sample was then analyzed on the described HPLC apparatus through a gradient method of 5% ACN to 95% ACN over a period of 20 minutes (Figure 5.1).
Figure 5.1: A 5-95% run of cefuroxime on HPLC

The 5% to 95% range was chosen to limit usage of the end ranges of column absorbance and therefore enhance the lifetime of the column. This HPLC trace provides a general range for the determination of the second method attempt. The location of the peak in the original run determines the second run, which is prepared with a range of 15% change in ACN over a space of 10 minutes (Figure 5.2).
Figure 5.2: A first attempt at a “15% method” for cefuroxime
The mobile phase composition was adjusted until the major eluent peak appeared more than 5 minutes after the solvent injection peak, to be assured of peak resolution from the solvent front (Figure 5.3).

Once this resolution was achieved, the method was determined to be acceptable. Analytical wavelength for each drug was determined as the wavelength which corresponded with the maximum absorbance response compared to the baseline noise for the sample peak, as provided by the attached photodiode array detector. Following development of the method, the process was analyzed for reproducibility through generation of a standard curve consisting of at least five varying injection quantities; an

**Figure 5.3: Acceptable method for cefuroxime analysis on HPLC**
R² value of greater than 0.99 was achieved when correlating micrograms injected and absorbance reported.

Antibiotics, the OC System, and Clinical Need

The following sections each discuss a different bioactive agent. Each of the drugs discussed was chosen for method development based on clinical need. The myriad drugs tested show the modularity of the system, and therefore the platform nature of the OC technology. A brief outline is given of the clinical use of the drugs as well as an overview of HPLC methodology for each. The results of method development and release testing from the OC system are presented subsequently. Finally, release eluents of applicable drugs were efficacy tested with a model cell line to illustrate release and maintenance of drug activity.

Cefuroxime

Cefuroxime is a member of the cephalosporin family, which displays activity against both gram-positive and gram-negative bacteria. The drug is usually well tolerated by patients and is used to treat a wide range of diseases, especially those where penicillinases are not advised for use or the patient exhibits allergic symptoms. Cefuroxime is a well-characterized drug with many methods for quantification and has a prominent chromophore with peak UV absorbance in the 270-280 nm range, making it acceptable for HPLC analysis. The drug is usually used in salt form, with cefuroxime
sodium being prevalent. This form is very hydrophilic and, for this reason, RP-HPLC can be used to quantitate drug presence in solution samples.

Cefuroxime sodium salt was determined to be best analyzed through the gradient method of 20 to 35% ACN over a time of 10 minutes. With this method, a peak was found at approximately 7.25 minutes. The best absorbance for analysis for this peak was determined to be 275 nm. A standard curve was prepared of a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.9999. A standard curve is shown in Figure 5.4.

![Figure 5.4: Cefuroxime standard curve at 275 nm](image)

From this standard curve, cefuroxime was determined to be analyzable using HPLC, allowing quantification of release from the OC4 system. Batch release studies were prepared in a method similar to previously described for doxycycline testing. Gels were
prepared used G4A as a diluent. A 2%, 5% and 10% loaded cefuroxime gel was prepared, to allow comparison of drug content effect on release. The results of the batch release studies can be seen in Figure 5.5.

![Figure 5.5: Release of cefuroxime from OC4 gel systems (n=3, error bars indicate 1 standard deviation)](image)

Interestingly, cefuroxime was initially quickly released from the OC4 depot. This is not surprising, given it is a highly water-soluble drug and the OC4 system is quick releasing. Also, no additives were used to control this burst. Even with this quick drug release, a portion of the loaded drug was released over a period of days following the initial burst in each of the three cases. Drug release was also relatively similar across specimens of each of the experimental groups. More drug was found in cumulative release measurements...
than was expected in the theoretical mass calculations of the system, which points to a need to better control the mixing process of the gel preparation. Perhaps most interesting is that as the weight percentage of drug in the gel was increased, the rapidity of drug release was also increased. This phenomenon was seen in other experimental groups, as well as in doxycycline, and points to the fact that drug concentrations must be tightly controlled in depot formulations.

To further illustrate the ability of this system to combat infection, eluents were sampled and used to treat populations of microbiological growths. *Staphylococcus aureus* (*S. aureus*, Moltox) was used as a model organism. *S. aureus* was thawed in tryptic soy broth and cultured at 30 ºC according to manufacturer instructions. All eluent samples were removed from storage (2 – 8 ºC). At that time, 15 µL of each sample was added to 5 milliliters of broth inoculated with *S. aureus* according to 0.5 McFarland Standard (0.05 absorbance reading). Controls consisted of 5 milliliters inoculated broth. Samples were vortexed gently and placed into a 30 ºC incubator overnight. After approximately 24 hours, relative absorbance was recorded for each sample using a spectrophotometer (600 nm wavelength). Relative absorbance was related to percent inhibition through use of controls. Results from 2% cefuroxime eluents can be seen in Table 5.1.
Table 5.1: Percent Inhibition of \textit{S. Aureus} by Cefuroxime Eluents

<table>
<thead>
<tr>
<th>Eluent ID</th>
<th>Drug</th>
<th>Measured Concentration after Dilution (HPLC)</th>
<th>Raw Optical Density (OD)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>-</td>
<td>-</td>
<td>0.982</td>
<td>0.00%</td>
</tr>
<tr>
<td>Control B</td>
<td>-</td>
<td>-</td>
<td>1.013</td>
<td>0.00%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Cefuroxime</td>
<td>4.15</td>
<td>0.052</td>
<td>94.79%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Cefuroxime</td>
<td>0.84</td>
<td>0.323</td>
<td>67.62%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Cefuroxime</td>
<td>0.33</td>
<td>0.662</td>
<td>33.63%</td>
</tr>
<tr>
<td>Day 4</td>
<td>Cefuroxime</td>
<td>0.12</td>
<td>0.795</td>
<td>20.30%</td>
</tr>
<tr>
<td>Day 5.5</td>
<td>Cefuroxime</td>
<td>0.08</td>
<td>0.88</td>
<td>11.78%</td>
</tr>
<tr>
<td>Day 7</td>
<td>Cefuroxime</td>
<td>0.02</td>
<td>0.991</td>
<td>0.65%</td>
</tr>
</tbody>
</table>

As can be seen from Table VI, clinically relevant cefuroxime dosing is delivered over multiple days, with the best effect coming on days 1 and 2. Inhibitory effects are seen until approximately day 7.

\textit{Clindamycin}

Clindamycin is a lincosamide that is finding a wider use in today’s clinic due to the wide range of efficacy for the drug\textsuperscript{289}. It can be used to treat infections from staphylococcal to anaerobic to even some protozoal infections\textsuperscript{290}. Clindamycin is supplied as a hydrophilic salt, traditionally as a hydrochloride. With this enhanced solubility, RP-HPLC is often employed with detection around 200 nm\textsuperscript{289, 290}. Clindamycin HPLC methods are usually isocratic\textsuperscript{289, 290} while gradient methods are useful especially as clindamycin is often used in combination treatments\textsuperscript{285}.

The clindamycin hydrochloride analysis method was determined to be 20\% to 35\% ACN over a period of 10 minutes. With this method, a peak was found at approximately 8.15 minutes. This peak was also determined to have a best absorbance for analysis at 210
nm. A standard curve was then prepared of a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.99999. A standard curve is shown in Figure 5.6.

![Figure 5.6: Clindamycin standard curve at 210 nm](image)

From this work, clindamycin was chosen for release analysis using OC4. Clindamycin was examined at concentrations of 5 and 10% by weight. Results can be seen in Figure 5.7.
Clindamycin shows a well-controlled release from the OC4 system following the inherent first day release of the system. Again, a higher release of drug occurred than was expected through theoretical measurements, pointing to a need to better control and monitor mixing conditions. These promising results were further confirmed through microbiological testing in a manner consistent with that outlined previously. The results of testing the 10% loaded gel system can be seen in Table 5.2.

*Figure 5.7: Release of clindamycin from OC4 gel systems (n=3, error bars indicate 1 standard deviation)*
Table 5.2: Percent Inhibition of *S. Aureus* by Clindamycin Eluents

<table>
<thead>
<tr>
<th>Eluent ID</th>
<th>Drug</th>
<th>Concentration</th>
<th>Raw OD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>-</td>
<td>-</td>
<td>0.876</td>
<td>0.00%</td>
</tr>
<tr>
<td>Control B</td>
<td>-</td>
<td>ug/mL</td>
<td>0.944</td>
<td>0.00%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Clindamycin</td>
<td>21.9</td>
<td>0.124</td>
<td>86.37%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Clindamycin</td>
<td>3.5</td>
<td>0.582</td>
<td>36.04%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Clindamycin</td>
<td>2.2</td>
<td>0.124</td>
<td>86.37%</td>
</tr>
<tr>
<td>Day 4</td>
<td>Clindamycin</td>
<td>2.2</td>
<td>0.154</td>
<td>83.08%</td>
</tr>
<tr>
<td>Day 8</td>
<td>Clindamycin</td>
<td>4.0</td>
<td>0.123</td>
<td>86.48%</td>
</tr>
</tbody>
</table>

From this data it can be assumed that a clinically relevant amount of efficacious clindamycin is delivered over a period of 7 days when the 10% loaded clindamycin gel is observed *in vitro*.

**Dicloxacillin**

Dicloxacillin is a modern member of the penicillin family. It is penicillinase-resistant, allowing use in indications where other members of the group are not effective\(^2\). It is a highly polar compound which causes difficulty with extraction in many processes\(^2\). Previous work with this drug has involved reverse phase columns and has used detection wavelengths between 220 and 240 nm\(^2\).\(^3\)\(^4\).

Dicloxacillin salt monohydrate was determined to be best analyzed through the gradient method of 45 to 60% ACN over a time of 10 minutes. With this method, a peak was found at approximately 7.33 minutes. This peak was also determined to have a best absorbance for analysis at 225 nm. A standard curve was then prepared of a sample
solution injected at different injection volumes, resulting in an $R^2$ value of 0.999. The standard curve is shown in Figure 5.8.

![Dicloxacillin standard curve at 225 nm](image)

**Figure 5.8: Dicloxacillin standard curve at 225 nm**

Following the development of this standard curve, dicloxacillin was examined for release from the OC4 system. Dicloxacillin was tested at 2, 5 and 10 weight percent loadings. This data is displayed in Figure 5.9.
Figure 5.9: Release of dicloxacillin from OC4 gel systems (n=3, error bars indicate 1 standard deviation)

The data shows that dicloxacillin was released from the OC system. Also, dicloxacillin was analyzed via microbiological assay. The concentrations measured from the 5% weight loaded eluents are listed in Table 5.3.

Table 5.3: Percent Inhibition of S. Aureus by Dicloxacillin Eluents

<table>
<thead>
<tr>
<th>Eluent ID</th>
<th>Drug</th>
<th>Measured Concentration after Dilution (HPLC)</th>
<th>Raw OD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>-</td>
<td></td>
<td>0.954</td>
<td>0.00%</td>
</tr>
<tr>
<td>Control B</td>
<td>-</td>
<td></td>
<td>0.967</td>
<td>0.00%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Dicloxacillin</td>
<td>12.98</td>
<td>0.07</td>
<td>92.71%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Dicloxacillin</td>
<td>3.22</td>
<td>0.204</td>
<td>78.76%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Dicloxacillin</td>
<td>1.23</td>
<td>0.021</td>
<td>97.81%</td>
</tr>
<tr>
<td>Day 4</td>
<td>Dicloxacillin</td>
<td>0.56</td>
<td>0.075</td>
<td>92.19%</td>
</tr>
<tr>
<td>Day 5.5</td>
<td>Dicloxacillin</td>
<td>0.26</td>
<td>0.314</td>
<td>67.31%</td>
</tr>
<tr>
<td>Day 7</td>
<td>Dicloxacillin</td>
<td>0.08</td>
<td>0.639</td>
<td>33.47%</td>
</tr>
</tbody>
</table>
This data suggests that a clinically relevant amount of efficacious dicloxacillin is delivered over a period of 7 days when the 5% loaded clindamycin gel is observed in vitro.

**Metronidazole**

This cytostatic drug has a wide range of uses within the clinic. Metronidazole is prescribed for conditions ranging from rosacea to bacterial vaginosis\(^\text{295}\). The drug has also been shown to sensitize tumors to radiotherapy, beginning its use in oncology\(^\text{296}\). For these conditions, the drug is often carried within a gel\(^\text{297}\), with release from the gel being an important experimental variable. For this reason, HPLC is often employed as the drug is water soluble, has a strong chromophore, and is used in the described wide variety of applications. Detection for this drug is performed over a wide range, from 250 to 350 nm\(^\text{295-297}\).

Metronidazole-free base was determined to be best analyzed through an isocratic method of 3% ACN over a time of 10 minutes. Gradient methods proved difficult due to the high water solubility of the drug, and a percentage outside the 5 to 95% preferred range was required. This method exhibits the effectiveness of isocratic methods with the described HPLC apparatus. With this method, a peak was found at approximately 7.30 minutes. This peak was also determined to have a best absorbance for analysis at 275 nm. A standard curve was then prepared of a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.999. The standard curve is shown in Figure 5.10.
Following the successful standard curve development, release was quantified from a 5 and 10 weight percentage OC delivery system. The results of this analysis can be seen in Figure 5.11.
Figure 5.11: Release of metronidazole from OC4 gel systems (n=3, error bars indicate 1 standard deviation)

Metronidazole demonstrated one of the better controlled releases from OC4 amongst the drugs observed; it was released in a controlled manner for longer than 1 week. Also, the opposite effect of drug loading was seen with metronidazole, where the higher loaded drug amount had a slower release profile than the lower, 5%, loaded construct. These differences are interesting to note as this drug was analyzed as a free base rather than a salt. Also, the drug involved has an inherent charge. Both of these facts can affect drug release and should be considered when using data from one drug to predict release of a different drug.
After these release profiles were prepared, metronidazole was examined by microbiological assay as well. Metronidazole is not effective at inhibition of *S. Aureus* so a different model organism was employed. *Veillonella parvula* was employed, as metronidazole is known to be effective against this bacterium, which is present in many systemic infections. *Veillonella parvula* was thawed in reinforced clostridial broth and cultured at 37 ºC with 5.0% CO$_2$ present, according to manufacturer instruction. Broth was inoculated with *V. parvula* according to 0.5 McFarland standard (0.05 absorbance reading). Inoculated broth (5.0 milliliters) was placed in a culture tube for each sample tested. Each eluent was stored at 2 - 8ºC prior to experimentation. All samples were removed from storage; 100µL of each sample was placed into a separate culture tube containing *V. parvula* (prepared as described earlier). Samples were vortexed gently and placed into a 37 ºC incubator with the presence of 5.0% CO$_2$ overnight. After approximately 24 hours, relative absorbance was recorded for each sample using a spectrophotometer (600 nm wavelength). Relative absorbance was related to percent inhibition through use of controls. The results from both 5 and 10 weight percent metronidazole eluents can be seen in Table 5.4.
Table 5.4: Percent Inhibition of *V. parvula* by Metronidazole Eluents

<table>
<thead>
<tr>
<th>Eluent ID</th>
<th>Drug</th>
<th>Concentration</th>
<th>Raw OD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>-</td>
<td></td>
<td>1</td>
<td>0.00%</td>
</tr>
<tr>
<td>Control B</td>
<td>-</td>
<td>ug/mL</td>
<td>1.016</td>
<td>0.00%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Metronidazole</td>
<td>68.8</td>
<td>0.04</td>
<td>96.03%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Metronidazole</td>
<td>18.3</td>
<td>0.062</td>
<td>93.85%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Metronidazole</td>
<td>8.7</td>
<td>0.036</td>
<td>96.43%</td>
</tr>
<tr>
<td>Day 6</td>
<td>Metronidazole</td>
<td>5.2</td>
<td>0.05</td>
<td>95.04%</td>
</tr>
<tr>
<td>Day 7</td>
<td>Metronidazole</td>
<td>1.0</td>
<td>0.487</td>
<td>51.69%</td>
</tr>
<tr>
<td>Day 8</td>
<td>Metronidazole</td>
<td>0.5</td>
<td>0.973</td>
<td>3.47%</td>
</tr>
<tr>
<td>Day 9</td>
<td>Metronidazole</td>
<td>0.2</td>
<td>1.072</td>
<td>-6.35%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eluent ID</th>
<th>Drug</th>
<th>Concentration</th>
<th>Raw OD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>-</td>
<td></td>
<td>0.452</td>
<td>0.00%</td>
</tr>
<tr>
<td>Control B</td>
<td>-</td>
<td>ug/mL</td>
<td>0.49</td>
<td>0.00%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Metronidazole</td>
<td>88.7</td>
<td>0.011</td>
<td>97.66%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Metronidazole</td>
<td>28.0</td>
<td>0.011</td>
<td>97.66%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Metronidazole</td>
<td>26.7</td>
<td>0.026</td>
<td>94.48%</td>
</tr>
<tr>
<td>Day 6</td>
<td>Metronidazole</td>
<td>19.1</td>
<td>0.001</td>
<td>99.79%</td>
</tr>
<tr>
<td>Day 7</td>
<td>Metronidazole</td>
<td>6.3</td>
<td>0.035</td>
<td>92.57%</td>
</tr>
<tr>
<td>Day 8</td>
<td>Metronidazole</td>
<td>4.1</td>
<td>0.018</td>
<td>96.18%</td>
</tr>
<tr>
<td>Day 9</td>
<td>Metronidazole</td>
<td>4.7</td>
<td>0.02</td>
<td>95.75%</td>
</tr>
</tbody>
</table>

As can be seen from this data, an efficacious amount of drug can be delivered for up to 1 week from a 5% loaded depot, and a 10% loaded depot delivers efficacious levels of drug for longer than 9 days.

*Paclitaxel*

Taxanes, and paclitaxel in particular, have taken a leading role in chemotherapy over the recent decade. This expansion of use is largely due to the efficacy of paclitaxel in treatment of a wide range of solid tumors. This drug moves to block mitotic activity.
by interference with the formation of microtubules\textsuperscript{299}, thus preventing cancer cell division. However, due to the prevalence of significant side effects, paclitaxel is almost always used in a low dose compared to that of other bioactive agents\textsuperscript{300}. This need for a precise drug concentration combined with the important nature of the drug indications, make paclitaxel an important drug for quantification. HPLC is uniquely suited for the low levels of drug used as well as the common use of paclitaxel with other drugs and excipients such as Chremophor EL\textsuperscript{298,301}. The hydrophobic nature of the drug does necessitate different mobile phase ratios than the other described drugs, but hydrophobic columns can be used and detection is usually around 230 nm\textsuperscript{298-301}.

Paclitaxel-free base, free of excipients, was determined to be best analyzed through the gradient method of 50 to 65\% ACN over a time of 10 minutes. With this method, a peak was found at approximately 6.55 minutes. This peak was also determined to have a best absorbance for analysis at 230 nm. A standard curve was then prepared using a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.999. The standard curve is presented in Figure 5.12.
Figure 5.12: Paclitaxel standard curve at 230 nm

This method was repeatable; however paclitaxel release in aqueous systems reveals poor results. The largest obstacle to testing in similar manners to those used for previous drugs lies in the lipophilic (non-water soluble) nature of the drug. For this reason, release studies performed in the traditional manner resulted in sub-analyzable amounts of drug release. It was postulated that the introduction of a non-aqueous solvent component, such as ACN, into the release medium could allow visualization of paclitaxel release by increasing the solubility of the drug in the medium. However, this idea was rejected due to two main concerns; (1) the drug medium would be less indicative of the physiological environment and (2) the introduction of additional solvents could adversely affect the degradation of the OC system and exhibit an inaccurate release profile. For these reasons, the only applicable method of analysis was that of an extraction study. To perform an extraction study, the extraction must be clarified and the efficiency must be
determined. To determine the extraction efficiency of paclitaxel from the OC system, three samples were prepared of 1 weight percent paclitaxel. Each of these samples was then submersed in 5 milliliters of acetonitrile (ACN) and placed in a 37°C incubator shaker. After 90 minutes the gel and drug were completely dissolved. A volume of 5 milliliters of HPLC grade water was added to each of the extraction vials. Following this addition, the solution became a cloudy mixture due to solubility issues with both the drug and polymer. Subsequently, the samples were centrifuged at 10,000 RPM for 20 minutes. A clear supernatant was achieved, extracted and filtered through a 0.45 µm Acrodisc® filter. These three eluents were then run in triplicate for each extraction using the HPLC. Results of this analysis are shown in Figure 5.13.

![Figure 5.13: Paclitaxel extraction efficiency study (n=3)](image-url)
Figure 38 shows that the paclitaxel extraction is relatively consistent with an efficiency of 42.85 percent (n=9, 1 standard deviation = 1.04). However, due to this low efficiency it was decided to postpone studies where release would be determined by the remaining drug in the gel.

Tobramycin

Many serious infections within the body require specialized antibiotics. Tobramycin is often used to combat difficult infection such as osteomyelitis and other serious gram negative bacillary infections. This aminoglycoside is similar chemically to a sugar complex but presents challenges for HPLC analysis as it lacks a chromophore. Because of this, pre-column derivatization techniques are often employed to make this drug visible to an associated UV detector. The method used in this test is similar to that presented by Lai of Varian chromatography systems. The pre-column derivatization employed involved the preparation of a 0.25 w/v solution of tobramycin in a 60/40 v/v ACN/water solvent. Simultaneously, a solution of orthopthaldehyde (OPA) in 0.1 v/v% 2-mercaptoethanol/borate buffer (0.4 M, 10.4 pH) was prepared. These two solutions were combined in a 1:1 volume ratio and allowed to react for 1 hour. At 1 hour, the solution was filtered through 0.45 µm filter and analyzed via HPLC.

Tobramycin sulfate salt was successfully prepared for HPLC analysis through a pre-column derivatization as described. A pre-column derivatization was required due to the lack of a chromophore on the tobramycin structure. Analysis of the tobramycin complex
was determined to be best performed using the gradient method of 5 to 95% ACN over a time of 20 minutes. With this method, a peak was found at approximately 6.15 minutes. This peak was also determined to have a best absorbance for analysis at 254 nm. Other peaks, resulting from the agents involved in the pre-column derivatization, present in chromatogram were omitted from standard curve analysis. A standard curve was prepared of a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.99. The standard curve is displayed in Figure 5.14.

![Figure 5.14: Tobramycin standard curve at 254 nm](image)

The reaction used to prepare the HPLC visible complex is time sensitive, so analysis of tobramycin visibility was performed. A sample was tested for response at 30, 90, 150 and 320 minutes post reaction initiation. Results can be seen in Figure 5.15. From this information it was determined that analysis should take place in the first hour following the pre-column derivatization to achieve the best results and a repeatable method.
Figure 5.15: Tobramycin time complex stability study
Following this evaluation, a release study was performed for tobramycin from OC4 at both 5 and 10 weight percent mixtures. The results of this work can be seen in Figure 5.16.

*Figure 5.16: Release of tobramycin from OC4 gel systems (n=3, error bars indicate 1 standard deviation)*

The results from this study are interesting in that, while tobramycin releases from the OC system, the results are different than those of any other drug studied. Before much is made of the difference however, it should be noted that much of the abnormality can be explained by the necessary use of a pre-column derivatization. The wide variation seen in post day 1 samples can easily be explained; samples could not be run at the exact same time due to the use of an auto sampler system. For this reason, sample 1 and sample 3 of a triplicate were analyzed as much as an hour apart which, as seen in Figure 40, can cause
considerable differences in HPLC values reported. Also, the day 1 results should be carefully scrutinized as the low values point to difficulty with the pre-column derivatization. With these items in mind, tobramycin can be incorporated into and released from the OC system, but a better method of analysis should be found to allow quantification.

Carboplatin

Carboplatin is highly interesting for this work due to the fact that it is both a chemotherapeutic and a water soluble drug. As discussed previously in Chapter 1, carboplatin is employed in a wide array of tumor treatments and would be an ideal model chemotherapeutic for such a platform technology. Interestingly, carboplatin and the other platinum drugs show great difficulty in analysis through RP-HPLC. Their hydrophilic nature, as well as the platinum complex, makes binding to hydrophobic C8 and C18 columns difficult. With this knowledge in hand, a reverse-phase HPLC method was sought for carboplatin.

Carboplatin-free base was determined to be best analyzed through an isocratic method of 5% ACN over a time of 10 minutes. Gradient methods proved difficult due to the high water solubility of the drug, and a percentage of 5% was required. Even with this highly aqueous mobile phase, the retention times were barely resolved from the solvent peak. With this method, a peak was found at approximately 2.22 minutes. Despite attempts at other mobile phase compositions, no better resolution was found. The peak was
determined to be capable of analysis and was determined to have a best absorbance at 240 nm. A standard curve was then prepared of a sample solution injected at different injection volumes, resulting in an $R^2$ value of 0.9999. A standard curve is shown in Figure 5.17. 

![Figure 5.17: Carboplatin standard curve at 240 nm](image)

Despite the difficulties with resolving the carboplatin from the solvent injection front, an excellent standard curve was prepared, lending credence to the acceptability of the method. This success lead to the incorporation of carboplatin in an OC9/PEG system both with and without the additive SW2. These samples were subjected to a typical release study, and the results of this study can be seen in Figure 5.18.
Figure 5.18: Release of carboplatin from OC9 gel systems (n=3, error bars indicate 1 standard deviation)

These exciting results indicate that carboplatin can be released from the OC systems and the release can also be controlled. Both groups have an acceptable amount of standard deviation within readouts at specific time points, but more interesting is the fact that the inclusion of the SW2 additive in the system allows a semi-linear release of the drug, visualized alone in Figure 5.19.
Figure 5.19: Release of carboplatin from an OC9/PEG/SW2 gel system (n=3, error bars indicate 1 standard deviation)

This release shows great promise for treatments of narrow therapeutic range drugs such as chemotherapeutics. Specifically, if this *in vitro* data could be carried over into an *in vivo* setting, such a treatment could allow a constant exposure of the tumor to drug as well as a mitigation of side effects seen by the patient.

**Conclusion**

The data presented in this chapter points to the potential of the OC system as a platform delivery system. Multiple drugs, antibiotics, antifungals, and chemotherapeutics, of much different chemistry were incorporated and released from the OC system. Also seen form the data, multiple drugs can be analyzed from a single HPLC apparatus.
Furthermore, each drug has a well-defined and reproducible analytical method. Each method has been determined to be efficient for the respective drug, and robust with an $R^2$ value of greater than 0.99.

Of most value within this chapter are the \textit{in vitro} release results of carboplatin from the OC9/PEG/SW2 system. This relatively linear release over 3 weeks, a typical chemotherapeutic regimen, could provide a very important approach to treatment of solid tumors. However, this data is not valuable if it cannot translate into \textit{in vivo} models.
CHAPTER SIX

THE USE OF THE OC SYSTEM TO DELIVER CARBOPLATIN IN AN ATHYMIC NUDE MOUSE MODEL

In previous chapters the ground work has been laid for the translation of a promising \textit{in vitro} performing system to the \textit{in vivo} setting. The idea of being able to introduce a system capable of continuously delivering a chemotherapeutic agent while maintaining efficacious drug concentrations holds great promise in the field of chemotherapy. To examine the potential efficacy of the OC system, further testing was required beyond the previously described characterization. This chapter outlines the use of the OC system in delivering carboplatin in an athymic mouse model.

\textit{The Murine Model and the Relation to the OC System}

The murine model is a typical model used for early \textit{in vivo} studies. A very robust and cost-effective model, mice can be used for protocols ranging from toxicity studies to tissue response visualization. For use with the OC system, the vision is to use a murine model for a tumor response study. Specifically, tumors will be induced in the mice, treated with the OC system, and monitored for tumor response. While a straight-forward plan, many variables must be considered, starting with the specific mouse strain upon which to perform studies.

128
The mouse strain chosen for this work was the Athymic Nude-Foxn1\textsuperscript{nu} (Harlan Laboratories, USA). These mice present an interesting combination of attributes as they both lack a thymus, and therefore lack lymphocytes, but also are hairless. Both of these attributes are important for the proposed study and present advantages over other strains. The genetic manipulation of the mice to prevent the formation of a thymus is valuable for induction of tumors. The lack of T and B cells prevents the mouse immune system from attempting to prevent the adherence of tumor cells implanted into the body. Accordingly, attribute cancer cell lines such as OVCAR-3 can be injected into athymic mice and tumors can be cultivated in the subjects\textsuperscript{305}. This tumorigenicity allows early stage tumor treatment models before proceeding to larger, more costly, animals.

The induced genetic mutation of athymic mice is the hairless nature, or nudity, of the strain. This lack of hair is valuable in that it allows direct observation of the epidermis of the subject. This can be valuable for monitoring of injection sites and surrounding tissue for inflammatory and immune response as well as the observation of subcutaneous artifacts. These artifacts can be either the induced tumors or devices implanted by researchers. In the instance of this work, the formation and macroscopic stability of the OC system can be observed \textit{in vivo} following subcutaneous implantation. Female mice were selected because an ovarian cancer cell line was used. For all these reasons, the female Athymic Nude-Foxn1\textsuperscript{nu} murine model was chosen for this work.
Following the identification of the animal model for this project, the assays were identified. First, following inducement of tumors, tumor progression would be monitored. Many studies use methods such as fluorescing tumor lines and systems such as the IVIS XR Lumina (Perkin-Elmer, USA) to quantify the relative florescence between animals. The use of this system however has many potential pitfalls, and a simpler, transcutaneous caliper measurement was identified. Not only is this method more cost effective and robust, it has been employed successfully by our group in past studies. For these reasons, tumor size as measured by ellipsoid caliper measurement was chosen for tumor progression monitoring. The ellipsoid caliper method includes the taking of 6 diameter measurements around the circumference of the ellipsoid shaped object and the averaging of these 6 measurements to reach a single composite value.

Gel permeation chromatography (GPC) was chosen to monitor the degradation of the OC system following implantation. Dichloromethane (DCM) was identified as a solvent capable of solubilizing the majority of the OC system and allowing quantification of molecular weight before and after insertion. Also, histology was chosen to help identify activity in the tumor microenvironment. Following excision and immersion fixation in formalin, tumors were to be processed and examined using two immunohistological stains. Apoptosis and cell proliferation at time of excision were identified as important variables showing tumor behavior and progression. Apoptosis imaging would be performed using the TUNEL assay kit (Invitrogen, USA) and cell proliferation analysis would be performed using the PCNA visualization kit (Invitrogen, USA).
With thought given to the eventual study, a pilot study was conducted to address what level of drug would be tolerated by the animals when delivered through the OC system. This preliminary work was also used to verify the assays chosen for the study as well as evaluate other potential metrics.

**Preliminary Work**

To most effectively treat the induced tumors, the maximum tolerated dose of carboplatin capable of being delivered from the OC system was determined. Such a dose would be the optimal concentration to use for the treatment of animals where a tumor had been induced as it would be the dose most likely to have an effect on the tumor without causing systemic morbid toxicity to the subjects within the study. For this reason a five mouse study was undertaken to determine an approximation of the therapeutic window.

To determine the concentration required, the literature was surveyed for the median lethal dose (LD$_{50}$). Literature values generally agreed with mouse intravenous LD$_{50}$ values of around 90 mg/kg and intraperitoneal LD$_{50}$ values of 150 mg/kg$^{307}$. As the desired site of implantation was subcutaneous, the intraperitoneal value was chosen as a better predicate value to use for these studies. Thus, using a value of 150 mg/kg and an average female mouse mass of 20 grams (0.02 kg), an LD$_{50}$ value was calculated at 3 mg per mouse. Using this as the estimated LD$_{50}$ values, mice were chosen to be tested at 15 mg, 10 mg and 3 mg if the 15 and 10 mg mice showed toxicity.
Initially, the 15 mg subject was chosen for initial testing. To calculate the volume of OC system with 5 wt% carboplatin required to reach this dosage, the density of the OC system was measured. This measurement yielded a density of 1.142 g/ml. Using this density, a 250 microliter injection was calculated to deliver 14.275 mg of drug. Due to the limitations of the injection system in quantitating delivery, a 250 µl injection was accepted as a reasonable amount to allow accurate delivery rather than an injection for exactly 15 milligrams of carboplatin.

With the injection volume calculated at 250 µl, gel preparation was initiated. OC9, PEG 400, SW2 and carboplatin were all removed from storage and placed in a chemical hood. Materials were mixed in a method similar to previously described methodology with minor exceptions. In order to allow injection into the mice subjects, sterilization was necessary. Sterilization by filtration is a common method for injectable elements, but the viscous nature of the OC system makes this problematic, time consuming, and wasteful. Gamma irradiation and ethylene oxide sterilization are options for larger scale batches and studies, but were not viable scenarios for the given work. For this reason, dry heat sterilization was chosen. All polymeric components were checked for viability at 130°C, and found to be acceptable at this temperature. The literature available for the drug carboplatin did not indicate heat stability at this temperature and was therefore not subjected to the heat sterilization, but rather carboplatin was assumed sterile due to the nature of the drug. Thus, the polymeric components were subjected to a 130°C 1-hour cycle in a dry heat oven. Following this treatment, the gel was immediately transported
to an isopropyl alcohol and irradiation sterilized biosafety cabinet and allowed to cool to room temperature. Following cooling, the carboplatin was introduced into the mixture and the system was mixed thoroughly by pestle and mortar within a sterile field. Following homogenous mixing, the system was transferred to the non-needle end of a sterile 1 ml BD luer-lok syringe capped with a 16 gauge needle and allowed to flow to fill the syringe.

Material was transferred the following day to Godley-Snell Research Center (GSRC, Clemson University, Clemson, SC) where the subjects were housed. Mouse A was anesthetized and injected in the subcutaneous space of the abdomen. The injection was performed without issue and the system visibly formed subcutaneously through the hairless mouse skin, see Figure 6.1. Seepage through the injection site was present but minimal. The mouse regained consciousness and was mobile and unaffected.
The subject was checked and examined on day 1 and no ill effects were noted. The mouse was visually checked each subsequent day with no illness noted. However, on day 7 the animal was found dead. The mouse had perished quickly and the corpse was in poor condition when found. Internal organs were misshapen and swollen; the liver of mouse A is presented in Figure 6.2 as an example.
Figure 6.2: Liver from Mouse A swollen and visibly diseased

The depot was not present in large quantities in the necropsy and therefore is postulated to have dissipated. Such rapid dissipation would explain the rapid exposure of the internal organs to carboplatin and the subsequent rapid deterioration of the subject. No signs of systemic infection were seen by the attending physician and cause of death was suggested as carboplatin toxicity. Interestingly, the mouse appeared to have been accepting nourishment until close to the time of death. The mouse had only lost ~2 grams despite the poor condition of the corpse, and the gastrointestinal (GI) tract showed fecal emboli throughout the entire system. This further strengthens the argument of a rapid degeneration of the animal.
In order to examine the behavior of the gel system \textit{in vivo}, HPLC analysis of the harvested remnants was employed. For this analysis the small amount of gel retrieved from the corpse was placed in a vacuum box and dried to a constant weight. Once constant weight was achieved, the gel/tissue complex was subjected to a drug extraction as described in previous chapters. The resultant eluent was then analyzed via HPLC. A peak was seen at a retention time similar to the carboplatin \textit{in vitro} studies, indicating that there was 0.39 mg of carboplatin in the 82.4 mg sample (0.5 wt%). However, there were issues with the given analysis. These issues include: (1) the assumption that the excised mass was entirely gel, which is unlikely as tissue was intimately joined to the gel sample, even after cleaning and (2) the assumption that the entire peak response seen in the chromatogram was carboplatin. To address concern (2), a photodiode array detector (PDA) was employed to compare the nature of the response peak generated to that of the standard curve employed in the \textit{in vitro} studies. A response of “not identical” was the outcome of the PDA spectrum comparison. This does not indicate that the compounds represented by the PDA are completely different, simply that the compounds cannot be claimed as identical. Hence, the carboplatin extracted from the gel could be degraded from the \textit{in vivo} experience, or even the extraction preparation, and caused an altered PDA signal. Regardless, the HPLC analysis of this gel was determined to be non-viable and the decision was made to perform future evaluation of gel using GPC to examine molecular weight effects over the course of the study.
Following the toxic exposure visualized in mouse A, mouse number B was treated with a 175 µl injection of the OC system. This treatment equated to roughly 10 milligrams of dose, or greater than 3x the literature LD$_{50}$. The OC system was prepared in standard fashion as previously described; however, a smaller gauge needle (20 gauge) was employed to create a smaller incision site in the subject. Injection was also performed in the rear flank in this subject, to attempt to limit the body cavity volume exposed, see Figure 6.3.

Mouse B awoke from treatment and was ambulatory within seconds. The subject never experienced any evident ill effects from treatment and survived with no weight loss for

Figure 6.3: Hind flank placement of polymer depot (Mouse B 6 days post implantation)
28 days. No signs of infection or injection site irritation were seen during study. The gel depot was evident and in place at the injection site for the complete duration of the study, see Figure 6.4. No internal organ damage was visualized upon scheduled necropsy.

Following this promising result, mouse C was treated with an identical 10 mg delivery sample. The mouse appeared healthy for the first 5 days but exhibited mass loss at day 5 and was found to have lost ~40% of body mass by day 6. The subject was euthanized according to Institutional Review Board protocol using isofluorine gas. During necropsy no internal organ damage was noted nor any signs of infection. The polymer depot was also perfectly formed and intact, as can be seen in Figure 6.5.
The cause of death was deemed to be lack of nutritional intake, with probable cause being lack of appetite as a side effect of treatment.

Following this subject loss, mice D and E were treated with 50 µl injections, which deliver approximately 3 mg, or roughly the published LD$_{50}$, of the drug. Neither mouse exhibited any ill effects throughout the study and maintained weight for the duration of the study. Both polymer depots remained formed in the hind flank for the duration of the study. Following sacrifice, liver and kidney from mouse D were immersion-fixed in formalin (10%), and subsequently prepared for staining. Both organs were stained with
hematoxylin and eosin (H&E) to allow visualization of organ response at a cellular level in both tissues. Images taken from both organs can be seen below at objective magnifications of 10x and 40x in Figure 6.6.

Figure 6.6: In clockwise order from top-left (1) Mouse D kidney at 40x (2) Mouse D kidney at 10x (3) Mouse D liver at 10x (4) Mouse D liver at 40x

As can be seen, and confirmed by a licensed pathologist, no evidence of toxicity is evident. This indicates that the carboplatin released from the 3 milligram systems was not distributed in large enough concentrations systemically to cause negative liver or kidney response on a cellular level.
To further understand the behavior of the OC system in this model, gel harvested from mouse E was subjected to GPC analysis with DCM as the mobile phase. A physically cleaned gel sample from mouse E was prepared along with four other samples via a dissolution and filtration process (0.45 µm acrodisc filter). The other four samples examined were (1) a remnant sample of the prepared gel that had not been subjected to the *in vivo* environment, (2) a neat (polymer only) OC9 polymer sample, (3) a neat SW2 polymer sample, and (4) neat PEG400. As expected, the glycolide heavy SW2 would not dissolve in the DCM solvent of the GPC (a fluorinated solvent, such as hexafluoroisopropanol (HFIP), based GPC system was not available for analysis). Therefore prior to filtration, insolubilized material could be visually seen in both OC system samples and the neat SW2 sample. Following filtration all samples were run. Results can be seen below.

![Figure 6.7: GPC results of OC system following mixing but prior to injection](image)

<table>
<thead>
<tr>
<th>Label</th>
<th>Elution Volume (cm³)</th>
<th>Retention Time (min)</th>
<th>RT (min)</th>
<th>Mh</th>
<th>Mw</th>
<th>Mz</th>
<th>Mw+1</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.700</td>
<td>33.700</td>
<td>25.700</td>
<td>1308.8</td>
<td>4756</td>
<td>985</td>
<td>14756</td>
<td>36516</td>
</tr>
</tbody>
</table>

*Figure 6.7: GPC results of OC system following mixing but prior to injection*
Figure 6.8: GPC results of OC system extracted from murine model

<table>
<thead>
<tr>
<th>Data Name</th>
<th>Elution Volume (ml)</th>
<th>Retention Time (min)</th>
<th>Adjusted RT (min)</th>
<th>Mn</th>
<th>Mn0</th>
<th>Mw</th>
<th>Mw0</th>
<th>Mw1</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.812</td>
<td>31.812</td>
<td>31.812</td>
<td>0.00</td>
<td>12500</td>
<td>8803</td>
<td>23000</td>
<td>51711</td>
<td>1.0965</td>
</tr>
</tbody>
</table>

Figure 6.9: GPC results of neat OC9 polymer

<table>
<thead>
<tr>
<th>Data Name</th>
<th>Elution Volume (ml)</th>
<th>Retention Time (min)</th>
<th>Adjusted RT (min)</th>
<th>Mn</th>
<th>Mw</th>
<th>Mw0</th>
<th>Mw1</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.119</td>
<td>30.119</td>
<td>30.118</td>
<td>1805</td>
<td>2564</td>
<td>19017</td>
<td>62117</td>
<td>4-48102</td>
</tr>
</tbody>
</table>

142
Figure 6.10: GPC results of neat PEG 400

Figure 6.11: GPC Results of neat SW2 (no peak present)
From these results it can be seen that the OC system does experience degradation when subjected to the *in vivo* environment. The neat OC9 polymer exhibits an $M_n$ of 14185 while the post-*in vivo* sample exhibits an $M_n$ of 8393. This relates to the *in vitro* degradation numbers referenced in earlier chapters and suggests that the polymer composition is biodegradable. Also, the PEG peak present in the pre-*in vivo* gel (Figure 50) is not present in the post-*in vivo* gel. This strongly indicates that the PEG had left the construct. Figure 54 is included to show that all variables have been considered and that the post-*in vivo* peak cannot be any polymer other than OC9, as SW2 is not visible on a DCM GPC. A summary of the results of the preliminary work are presented in Table 6.1.

**Table 6.1: Results of Preliminary Animal Studies**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Dose</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15 mg/ 250 µl</td>
<td>Found dead on day 7. Massive apparent damage to internal organs.</td>
</tr>
<tr>
<td>B</td>
<td>10 mg/ 175 µl</td>
<td>Survived entire 21 days with no ill effects.</td>
</tr>
<tr>
<td>C</td>
<td>10 mg/ 175 µl</td>
<td>Euthanized on day 6 following ~40% loss of body mass due to lack of nutrition.</td>
</tr>
<tr>
<td>D</td>
<td>3 mg/ 50 µl</td>
<td>Survived entire 21 days with no ill effects.</td>
</tr>
<tr>
<td>E</td>
<td>3 mg/50 µl</td>
<td>Survived entire 21 days with no ill effects.</td>
</tr>
</tbody>
</table>

From this work it was evident that no clear window of treatment could be identified, especially in this statistically underpowered study. While promising results were seen with mouse C and in the 3 mg mice, no clear window of dosing was found. Also, a
dosing above 3 milligrams would raise the probability of success in tumor response model studies. For this reason a larger pilot study was conducted.

Cell Culturing and Testing of Efficacy with Carboplatin Eluents

Concurrently with other preliminary activities, OVCAR-3 cells were acquired from ATCC (USA). The specific cell line acquired was NIH:OVCAR-3, ATCC catalog No. HTB-161. These ovarian adenocarcinoma cells were sourced originally from humans and have been used in multiple studies for xenografts over the past 30 years. In one study provided by ATCC, tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with $10^7$ cells. These cells are a biosafety hazard. The cells were provided frozen and thus were thawed to allow for use. Thawing was performed by gentle agitation in a 37°C water bath, and vial was decontaminated using 70% ethanol. Vial contents were then transferred to a centrifuge tube containing 9.0 ml complete culture medium and spun at approximately 125 xg for 5 to 7 minutes. Pellets were resuspended with medium prepared as per manufacturer’s instruction. Medium for this cell line was prepared through combining ATCC formulated RPMI1640 Medium with 0.01 mg/ml bovine insulin and fetal bovine serum to a final concentration of 20%. Cells were then incubated at 37°C in a 5% CO$_2$. Medium was renewed every 2 to 3 days during cell use.

Following the successful cultivation of cells, OVCAR-3 cells were plated at a seeding density of 37,500 cells/cm$^2$ in a 12 well plate. Once seeded the cells were exposed to free
carboplatin in aqueous solution at a calculated 2x the half maximal inhibitory concentration (IC<sub>50</sub>). The cells were monitored against controls through photospectrometry. Using this methodology, a 66% decrease was seen in cell count against the control. From this data it is reasonable to assume that the OVCAR-3 cells can be cultured in sufficient numbers for implantation and will likely be susceptible to carboplatin presence in vivo. The cell line was frozen for maintenance until a tumor study could be mounted.

**Full Pilot Study**

In order to better define the upper threshold of the therapeutic window, a pilot study was performed. In this study 20 mice were divided into 5 groups as seen in Table 6.2.

**Table 6.2: Groups and Subjects for the Pilot Study**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mouse</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,2,3,4</td>
<td>No treatment received</td>
</tr>
<tr>
<td>2</td>
<td>5,6,7,8</td>
<td>175 µl of unloaded OC system for each subject</td>
</tr>
<tr>
<td>3</td>
<td>9,10,11,12</td>
<td>175 µl of 5wt% loaded carboplatin (~10 mg dose)</td>
</tr>
<tr>
<td>4</td>
<td>13,14,15,16</td>
<td>150 µl of 5wt% loaded carboplatin (~8 mg dose)</td>
</tr>
<tr>
<td>5</td>
<td>17,18,19,20</td>
<td>100 µl of 5wt% loaded carboplatin (~6 mg dose)</td>
</tr>
</tbody>
</table>
The study was conducted in such a way as to examine the OC system independent of carboplatin, as well as at three levels of carboplatin loading. Thus, the OC system could be ruled out of serious consideration as a toxin and the experimental variable, drug loading, could also be examined.

To quantify differences between groups, four variables were identified for monitoring during the proposed study. Animal mass, gel molecular weight, complete blood count (CBC), and liver/kidney histology were all identified as important metrics of performance. Animal mass was measured in grams weekly and at death as a measure of health and nutritional intake. CBC testing was performed on animals where possible to determine whether anemic conditions were present as a result of treatment as well as immune response and lymphocyte presence in the circulating blood. GPC and histology were performed on every animal as previously described in the preliminary studies.

A single batch of OC system was prepared for the entire animal study, using described methodology. The delivery system had never been prepared on this scale before but no significant issues were seen during preparation. Gel was divided into syringes for each separate group. It should be noted that the placebo gel for group 2 was segregated prior to the integration of the carboplatin to assure absence of the bioactive agent. All twenty treatments were performed in succession and no significant difficulties were seen during injection. Needles were not changed in between mice of the same group to preserve gel. All animals awoke from anesthesia and showed no visible signs of impediment.
No difficulties were seen until day 6 when two mice, #15 and #16 were found to have expired overnight. In the next two days, Mice #11, 12, 19 and 20 were all either found dead or euthanized due to veterinary concerns as per protocol. The remaining 14 mice showed no signs of afflictions or weight loss, with many of the experimental mice gaining weight as can be seen in Table 6.3. Within the table, groups are color coded for easier identification.

**Table 6.3: Subject Weights during Course of Study**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Initial Weight (g)</th>
<th>Weight Day 7 (or prior)</th>
<th>Weight Day 14</th>
<th>Weight Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>21</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>24</td>
<td>26</td>
<td>24</td>
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<tr>
<td>4</td>
<td>22</td>
<td>23</td>
<td>25</td>
<td>25</td>
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<tr>
<td>5</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>22</td>
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<tr>
<td>6</td>
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<td>23</td>
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<td>9</td>
<td>21</td>
<td>21</td>
<td>22</td>
<td>21</td>
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<tr>
<td>10</td>
<td>23</td>
<td>24</td>
<td>26</td>
<td>26</td>
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<tr>
<td>11</td>
<td>24</td>
<td>14*</td>
<td>-</td>
<td>-</td>
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<tr>
<td>12</td>
<td>24</td>
<td>16*</td>
<td>-</td>
<td>-</td>
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<td>21</td>
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<td>21</td>
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<td>21</td>
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<tr>
<td>15</td>
<td>19</td>
<td>13†</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>23</td>
<td>16†</td>
<td>-</td>
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<td>17</td>
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<td>23</td>
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<td>18</td>
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<td>25</td>
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<tr>
<td>19</td>
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<td>15†</td>
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<td>-</td>
</tr>
<tr>
<td>20</td>
<td>22</td>
<td>13*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Mice euthanized at or before day 7 – Weight taken at euthanasia
† Mice found dead at or before day 7 – Weight taken when found
GPC was used to examine the effect on polymer molecular weight following in vivo implantation. This data can be seen in Table 6.4. It should be noted that there are no values for group 1 as no polymer was present in these animals.

**Table 6.4: Results of GPC Data from Mice Within The Study**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Final Viable Day</th>
<th>Molecular Weight ($M_n$)</th>
<th>Group Average (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>9623</td>
<td>9833.25</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>9445</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>9832</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>1218</td>
<td>7563.25</td>
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<tr>
<td>10</td>
<td>21</td>
<td>9465</td>
<td></td>
</tr>
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<td>11</td>
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<td>10488</td>
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<td>12</td>
<td>7*</td>
<td>9082</td>
<td></td>
</tr>
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<td>21</td>
<td>1240</td>
<td>5846.5</td>
</tr>
<tr>
<td>14</td>
<td>21</td>
<td>7654</td>
<td></td>
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<tr>
<td>15</td>
<td>6†</td>
<td>6910</td>
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</tr>
<tr>
<td>16</td>
<td>5†</td>
<td>7582</td>
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</tr>
<tr>
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<td>21</td>
<td>7497</td>
<td>7805.25</td>
</tr>
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<td>7683</td>
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</tr>
<tr>
<td>19</td>
<td>7†</td>
<td>8470</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>6*</td>
<td>7571</td>
<td></td>
</tr>
</tbody>
</table>

*Mice euthanized † Mice found dead

The polymer groups which contained drug appear to have exhibited more degradation than the other groups present in the study. This point can likely be attributed to the fact that the presence of the bioactive agent lowered the local pH of the construct. A lower pH is known to increase hydrolysis and hence hydrolytic degradation. Also, as the drug
leaves the construct, the avenues and volume within the construct would increase, allowing water access and increasing hydrolysis. Perhaps the next most interesting facet is that the values of the polymer in the earlier perishing subjects does not vary significantly from those of the mice that survived the entire study. This is interesting as one would expect hydrolysis to be an ongoing process, with those samples exposed to hydrolytic conditions longer to be more degraded. There are two samples, from mice 9 and 13, that were notably degraded but that did run the entire course of the study; these are the exception to the rule. It would be interesting to see if the initial degradation is a factor influencing the PEG evacuating the construct. This could be a significant contributor to the hydrolytic process and is a common variable across all groups.

All mice were exsanguinated at euthanasia and blood was provided to AnMed medical center (Seneca, SC) for a complete blood count. Exsanguination was not possible with the three mice found dead, as noted in Tables 6.5 and 6.6 on the following pages, which show the results of the CBC analysis. Note that ‘-’ indicates that no blood was collected for a CBC and that ‘NR’ denotes complications with testing at the testing site resulted in no value being reported.
### Table 6.5: Selected Results of CBC Testing (Cell Count)

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>Blood Draw/CBC</th>
<th>WBC</th>
<th>RBC</th>
<th>HGB</th>
<th>PLT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4-12 Week old Females</td>
<td>5.7</td>
<td>8.4</td>
<td>14.4</td>
<td>813.6</td>
</tr>
<tr>
<td>Top End (1 STD)</td>
<td></td>
<td></td>
<td>7.4</td>
<td>9.1</td>
<td>15.3</td>
<td>924</td>
</tr>
<tr>
<td>Low End (1 STD)</td>
<td></td>
<td></td>
<td>4</td>
<td>7.7</td>
<td>13.5</td>
<td>703.2</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>Yes</td>
<td>2.5</td>
<td>5.44</td>
<td>12.3</td>
<td>586</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>Yes</td>
<td>7.9</td>
<td>5.96</td>
<td>12.1</td>
<td>777</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>Yes</td>
<td>8</td>
<td>7.22</td>
<td>12.2</td>
<td>773</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>Yes</td>
<td>6.3</td>
<td>7.38</td>
<td>12.3</td>
<td>523</td>
</tr>
<tr>
<td>5</td>
<td>Polymer Only</td>
<td>Yes</td>
<td>6.3</td>
<td>7.18</td>
<td>13.6</td>
<td>230</td>
</tr>
<tr>
<td>6</td>
<td>Polymer Only</td>
<td>Yes</td>
<td>5.3</td>
<td>6.58</td>
<td>12.2</td>
<td>953</td>
</tr>
<tr>
<td>7</td>
<td>Polymer Only</td>
<td>Yes</td>
<td>5.9</td>
<td>6.98</td>
<td>13.2</td>
<td>954</td>
</tr>
<tr>
<td>8</td>
<td>Polymer Only</td>
<td>Yes</td>
<td>7.1</td>
<td>6.16</td>
<td>11.2</td>
<td>545</td>
</tr>
<tr>
<td>9</td>
<td>10 mg Carbo</td>
<td>Yes</td>
<td>4.1</td>
<td>6.86</td>
<td>13</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>10 mg Carbo</td>
<td>Yes</td>
<td>5.9</td>
<td>6.44</td>
<td>12.1</td>
<td>440</td>
</tr>
<tr>
<td>11</td>
<td>10 mg Carbo</td>
<td>Yes</td>
<td>1.6</td>
<td>8.21</td>
<td>14.1</td>
<td>NR</td>
</tr>
<tr>
<td>12</td>
<td>10 mg Carbo</td>
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<td>0.6</td>
<td>7.38</td>
<td>12.5</td>
<td>126</td>
</tr>
<tr>
<td>13</td>
<td>8 mg Carbo</td>
<td>Yes</td>
<td>4.9</td>
<td>7.92</td>
<td>15</td>
<td>805</td>
</tr>
<tr>
<td>14</td>
<td>8 mg Carbo</td>
<td>Yes</td>
<td>4.2</td>
<td>6.07</td>
<td>10.9</td>
<td>640</td>
</tr>
<tr>
<td>15</td>
<td>8 mg Carbo</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>8 mg Carbo</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>6 mg Carbo</td>
<td>Yes</td>
<td>5.4</td>
<td>6.19</td>
<td>11.9</td>
<td>675</td>
</tr>
<tr>
<td>18</td>
<td>6 mg Carbo</td>
<td>Yes</td>
<td>6.4</td>
<td>7</td>
<td>14.4</td>
<td>551</td>
</tr>
<tr>
<td>19</td>
<td>6 mg Carbo</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>6 mg Carbo</td>
<td>Yes</td>
<td>4.8</td>
<td>7.7</td>
<td>13</td>
<td>NR</td>
</tr>
</tbody>
</table>

Note: WBC = White Blood Cell count, RBC = Red Blood Cell count, HGB = Hemoglobin count, PLT = Platelet count
Table 6.6: Selected Results of CBC Testing (White Blood Cell Makeup)

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>Segs#</th>
<th>Lymph#</th>
<th>Mono#</th>
<th>Eos#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference Values</td>
<td></td>
<td>18.90%</td>
<td>77.90%</td>
<td>1.50%</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>0.6</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>2.8</td>
<td>4.7</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>2.6</td>
<td>4.9</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>2.6</td>
<td>3.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>Polymer Only</td>
<td>1.8</td>
<td>4.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Polymer Only</td>
<td>3.3</td>
<td>1.7</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>Polymer Only</td>
<td>2.4</td>
<td>3.1</td>
<td>0.1</td>
<td>0.3</td>
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<tr>
<td>8</td>
<td>Polymer Only</td>
<td>2.8</td>
<td>0.1</td>
<td>4</td>
<td>0.2</td>
</tr>
<tr>
<td>9</td>
<td>10 mg Carbo</td>
<td>1.3</td>
<td>2.7</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
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Note: Segs# = Neutrophil count, Lymph# = Lymphocyte count, Mono# = Monocyte count, Eos# = Eosinophils count

Across this study, many items bear evaluation. From an experimental standpoint, it is evident that larger animal groups are required. In group 4, the 8 mg dosage group, only a single CBC was gained at the end of the study making comparisons across groups difficult if not impossible. With that said, some observations can be made about the data as a whole or between other groups. First, almost all mice, including the controls, exhibited anemic or close to anemic counts of red blood cells (RBCs). This can likely be
attributed to smaller sample groups but bears noting. Next, in the animals that perished but from which blood was taken (#11, 12, and 20), white blood cell (WBC) counts were low, especially in #11 and #12. This could indicate an increase in carboplatin release into the systemic tissue as the blood and circulatory system would be the primary carrier for this systemic distribution. Also, there were reduced platelet counts in the samples from #11, 12, and 20, further evidence of a potential systemic toxicity. Lastly and important to note, Table 6.6 data shows a marked increase of non-lymphocyte WBCs. The presence of large number of neutrophils (segs# as noted on CBC), monocytes, and eosinophils in the polymer control group 2, indicates an immune response outside the normal range.

While signs of irritation and immune response were not noted macroscopically, it appears that the introduction of large amounts of polymer into the mice did elicit an immune response. This should be carefully monitored in future studies to ensure the biocompatibility of the system.

Finally histological analysis was conducted on all twenty mouse liver and kidneys. Mice organs were excised and fixated using immersion techniques in 10% formalin. Tissues were then trimmed, transferred to cassettes and embedded in paraffin wax. Samples were then sectioned using a microtome and stained. Using H&E staining, tissue sections were examined in conjunction with a licensed pathologist. No differences nor signs of toxicity were seen between groups. Representative images from livers and kidneys of both groups are provided below.
Figure 6.12: Liver histology images (H&E staining) of mice 1, 5, 9, 16, and 20. Images are displayed clockwise in numeric order with mouse 1 in the top-left corner. (10x objective magnification)
Figure 6.13: Kidney histology images (H&E staining) of mice 1, 5, 9, 16, and 20. Images are displayed clockwise in numeric order with mouse 1 in the top-left corner. (10x objective magnification)
No signs of cellular toxicity was seen in either set of tissue samples. Normal cell activity was seen in the liver sections provided as well as in the kidney samples. Some tubular distortion can be seen in Figure 6.13, but this is not excessive for mouse kidney samples.

Discussion

This pilot study provides many answers as well as questions. The primary purpose of this study, to delineate an upper threshold of treatment, does not appear to have been met. Two mice perished from each of the experimental groups, which indicates that the LD$_{50}$ of the treatment is somewhere within the range analyzed. There were not, however, large enough groups of subjects to fully elucidate the differences between the experimental groups. It can be assumed however that at 3 mg dosing, as seen in the preliminary work, that reduced toxicity will be seen. Perhaps groups can tolerate 3 mg dosing from this system with limited morbidity, which would already be an improvement over the free drug LD$_{50}$ of 3 mg.

The desired OVCAR-3 strain of cells was successfully cultured. In addition, the cells could be cultured in the relatively large quantities required for injection and future tumor xenografting. Most importantly, the OVCAR-3 cells were shown in vitro to be susceptible to carboplatin treatment, indicating that a murine model using an OVCAR-3 xenograft would be able to show quantitative tumor regression when exposed to necessary quantities of carboplatin.
While the “polymer only” injected mice showed no signs of outward distress, irritation, or immune response, the CBC work on these mice showed some troubling facets. There were signs of a systemic immune response (eosinophil presence, etc.) which indicate that the materials were not fully tolerated. While no biomaterial can be fully inert, this is a part of the system which bears further scrutiny. Despite the presence of the immune response cells, no outward toxicity or debility was noted in the mice. It also bears noting that the depot themselves were roughly 1% of the mouse’s body mass, and so would represent a very large presence within the corpus. In future studies, histology can be used at the implant site to examine tissue response and ensure that the immune response is not overactive as well as check for the formation of fibrous capsules around the implant site.

The CBC results and histology samples are somewhat contradictory with respect to systemic toxicity. From the liver and kidney H&E stains, both organs appear to be functioning normally throughout the subjects of the study. However, the CBC white blood cell and platelets values indicate a different picture of decreased viability. The liver and kidney are described as the organs of toxicity for systemically distributed carboplatin. However, in the study they appear to be healthy even in otherwise clearly sick mice. The drug very likely is not being systemically administered in large quantities, but likely overwhelming local tissue in cases of morbidity and causing organ damage in those systems.
This hypothesis is further encouraged by the observation of the GI tract in the mice that perished within the study. All mice which were deceased prior to day 21 were found to have significant damage to the GI tract, some with visible blood drainage from the anus and vagina prior to death. These organs are among the areas most closely proximal to the injection site, and if excess drug was diffusing outward from the site, without reaching the liver or kidney, the GI tract would be a logical substitute for this toxicity. Also, disruption of the GI tract could cause animals to cease eating due to disrupted appetite. These mice in turn would become weaker and therefore become more likely to perish.

Furthermore, the mice were housed in pairs as behavioral science shows us that animals housed with a partner for interaction will more accurately portray a real world scenario. This is interesting when viewed in the light of the fact that all 6 mice that perished were sets of two housed together. Nowhere in the study was there a pair of mice where one died and one lived. Also, each of the mouse pairs that died were the later of the groups in terms of injection, and a single syringe and needle was used to inject each mouse. This could mean that gel and drug could have been left on the outside of the epidermis allowing the cage mates to lick/ingest material in normal cleaning and social interaction. The effect that these materials would have directly on the GI tract following ingestion is difficult to predict but could explain the significant differences in GI health between animals within the same groups.
This last thought touches on another facet of the study that requires attention, the injections into the animals. First, the use of the same needle for an entire group to save polymer introduced significant variables throughout the study. If a single animal had been diseased an entire experimental group could have been lost. Also, the luer-lock syringe set-up did not allow accurate injection as the 1 ml syringe was graduated in 0.02 ml or 20 µl markings. This made it very difficult to attain the desired accuracy for the system. Also, as these devices are designed for delivering liquids, the gels used in this study did provide some issues mechanically with injection. Lastly, the gels required loading the night before treatment and so the gel system was at rest in the needles overnight prior to treatment. It cannot be assured that the system did not self-segregate with the PEG 400 causing the preferentially held drug to migrate to the later injection areas, resulting in a larger than anticipated actual carboplatin dose in the later mice of a group. Further, it is assumed that the entire polymer system was mixed homogenously by hand using a mortar and pestle. This process had been shown to be effective in previous studies on a smaller scale, but could have been lacking with the large quantities required for this study.

It should be noted that a significant assumption from this study was the actual LD$_{50}$ value. No free carboplatin injections were performed and actual subcutaneous values are not readily available in the literature. The calculated value based on the IP values from the literature does provide an educated guess as to the value but intravenous mouse values are significantly lower (almost $\frac{1}{2}$) and as such the actual subcutaneous LD$_{50}$ could
be lower than expected. This would indicate that the system is more successful at increasing the maximum tolerated dose than initially thought.

This research does indeed combine the fields of controlled delivery and chemotherapy in a step forward. The results suggest an increase in the LD$_{50}$, albeit unquantified, of carboplatin tolerated within a mouse model. This in turn indirectly signifies that the drug is released in lower quantities over a period of time, allowing the mouse subjects to better tolerate the dose. The system however is far from complete with many significant hurdles remaining to make a clinically viable product.

The results of the study bring to mind significant questions, not the least of which is the validity of the mouse model. The behavioral aspects alone discussed previously make the results of the mouse model difficult to interpret, and also significant questions have been brought to light recently about the translational ability of murine research\textsuperscript{308}. The subject is becoming so prevalent that it has made its way into the mainstream media, recently appearing in the New York Times\textsuperscript{309}. While this study incorporated an adequate and cost effective model for early screening exercises, future project critical studies should be performed on larger more clinically relevant models.

One significant drawback of the mouse model is the inability to test and model more complex medical problems and treatments. For example, one method of chemotherapy gaining wide spread acceptance in the clinic is transarterial chemoembolization, which
murine models have difficulty modeling. In this procedure, the tumor is accessed through the main arterial stalk which supplies the vasculature of the malignancy. The agent is then delivered via a catheter, port or other device. The agent then uses the vasculature of the tumor itself to fully irrigate and perfuse the target and thus efficiently and accurately deliver the desired pharmaceutical payload. This delivery route affords many advantages over systemic delivery, such as efficiency and reduced systemic toxicity, as well as offers the best opportunity to distribute the maximum amount of drug to the malignant tissue. Multiple clinical studies have shown the potential efficacy of such a procedure against hepatocellular cancer. This procedure is performed traditionally with a concentrated drug solution, but recently microparticles and drug-eluting beads have been examined. One such product, HepaSphere®, uses microspheres to not only treat the malignancy but also to occlude the arterial blood flow and thus act as an anti-angiogenic. The ability to not only deliver desired agents as well as prevent primary blood supply to a region makes polymeric systems attractive in this application. Such methods hold great promise for treating mid to late stage cancers in difficult locations such as the hepatic system and could provide excellent uses of the OC system.

Before larger scale studies can be undertaken it is important to understand that the system still does require further screening in a mouse system. Further work should include a statistically relevant (n=18) second pilot study of mice injected with a 3 mg dose of the drug. This study would show the tolerance of this treatment and could be used to distinguish some of the behavioral/study design variables discovered. Also, concurrently
it is suggested to perform a maximum tolerated dose study aimed at the definition of the upper threshold of the therapeutic window for free carboplatin injections in the subcutaneous region for these mice. This would allow a better idea of the increased drug delivery capability of the system.

Following this, and assuming an acceptable rate of tolerance of the 3 mg mice across the group, the model would be ready for tumor bearing studies as suggested initially. Within this study further assays could be included to fully elucidate the biological processes under examination. First, histological analysis at the implant site is suggested, in addition to the PCNA and apoptosis tumor histology. This assessment would be valuable for understanding the cellular immune response elicited by the OC system. Also, organs such as the gastrointestinal tract and ovaries, in addition to the liver and kidneys, should be examined by histological means for signs of cellular distress. Also, perhaps most importantly, pharmacokinetics (PK) work should be performed to attempt to quantify the release of drug from the construct. If there is drug being released systemically, PK work would allow quantification and would provide an idea of the release rate of the bioactive agent from the construct.

Thus the OC system and similar technologies hold great promise in the ongoing battle against cancer. Such technology could one day not only increase patient compliance and reduce healthcare costs, but also make chemotherapy more tolerable to the patient. In effect, patient tolerance could be made similar to the administration of any common
antibiotic such as penicillin by maintenance of the drug concentration within the therapeutic window. This dissertation is meant to be a humble addition to the wealth of work being put forward in this area and, if successful in the improvement of one person’s quality of life or even the addition of ten minutes with loved ones, would be well worth the effort.
CHAPTER SEVEN

CONCLUSIONS

The presented work serves to further integrate the fields of chemotherapy and controlled delivery. The different combinations of the polymer, diluents and additives serve to provide a range of release of a water soluble drug from 3 to greater than 45 days, thus allowing the potential for addressing of a large number of clinical indications. Also, the OC system was shown capable of release of the chemotherapeutic carboplatin in a relatively linear manner over a period of 3 weeks, the course of a typical chemotherapeutic regimen. Finally, the OC polymer system was examined in an in vivo model and tolerance of the non-drug loaded system was exhibited. Preliminary work helped to show that mice would tolerate levels of treatment greater than the 3 mg indicated by the literature as well as the effective formation of the system in the subcutaneous region of all 20 mouse subjects. The system was also shown to be absorbable and that the absorbable by-products did not cause death or incapacitation in the 3 weeks of observation. A tumor cell line, OVCAR-3, was identified, cultured, and proven susceptible to the chosen drug carboplatin in vitro at 2x the half maximal inhibitory concentration.

The polymer system is shown to be promising for use with chemotherapy. The ability to replace once per 3 week exposure regimens with a constant release of drug holds great clinical potential. The ability to deliver drugs in this manner could not only increase tumor response to chemotherapy and increase survival metrics in patients, but also limit
the inherent side effects of such therapy. This work has laid the foundation for future, larger, statistically significant studies wherein the tumor response of the system can be measured.
The following items are suggested for future studies:

- A larger, more statistically significant, maximum tolerated dose study should be performed to further define the therapeutic window. A sample size of at least 18 specimens is suggested per group, with dosing ranging from 3 mg to 15 mg.

- A study should be performed to determine the free carboplatin, e.g. not in the OC depot, maximum tolerated dose to offer a quantifiable control group in future studies.

- Other assays should be incorporated in future studies. These assays include:
  - Immunohistochemistry at tumor sites to show cell proliferation (a metric of tumor growth) and cell apoptosis (a metric of anti-neoplastic effectiveness).

- Histology, including simple hematoxylin and eosinstaining, should also be performed on other organs beyond those examined in this research, such as the brain, gastrointestinal tract and ovaries as well as at the implant site.
Phamacokinetics should be examined for carboplatin release *in vivo* to allow quantification of release, examination of drug build-up in the surrounding tissue, and systemic circulation of drug.

A larger animal model should be investigated for future animal studies.

The OC system should be examined for treatment of other conditions, such as diabetes, patient compliance, inflammation reduction and long-term disease control, where a sustained release of a pharmaceutical agent could be helpful.
BIBLIOGRAPHY


27. Sriprasad S. “History of prostate cancer treatment” Surgical Oncology 18 (2009) 185-191


32. Denis L. “The role of active treatment in early prostate cancer” Radiotherapy and Oncology 57 (2000) 251-258


46. Jacob S, Ng W, Delaney G, Barton M. “Estimation of an Optimal Chemotherapy Utilisation Rate for Primary Malignant Brain Tumours: an Evidence-based Benchmark for Cancer Care” Clinical Oncology 23 (2011) 48-54


55. Sood AK. “Fifth International Conference on Ovarian Cancer: Challenges and opportunities” Gynecologic Oncology 97 (2005) 916 – 923


64. Frumovitz M. “Vascular endothelial growth factor (VEGF) pathway as a therapeutic target in gynecologic malignancies” Gynecologic Oncology 104 (2007) 768–778


174


93. Sakamoto A. and Iwamoto Y. “Current Status and Perspectives Regarding the Treatment of Osteosarcoma: Chemotherapy” Reviews on Recent Clinical Trials, 3 (2008) 228-231


109. Azambuja E, Fleck JF, Batista RG, Menna Barreto SS. “Bleomycin lung toxicity: who are the patients with increased risk?” Pulmonary Pharmacology & Therapeutics 18 (2005) 363–366


118. Matsui H. “Combination Chemotherapy with Methotrexate, Etoposide, and Actinomycin D for High-Risk Gestational Trophoblastic Tumors” Gynecologic Oncology 78 (2000) 28–31


120. Lasek W, Sora M, Wańkowicz A, Jakóbisiak M. “Combination of immunotherapy with cyclophosphamide actinomycin D chemotherapy potentiates antileukemic effect and reduces toxicity in a L1210 leukemia model in mice” Cancer Letters 89 (1995) 137-143


182. Tulandi T and DeCherney H. “Limiting access to letrozole—is it justified?” Fertility and Sterility 88 (2007) 779-780


218. Fukumora D and Jain R. “Tumor microvasculature and microenvironment: Targets for anti-angiogenesis and normalization” Microvascular Research 74 (2007) 72–84


285. Thomas L, Bizikova T, Minihan A. “In Vitro Elution and Antibacterial Activity of Clindamycin, Amikacin and Vancomycin from R-gel Polymer” Veterinary Surgery 40 (2011) 774-780


304. Lai F. “Enhancement of detection sensitivity of tobramycin using pre-column derivatization” LC Vairan Application Note Number 5 LC5:0991

305. Product Information Sheet for ATCC® HTB-161™ American Type Culture Collection (2011)


