MODULATION OF MULTIDRUG RESISTANCE IN CANCER USING POLYMER-BLEND NANOPARTICLES

Thesis Presented

by

Lilian Emilia van Vlerken

to

The Bouvé Graduate School of Health Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Science with Specialization in Pharmaceutics

NORTHEASTERN UNIVERSITY
BOSTON, MASSACHUSETTS

January 30th, 2008
Signature page 1 (Signed by Dean and Library)
Northeastern University
Bouve Graduate School of Health Sciences

Thesis title: Modulation of Multidrug Resistance in Cancer Using Polymer-Blend Nanoparticles

Author: Lilian Emilia van Vlerken

Program: Pharmaceutical Science with specialization in Pharmaceutics and Drug Delivery Systems

Approval for thesis requirements of the Doctor of Philosophy in Pharmaceutical Science

Thesis Committee (Chairman) ___________________________ Date:_______
__________________________ Date:_______
__________________________ Date:_______
__________________________ Date:_______
__________________________ Date:_______

Director of the Graduate School ___________________________ Date:_______
Dean ___________________________ Date:_______
Copy Deposited in Library ___________________________ Date:_______
Thesis title: Modulation of Multidrug Resistance in Cancer Using Polymer-Blend Nanoparticles

Author: Lilian Emilia van Vlerken

Program: Pharmaceutical Science with specialization in Pharmaceutics and Drug Delivery Systems

Approval for thesis requirements of the Doctor of Philosophy in Pharmaceutical Science

Chairman: __________________________ Date:_______

Thesis Committee __________________________ Date:_______
________________________________________ Date:_______
________________________________________ Date:_______
________________________________________ Date:_______

Director of the Graduate School __________________________ Date:_______
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>9</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>11</td>
</tr>
<tr>
<td>OBJECTIVE AND SPECIFIC AIMS</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER 1: MULTIDRUG RESISTANCE IN CANCER</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER 2: EVALUATION OF THE THERAPEUTIC EFFICACY OF COMBINATION PACLITAXEL AND CERAMIDE TO OVERCOME MDR IN IN-VITRO MODELS OF HUMAN BREAST AND OVARIAN CANCERS</td>
<td>37</td>
</tr>
<tr>
<td>CHAPTER 3: VERIFICATION OF THE HYPOTHESESIZED CELLULAR MECHANISM WHEREBY PACLITAXEL AND CERAMIDE CO-THERAPY OVERCOMES TUMOR MDR</td>
<td>65</td>
</tr>
<tr>
<td>CHAPTER 4: DEVELOPMENT AND CHARACTERIZATION OF A POLYMER-BLEND NANOPARTICLE DRUG DELIVERY SYSTEM FOR CO-ADMINISTRATION OF PACLITAXEL AND CERAMIDE CO-THERAPY THROUGH TEMPORAL-CONTROLLED RELEASE</td>
<td>85</td>
</tr>
<tr>
<td>CHAPTER 5: EVALUATION OF THE THERAPEUTIC EFFICACY AND SAFETY PROFILES OF PACLITAXEL AND CERAMIDE POLYMER-BLEND NANOPARTICLE THERAPY TO OVERCOME MDR IN IN-VIVO MODELS OF HUMAN BREAST AND OVARIAN CANCER</td>
<td>114</td>
</tr>
<tr>
<td>CHAPTER 6: EVALUATION OF THE BIODISTRIBUTION AND PHARMACOKINETIC PROFILES OF PACLITAXEL AND CERAMIDE ADMINISTERED IN POLYMER-BLEND NANOPARTICLE IN-VIVO IN TUMOR-BEARING ANIMALS</td>
<td>158</td>
</tr>
<tr>
<td>CONCLUDING REMARKS</td>
<td>179</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>181</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Cellular mechanism of MDR</td>
<td>17</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Ceramide synthesis and metabolism</td>
<td>21</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Tumor targeting by the EPR effect</td>
<td>27</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Chemical structures of polymers and drugs</td>
<td>29</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Multifunctional nanoparticles</td>
<td>33</td>
</tr>
<tr>
<td>Figure 6</td>
<td>MDR phenotyping: protein markers</td>
<td>44</td>
</tr>
<tr>
<td>Figure 7</td>
<td>MDR phenotyping: PTX resistance</td>
<td>45</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Potency of synthetic CER analogs</td>
<td>47</td>
</tr>
<tr>
<td>Figure 9</td>
<td>C6-CER dose response</td>
<td>48</td>
</tr>
<tr>
<td>Figure 10</td>
<td>C6-CER hepatotoxicity</td>
<td>50</td>
</tr>
<tr>
<td>Figure 11</td>
<td>PTX + CER <em>in-vitro</em> efficacy against MDR</td>
<td>52</td>
</tr>
<tr>
<td>Figure 12</td>
<td>PTX + CER <em>in-vitro</em> efficacy against wild-type cancer</td>
<td>54</td>
</tr>
<tr>
<td>Figure 13</td>
<td>PEO-PCL nanoparticle characterization: SEM and drug release</td>
<td>56</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Intracellular PEO-PCL nanoparticle trafficking</td>
<td>57</td>
</tr>
<tr>
<td>Figure 15</td>
<td>PTX + CER nanoparticle <em>in-vitro</em> efficacy against MDR</td>
<td>59</td>
</tr>
<tr>
<td>Figure 16</td>
<td>PTX + CER vs. PTX + TAM <em>in-vitro</em> efficacy</td>
<td>61</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Time-dependent intracellular drug accumulation</td>
<td>71</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Dose-dependent intracellular drug accumulation</td>
<td>72</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Intracellular retention/efflux of rhodamine-123</td>
<td>75</td>
</tr>
<tr>
<td>Figure 20</td>
<td><em>In-vitro</em> apoptotic activity: Yo-PRO and PI inclution</td>
<td>77</td>
</tr>
</tbody>
</table>
Figure 21 – *In-vitro* apoptotic activity: caspase-3 activity

Figure 22 – Caspase-dependent vs. –independent apoptotic activity

Figure 23 – *In-vitro* efficacy of temporally spaced drug administration

Figure 24 – Polymer-blend nanoparticle design

Figure 25 – Polymer-blend nanoparticle manufacturing

Figure 26 – SEM and TEM of polymer-blend nanoparticles

Figure 27 – *In-vitro* drug release from polymer-blend nanoparticles

Figure 28 – Polymer-blend film microscopy

Figure 29 – Drug compartmentalization in polymer-blend films

Figure 30 – *In-vitro* efficacy of polymer-blend nanoparticles

Figure 31 – Intracellular trafficking of polymer-blend nanoparticles

Figure 32 – *In-vivo* efficacy of polymer-blend nanoparticles: breast cancer

Figure 33 – Tumor volumes before and after treatment: breast cancer

Figure 34 – Final tumor mass after treatment: breast cancer

Figure 35 – *In-vivo* efficacy of polymer-blend nanoparticles: ovarian cancer

Figure 36 – Final tumor mass after treatment: ovarian cancer

Figure 37 – *In-vivo* apoptotic activity: TUNEL staining

Figure 38 – Tumor MDR phenotyping: P-glycoprotein

Figure 39 – Tumor MDR phenotyping: GCS

Figure 40 – Stroma vs. carcinoma cell composition of tumors

Figure 41 – Safety evaluation: body weight changes
**Figure 42** – Safety evaluation: white blood cell counts  
**Page 146**

**Figure 43** – Safety evaluation: serum ALT activity  
**Page 148**

**Figure 44** – Safety evaluation: serum LDH activity  
**Page 149**

**Figure 45** – Safety evaluation: liver histology  
**Page 151**

**Figure 46** – Blood and tumor PK of PTX + CER: MCF7 tumors  
**Page 162**

**Figure 47** – Qualitative tumor biodistribution: MCF7 tumors  
**Page 163**

**Figure 48** – Blood and tumor PK of PTX + CER: MCF7_{TR} tumors  
**Page 165**

**Figure 49** – Qualitative tumor biodistribution: MCF7_{TR} tumors  
**Page 166**

**Figure 50** – Tissue biodistribution of PTX + CER: MCF7 tumors  
**Page 171**

**Figure 51** – Tissue biodistribution of PTX + CER: MCF7_{TR} tumors  
**Page 172**
LIST OF TABLES

Table 1 – Size and zeta-potential of polymer-blend nanoparticles 95
Table 2 – ESCA of polymer-blend nanoparticles 98
Table 3 – Serum estradiol levels 124
Table 4 – Blood PK of PTX + CER 168
Table 5 – Tumor PK of PTX + CER 169
SUMMARY

The development of multidrug resistance (MDR) to a wide variety of chemotherapeutic agents is one of the most challenging aspects of cancer therapy, and is often the cause for poor patient prognosis, since it renders the cancer un-responsive to most chemotherapeutic options. Cases of MDR in the clinic are often seen in patients that have breast or ovarian cancer, and statistics point to the fact that more than 50% of these patients will develop multidrug resistance, particularly upon relapse. Current therapeutic strategies involve the use of highly toxic doses and combinations of chemotherapeutics, although frequently unsuccessful. Strategies to circumvent one common cellular mechanism whereby MDR arises, namely P-glycoprotein efflux, are undergoing clinical trials, albeit with little success to date, due to poor target responsiveness and high systemic toxicity.

As an alternate mechanism to overcome MDR, this work describes the development of a polymeric nanoparticle platform to deliver a combination therapy of the drug C6-ceramide, a synthetic analog of an endogenously occurring sphingolipid, together with the chemotherapeutic paclitaxel. This combination therapy aims to circumvent a second common cellular mechanism whereby MDR also can arise, namely the inhibition of apoptotic signaling. Drug delivery within polymeric nanoparticles furthermore enhances tumor-targeting of the therapeutic load, thereby resulting in increased therapeutic efficacy and a decrease in adverse side-effects. Moreover, evidence suggests that this nanoparticle strategy can simultaneously bypass P-glycoprotein efflux, thereby overcoming MDR by two main cellular mechanisms giving rise to the phenotype. For enhanced therapeutic efficacy, a nanoparticle system has been engineered that is
composed of a blend of two polymers, a pH-responsive polymer and a slow degrading polymer, in such a way that the release of the two therapeutic agents (ceramide and paclitaxel) is precisely tuned for optimal efficacy. The studies reveal that the therapy indeed proves most efficacious against tumors bearing the MDR phenotype, as revealed in both in-vivo models of breast as well as ovarian MDR cancer. Not surprisingly, the data supports the idea that this therapy achieves success by not only by prolonging drug retention in tumor and blood and enhancing tumor drug delivery through the physical properties of nanoparticles, but more importantly by overcoming the hypothesized cellular mechanisms of MDR through the combination therapy approach. Thus, hereby a novel therapeutic strategy is presented for the treatment of MDR cancer that shows promise for clinical success.
ACKNOWLEDGEMENTS

Many individuals have played key roles towards the achievement and completion of this project through the contribution of valuable resources, suggestions, support, and time who I would like to acknowledge. First and foremost, I would like to thank my advisor, Dr. Mansoor Amiji, for all the support, advice and opportunity that he has given me. Furthermore, I would like to thank my thesis committee members Dr. Ralph Loring, Dr. Robert Campbell, Dr. Tara Pouyani, and Dr. Michael Seiden for their support, advice, and generous allotment of time. I would also like to thank Dr. Michael Seiden and members of his group in the Hematology and Oncology Department at Mass. General Hospital, namely Jennifer Mahoney and Kate Wolak for their kind and generous share of cell lines and other valuable resources. In addition, many thanks to Dr. Zhenfeng Duan for his valuable support, suggestions, and share of resources. Moreover, I would like to thank Dr. Robert Langer of Mass. Institute of Technology and Dr. Steven Little of the University of Pittsburgh for providing us with poly(beta-amino ester). Much gratitude also goes out to Dr. Robert Campbell and members of his lab for sharing the use of their fluorescent microscope, as well as Dr. Barbara Waszczak and members of her lab for sharing the use of the cryostat. In addition, I would also like to thank William Fowle for help with electron microscopy characterization and Dr. Lara Gamble of the University of Washington for ESCA studies (under NIBIB grant EB-002027). I would like to thank Dr. Ed Luther and Mary Lee of Compucyte Corp. for their help with iCys microplate cytometry studies. Further, much gratitude to Dr. Bo Rueda and his lab members at the Mass. General Hospital for suggestions and help in the preparation of silastic estradiol implants. Additional gratitude goes out to Dr. Dinesh Shenoy and Dr. Shashi Mehta for
all their guidance, support, and advice throughout this entire project. Special thanks also go out to the IGERT Nanomedical Science and Technology program committee members; Dr. Sridhar Srinivas, Dr. Mansoor Amiji, Dr. Mary Jo Ondrechen, Dr. Sanjeev Mukerjee, and Dr. Gilda Barbarino, and colleagues of the IGERT program for the opportunity to join the program and support throughout. Particular gratitude hereby goes out to Dr. Dattatri Nagesha and Rita Kaderian, for all their help and support throughout this fellowship. Lastly, very special thanks to my family and friends and my colleagues and friends within the lab and department for all their support and guidance throughout this project.

**Financial Support Acknowledgments:** This project was supported by grants from the National Cancer Institute of the National Institutes of Health through R01-CA095522 and R01-CA119617. Additionally, Lilian E. van Vlerken was a Fellow in the IGERT Nanomedical Science and Technology training program, which is jointly funded by the National Cancer Institute and National Science Foundation under grant number CA0504331.
OBJECTIVE AND SPECIFIC AIMS

Development of acquired multidrug resistance (MDR) represents the single most important reason for failure of many chemotherapeutic agents in the clinical management of cancer. In this doctoral dissertation study, we have developed a multifunctional nanotherapeutic approach for overcoming MDR. Our innovative strategy is based on combining drug delivery optimization to solid tumors and reversal of cellular resistance by reinstatement of the apoptotic signal. We have developed and evaluated engineered nanocarrier systems using biodegradable polymer blends that contain a pro-apoptotic drug (paclitaxel, PTX) and an apoptotic modulator (C6-ceramide, CER) and examined the delivery potential, as well as, the efficacy in sensitive and resistant models of human breast (MCF-7) and ovarian (SKOV3) tumors.

The specific aims of this dissertation research were to:

Aim 1: Evaluate the therapeutic efficacy of a PTX/CER combination therapy to overcome multidrug resistance in-vitro in human breast and ovarian cancer models.

Aim 2: Evaluate and verify the proposed cellular mechanisms whereby the PTX/CER combination therapy overcomes multidrug resistance.

Aim 3: Develop and characterize a polymer blend nanoparticle drug delivery system for co-administration of the PTX/CER combination therapy based on optimum release kinetics of each agent.

Aim 4: Evaluate the therapeutic efficacy of the novel PTX/CER nanoparticle therapy to overcome MDR in-vivo in an orthotopic human breast cancer model.
Aim 5: Investigate the biodistribution, pharmacokinetics, and safety profile of the novel PTX/CER nanoparticle therapy in-vivo in an orthotopic human breast cancer model.
CHAPTER 1: MULTIDRUG RESISTANCE IN CANCER

I. Classification and Mechanisms of MDR in Cancer

In the battle against cancer, the development of MDR poses one of the most challenging threats to survival, and is commonly found to be the reason for tumor persistence despite invasive chemotherapy. As the term implies, MDR refers to a cross-resistance to structurally and functionally unrelated drugs, thereby rendering the tumor unresponsive to most chemotherapeutic options. In response, patients that present with the MDR phenotype are often given higher doses and/or combinations of chemotherapeutic drugs (Bast RC, et al. 2000), still often failing to eradicate the tumor entirely. And since doses of potent cytotoxic drugs cannot be limitlessly increased, therapeutic success at this stage hinges on therapeutic strategies that will circumvent the cellular mechanisms that give rise to MDR.

MDR is classified as “intrinsic” if, by genetic pre-disposition, the cancer is unresponsive to the drugs from the outset (Bast RC, et al. 2000). MDR can also be “acquired”, which occurs when the cancer becomes insensitive to the drugs upon relapse following initial sustained treatment (Bast RC, et al. 2000). Chemoresistance can generally result from either of two means, by a physical impairment to drug delivery to the tumor (Galmarini CM, et al. 2003), such as poor absorption, increased metabolism/excretion, or poor diffusion of drugs into the tumor mass, or, more challengingly, through intracellular mechanisms in the cancer cell itself (Bradley G, et al. 1988; Gottesman MM, et al. 2002; Kellen JA 2003). Alterations in the intracellular machinery of cancer cells is commonly implicated in the development of MDR, and,
often, more than one mechanism, either simultaneous or sequential, may be responsible for the MDR phenotype (Harris AL, et al. 1992; Kellen JA 2003). The most frequently occurring causes of MDR include overexpression of membrane-bound ATP-dependant drug efflux pumps from the ABC transporter family (most notably P-glycoprotein/MDR-1), modifications in drug metabolism through glutathione-S-transferase or cytochrome P450 activity, alterations in DNA repair mechanisms, and modifications of apoptotic signaling (Bradley G, et al. 1988; Harris AL, et al. 1992; Gottesman MM, et al. 2002) (Figure 1).

II. MDR Prevalence and Current Therapeutic Strategies

The MDR phenotype was first described in 1970, when Biedler and Riehm obtained a cell line that showed cross-resistance between unrelated classes of chemotherapeutic agents (Biedler JL, et al. 1970). Soon afterwards, the ABC-drug transporters, which included P-glycoprotein, were identified, and quickly became the diagnostic marker for MDR. While certain cancers, particularly colon, kidney, and adrenal adenocarcinomas, generally express high levels of MDR-1, the gene transcript encoding P-glycoprotein, intrinsically, most cancers, such as breast, lung, ovarian, prostate and head and neck, generally express low levels of MDR-1. A particularly striking observation was that MDR-1 mRNA levels drastically increased in these cancers following chemotherapy, and that the overall pattern of chemosensitivity closely matched MDR-1 expression, whereby kidney and colon cancers were intrinsically chemoresistant, while ovarian, breast, and small cell lung cancers reverted from chemosensitive to chemoresistant (Goldstein LJ, et al. 1989). These patterns were frequently observed in subsequent studies, thereby firmly establishing the link between P-glycoprotein, and
Figure 1 – Illustration of the main cellular mechanisms of MDR (adapted from Gottesman MM, et al. 2002)
related proteins of the ABC-transporter family, as the basis for MDR (Kartner N, et al.
2005).

Once the mechanism of MDR had been established, and based on this mechanism,

novel therapeutic strategies were enthusiastically aimed at blocking the ABC-drug efflux

transporters. Soon thereafter, agents such as verapamil, quinine, and cyclosporine-A with

prior approval for use in other medical purposes, were shown to block P-glycoprotein
effectively, and thus trials ensued to investigate their clinical use as MDR modulators
efficacy and high systemic toxicity of combination cytotoxic drugs and P-glycoprotein
modulators ultimately led to their failure in the clinic (Robert J, et al. 2003). Second
generation P-glycoprotein inhibitors were designed as analogs of the first generation
drugs, so that the systemic toxicity was minimized (Robert J, et al. 2003; Szakacs G, et al.
2006). For example, PSC-833, an analog of the immunosuppressant drug cyclosporine-A,
antagonized P-glycoprotein without inhibiting immune function. However, promising
early clinical trials ultimately failed again due to low transporter affinity and high
pharmacokinetic interactions with the chemotherapeutic drugs (Szakacs G, et al. 2006).
Third generation P-glycoprotein inhibitors were designed specifically for high target
affinity and low pharmacokinetic interactions, resulting in compounds such as LY335979
and GF-120918 that showed nanomolar target affinity (Robert J, et al. 2003). However,
clinical success of even these compounds remains inconclusive, and the strong affinity of
these antagonists to P-glycoprotein, a transporter with important endogenous properties

The lack of clinical success with these prior generations of MDR modulators strengthened the idea that MDR in cancer is in fact due to other mechanisms besides drug efflux. In recent years, basic research and clinical evidence has suggested the involvement of other mechanisms of MDR within the cancer cell that give rise to the MDR phenotype. The role of DNA repair following damage by certain chemotherapeutic classes through topoisomerase I and II activity, and neutralization of electrophilic drugs by glutathione-s-transferase have been reported as mechanisms whereby the cancer cells develop chemoresistance (Harris AL, et al. 1992). Similarly, modulation of the programmed cell death (apoptosis) pathway has emerged with clear importance as an alternative strategy whereby cancers become chemoresistant. Apoptosis is an integral part of the cytotoxic action of many anticancer agents (Lutzker SG, et al. 1996); therefore, it is not surprising that the inhibition of apoptotic signaling leads to a resistance in the chemotherapeutic response. Deregulation of several key apoptosis modulating factors has been described in various experimental cases of MDR (Tolomeo M, et al. 2002; Pommier Y, et al. 2004), including functional upregulation or overexpression of p21, Bcl-2, and Bcl-XL, and/or downregulation of the classic oncogenic mediator, p53. As a result, MDR modulation strategies are increasingly looking away from the ABC-transporter paradigm and towards modulation of apoptotic signaling. Several apoptosis modulating strategies (e.g., protein tyrosine kinases PKI166 and ST1571, BCL-2 antisense G-3139, and retinoids 9-cis-RA and AM-580) are currently in clinical trials, and their efficacy in MDR modulation is largely under preclinical investigation (Tolomeo M, et al. 2002).
Currently, clinical relevance of MDR is most notable in the treatment of breast and ovarian cancer, where large percentages (~30% for ovarian cancer) of women will have resistant tumors at onset and almost all of them will become resistant upon relapse. Paclitaxel (PTX), an anti-tumor chemotherapeutic agent originally derived from the bark of the Pacific yew tree (Taxus brevifolia) (Wani M C, et al. 1971), is widely used in the treatment of solid tumors, particularly as the first-line drug in treatment of cancers of the breast and ovaries (Penson RT, et al. 1999; Khayat D, et al. 2000). PTX exerts its cytotoxicity though several mechanisms (Yusuf RZ, et al. 2003), but classically by binding tubulin, thereby inducing tubulin hyperpolymerization resulting in super-stable microtubules, which interferes with mitotic spindle function and ultimately arrests cells in the G2/M phase of mitosis, resulting in apoptosis (Bhalla KN 2003). PTX resistance has been shown to largely result from either P-glycoprotein expression, alterations in beta-tubulin, alterations in cytokine levels, and alterations in apoptotic signaling (Yusuf RZ, et al. 2003). Although it is understood that the cytotoxic action of PTX results in activation of the apoptotic signaling cascade, recent studies suggest that PTX therapy specifically results in accumulation of endogenous ceramide, a lipid with function as a cellular second messenger in apoptosis (Charles A G, et al. 2001).

III. Spingolipids in Apoptotic Signaling

Ceramide (CER), a naturally occurring sphingolipid, is derived intracellularly by hydrolysis of the lipid sphingomyelin, or by de-novo synthesis through N-acylation of sphinganine (Senchenkov A, et al. 2001) (Figure 2). Accumulation of endogenous CER, produced by either hydrolysis or de novo formation, is known to result in response to several stimuli, such as growth factor deprivation, pro-inflammatory signals, exposure to
Figure 2 – Ceramide synthesis and metabolism pathways. [adapted from Perry DK, and Hannun YA, (1998)]
increased temperature and radiation, and other stressors such as chemotherapeutics and related cytotoxic agents (Senchenkov A, et al. 2001; Kolesnick R 2002). Among such stimuli, PTX has been shown to elevate intracellular CER levels in breast cancer cells (Charles A G, et al. 2001). Intracellular CER is implicated in the cellular responses to stress, such as apoptosis and cell cycle arrest (Charles A G, et al. 2001; Selzner M, et al. 2001; Sietsma H, et al. 2001; Tilly JL, et al. 2002), where CER functions as a second messenger in the signaling cascade that initiates these responses. In fact, studies have shown that administration of exogenous CER analogs, particularly C2- and C6-ceramide, encourages cell death by apoptosis and inhibition of tumor growth in several tumor models (Selzner M, et al. 2001; Sietsma H, et al. 2001; von Haefen C, et al. 2002; Radin N S 2003; Reynolds C P, et al. 2004). In the cell, CER can subsequently be further metabolized by the enzyme glucosylceramide synthase (GCS) to yield glucosylceramide (gluCER) (Figure 2), a glycosylated form of CER that does not have pro-apoptotic activity (Lavie Y, et al. 1996; Lucci A, et al. 1998; Liu Y-Y, et al. 2001; Morjani H, et al. 2001; Morjani H, et al. 2001; Itoh M, et al. 2003). Several MDR tumor cell lines have exhibited elevated levels of non-cytotoxic gluCER and corresponding elevated levels of GCS (Lavie Y, et al. 1997; Lucci A, et al. 1998; Liu Y-Y, et al. 2001; Morjani H, et al. 2001; Itoh M, et al. 2003), and clinical studies have noted elevation of gluCER levels in tumor specimens of breast cancer and melanomas that were poorly responsive to chemotherapy (Lucci A, et al. 1998). These findings not only suggest the importance of CER in the mediation of the cytotoxic response to anti-tumor chemotherapeutics, but they also suggest that inhibition of apoptotic signaling may be an important mechanism whereby tumors develop MDR.
Apoptotic signaling is classically divided into external and intrinsic pathways (Gulbins E, et al. 2000), wherein the external pathway involves activation of death receptors, FasR and TNF$\alpha$R, leading to activation of initiator caspases 8/10 (Gulbins E, et al. 2000; Degterev A, et al. 2003), while the intrinsic pathway leads to depolarization of the mitochondrial membrane potential leading to cytochrome c release and activation of caspase 9 (Gulbins E, et al. 2000; Degterev A, et al. 2003). Ultimately, while both pathways lead to activation of effector caspases, primarily caspase 3, 6, and 7, the pathways can operate in a harmony whereby commonality and communication between apoptotic mediators can lead to cross-activation (Gulbins E, et al. 2000; Degterev A, et al. 2003).

Various implications of CER in apoptotic signaling have placed it both in the mitochondrial pathway, by suppressing Bcl-2 at the mitochondria leading to cytochrome c release and formation of the caspase 9 apoptosome, and in the death receptor pathway of apoptosis whereby regulation of CER was stimulated by activation of the Fas-receptor and the TNF$\alpha$ – receptor (Obeid LM, et al. 1993; Pushkareva M, et al. 1995; Kolesnick RN, et al. 1998). Recent reports have even implicated the role of CER in the formation of channels through the mitochondrial membrane, aiding in mitochondrial membrane depolarization (Siskind LJ 2005), although these reports have not yet fully been verified. Definitive evidence of the importance of CER in apoptotic signaling came from acid-sphingomyelinase knockout mouse models that were deficient in apoptotic signaling (Santana P, et al. 1996). The complexity of CER in apoptotic signaling is further promoted by recent reports, which implicate CER in non-caspase dependent apoptosis, an apoptotic pathway that resembles necrosis, and is characterized by the formation of
reactive oxygen species (Maurer BJ, et al. 1999; Kim WH, et al. 2005; Granot T, et al. 2006). While the importance of CER in the apoptotic response is well established, recent reports have linked glucosylceramide synthase (GCS), the enzyme responsible for bioactivation of CER to an apoptotically defunct moiety, gluCER, to P-glycoprotein expression in MDR cancers (Lavie Y, et al. 1997; Shabbits JA, et al. 2002; Gouaze V, et al. 2005), suggesting a potential co-regulation of these two separate cellular mechanisms in the MDR occurrence.

IV. Enhancing Drug Delivery to Tumors – Role of Nanotechnology

While the development of MDR poses a great threat to survival of cancer patients, drug delivery to solid tumors in and of itself is a significant challenge that also determines survival outcome. A major barrier to successful anti-cancer therapy is the challenge of delivering the required therapeutic concentration to the tumor site while minimizing undesirable side effects resulting from systemic administration. Site-specific drug delivery systems increase the therapeutic benefit by delivering a greater fraction of the dose at the target site, which minimizes the amount of therapeutic that accumulates at non-specific targets. Drug delivery throughout the tumor mass is crucial for the treatment to be effective, since residual cancer cell survival can promote re-growth and often becomes the cause for drug resistance (Bast RC, et al. 2000). Systemic administration of chemotherapeutics requires the drug to pass through 3 main stages (Jang SH, et al. 2003). First the drug must be transported within the blood vessels under minimal excretion, metabolism, and uptake by non-specific sites, in order to reach the tumor site. Secondly, the drug must transport across the blood vessel wall into the surrounding tissues (Jang SH, et al. 2003). And lastly, the drug must transport through the interstitial space within the
tumor mass and be taken up by the cells. Success of all of these processes is determined by the physicochemical properties of the drug, such as size, diffusivity, and protein binding affinity; drugs with a high molecular weight (size), poor diffusivity across biological membranes, and high affinity for protein binding, will have difficulty reaching the tumor site and entering the cells within the tumor mass. However, even if a drug complies with perfect physiochemical properties, its success through these three properties is not guaranteed. As it turns out, tumors possess additional physiologic hurdles that limit drugs reaching their target following systemic administration. First, tumors possess a very dense, tortuous, and heterogeneous vasculature, which affects targeting and extravasation of the drug within various regions of the tumor mass differently (Jang SH, et al. 2003). Only once the tumor has surpassed a cutoff size of 2 mm, will it promote angiogenesis and form its own blood supply (Jang SH, et al. 2003). The result is that the tumor mass can be divided into 4 distinct regions of vasculature (Jang SH, et al. 2003). The center of the mass is an avascular necrotic region. This is surrounded by a semi-necrotic region with unbranched capillaries. Moving outward, the next region is characterized by microvasculature that is mostly venular, and so has very few arterioles. And at the periphery the tumor mass has regular blood flow, consisting of both arteries and veins. One can envision the difficulty for a small molecule drug then to find its way beyond the periphery of the tumor mass, since the vasculature becomes sparser moving in towards the center. However, in addition to the vascular hurdle, there is an increase in interstitial pressure from the periphery to the core of the tumor that makes it difficult for a small molecule drug to traverse throughout the tumor mass (Jang SH, et al. 2003). The result of this pressure buildup is that the net convective flow of the
tumor mass is outward, thereby pushing small molecule drugs out of the mass (Jang SH, et al. 2003). The third main hurdle to tumor drug delivery is the presence of a dense extracellular matrix between cancer cells in the tumor mass (Jang SH, et al. 2003). This extracellular matrix is mainly composed of fibrous proteins, such as collagen and elastin, and polysaccharides, such as hyaluronan and proteoglycan, inherently present to stabilize spatial relationships between cells, to regulate intercellular macromolecule transport, and to prevent bacterial invasion, but, unfortunately, in this manner it also acts as a physical barrier preventing movement of small molecule drugs through the tumor mass (Jang SH, et al. 2003). Together these hurdles greatly hinder chemotherapeutic drugs from entering and/or traversing throughout the tumor mass, thereby resulting in an ineffective treatment.

For colloidal carriers, such as liposomes and polymeric nanoparticles, however, these barriers can be overcome due to the properties of tumor neo-vasculature. Angiogenesis (the formation of new vasculature) associated with rapid growth of the tumor mass results in fenestrations in tumor capillaries (Maeda H, et al. 2000). These fenestrations are resultant of gaps between adjacent endothelial cells, and average a size around 200-400nm (Maeda H, et al. 2000). In addition, the lymphatic system, which usually runs alongside the blood circulation to clear macromolecules from tissues, is impaired in solid tumors. This lack of lymphatic drainage in the tumor mass then results in decreased clearance of macromolecules (>40 kDa) (Maeda H, et al. 2000). Together, these two physiologic parameters of the tumor mass, termed the enhanced permeability and retention (EPR) effect (Maeda H, et al. 2000), can be taken advantage of to improve drug delivery to tumors mediated by nanocarriers. In this mechanism, as illustrated in Figure 3, nanoparticulate carriers can deliver the drug specifically to the tumor site, since
Figure 3 – Passive tumor targeting by nanoparticles and the EPR effect.
their size allows for extravasation at the tumor mass mainly through the vascular fenestrations, and retention in the tumor mass due to the lack of lymphatic drainage. Hereby a system exists to not only increase tumor-specific drug targeting which reduces hazardous side-effects, but also to overcome the physical barriers to tumor drug delivery.

V. Polymeric Nanoparticles in Drug Delivery

Biodegradable polymers such as poly(epsilon-caprolactone) (PCL) (Figure 4c) and poly(D,L-lactic-co-glycolic acid) (PLGA) (Figure 4d), are useful materials to formulate drug delivery carriers for tumor targeted delivery. Biocompatibility and degradation methods of these polymers have been widely studied (Pitt CG, et al. 1981; Woodward S C, et al. 1985; Jain RA 2000; Serrano MC, et al. 2004), and found to be non-toxic, leading to the US Food and Drug Administration approval and acceptance for medical applications. Additionally, these polymers offer an advantage for drug delivery, whereby they efficiently encapsulate hydrophobic compounds, and slow degradation of the particle allows for extended release of the drug (Uhrich K E, et al. 1999). Surface modification of the nanoparticles with a poly(ethylene oxide)-poly(propylene oxide) triblock copolymer (PEO-PPO-PEO, Pluronic®) improves the stability of the nanoparticle in the aqueous environment of the body, while decreasing immune activation, repelling plasma proteins and decreasing reticulo-endothelial uptake leading to an increase in circulation time and passive tumor targeting by the EPR effect (Gref R, et al. 1997). Previous studies from our group have shown that PTX-containing PEO-PCL nanoparticles remain stable in-vivo, and retain their Pluronic® surface layer to increase the circulating half-life and plasma residence time of PTX from a fraction of an hour to 25.3 hours and 24.0 hours, respectively, alongside a nearly 8-fold decrease in total body
Figure 4 – Chemical structures of the therapeutics a) ceramide (CER), and b) paclitaxel (PTX) along with the polymers c) poly(epsilon caprolactone) (PCL), d) poly(lactic co-glycolic acid) (PLGA) and e) poly(beta-amino ester) (PbAE).
clearance of the drug (Shenoy D, et al. 2005b; Shenoy D B, et al. 2005). The concentration of PTX inside the tumor mass of mice-bearing human ovarian carcinoma (SKOV3) xenografts, as a result, was 8.7-fold higher 5 hours post intravenous administration as compared to mice treated with paclitaxel in aqueous solution (Shenoy D, et al. 2005b).

On the other spectrum, pH-responsive polymers offer an advantage in tumor targeting, in that they allow for triggered release specifically within the acidic environment (~pH 6.5) of the tumor mass. Dr. Robert Langer’s group at MIT has synthesized a unique library of polymers, classified as poly(beta amino esters) (PbAE) (Figure 4e), by reacting diol-diacrylates with primary and secondary amines (Lynn DM, et al. 2001). One of PbAE members, selected for these studies, has unique pH-solubility properties; the polymer remains intact at pH 7.4, but rapidly dissolves at pH 6.5. Interestingly, this polymer efficiently encapsulates hydrophobic compounds. Our group has previously developed PEO-PbAE nanoparticles to deliver PTX as a novel stimuli-responsive drug delivery system to an in-vivo ovarian model of cancer, and showed that the particles released their drug load not only in a pH-specific manner upon internalization into the tumor cells, but that the resultant therapeutic efficacy increased at least two-fold over that of a non-pH responsive PEO-PCL nanoparticles (Shenoy D, et al. 2005a; Devalapally H, et al. 2006).

VI. Current Nanoparticle Strategies to Overcome MDR in Cancer

Few groups have investigated the use of nanoparticles in the treatment of MDR, and those that have focused on facilitating the delivery of chemotherapeutic drugs past the P-
glycoprotein pump, thereby evading drug efflux, leading to enhanced chemosensitivity. Couvreur’s group published the first reports utilizing poly(alkylcyanoacrylate) nanoparticles to chemosensitize MDR hepatocellular carcinoma and MDR leukaemia models to the cytotoxic action of doxorubicin (de Verdiere AC, et al. 1997; Barraud L, et al. 2005). Lamprecht and Benoit used lipid nanocarriers to overcome etoposide-resistance \textit{in-vitro} in MDR glioma, but interestingly attributed the chemosensitization seen in their system to inhibition of P-glycoprotein by the poly(ethylene glycol)-based surfactant coating on particles, rather than a bypass of the transporter (Lamprecht A, et al. 2006). Koziara \textit{et al.} delivered paclitaxel in wax nanoparticles to chemosensitize MDR in colorectal cancer \textit{in-vivo}, by evading P-glycoprotein mediated drug efflux (Koziara JM, et al. 2006). Lastly, Wong \textit{et al.} have used polymer-lipid nanoparticles to enhance doxorubicin uptake and retention in MDR breast cancer cells by evading P-glycoprotein efflux (Wong HL, et al. 2006). To date, however, the use of nanoparticles has not been investigated as a therapeutic approach to overcome alternate, or simultaneously multiple mechanisms of MDR, supporting the novelty of the therapeutic approach designed in this work.

\textbf{VII. Multifunctional Nanoparticles in Tumor Drug Delivery}

Polymer chemistry allows for many variations, whereby polymeric nanoparticles can be easily manipulated without loss of their desired physical, chemical, and biological properties. In one manner, this principle can be utilized to greatly improve the function of the nanoparticle in cancer therapy through attachment of tumor-specific targeting moieties, such as antibodies or receptor ligands directed at cell surface markers unique to the cancer cell. Alternately, this principle can be utilized to improve the function of the
nanoparticle for simultaneous delivery of a combination of drugs to the cancer cell, creating a multivalent therapeutic strategy. Such manipulations of the nanoparticle formulation allow for multi-functionalization directed at enhancing cancer therapy, as illustrated in Figure 5.

The versatility of nanocarrier platforms opens up possibilities to incorporate combination therapies into a single drug delivery system. Combination drug therapy for cancer was first proposed in a legendary move by Drs. Frei, Freireich, and Holland who revolutionized cancer therapy by implementing combination chemotherapy to treat acute lymphoblastic leukemia (ALL), a malignancy that prior to 1950 was largely incurable (Frei, et al. 1958). In this case, it was hypothesized that concurrent use of multiple drugs with differing mechanisms of action would circumvent the development of drug resistance, the likely cause for prior therapeutic failure in ALL (Frei, et al. 1958). The success of this strategy caused the approach to quickly gain widespread acknowledgement to become a common consideration in current cancer therapy. Furthermore, the idea has been extended beyond combination chemotherapy to combine drugs with entirely distinct pharmacological targets, e.g., combinations of chemotherapeutic agents with angiogenesis inhibitors, protease inhibitors, immunotherapeutics, hormone therapeutics, and modulators of multidrug resistance, therapies largely stemming from advances in cancer molecular and cell biology leading to alternate therapeutic targets.

One prime example where such multifunctional nanoparticles benefit combination drug therapy is given by Sengupta et al. (2005). Research had alluded to the fact that simultaneous administration of angiogenesis inhibitors and chemotherapeutics may
Figure 5 – Illustration of multifunctional polymeric nanoparticles that can combine combination drug therapies and/or tumor targeting moieties.
actually cause detrimental effects, where a breakdown of vascularity not only prevents the chemotherapy from accumulating throughout the tumor site, but that it can also lead to tumor hypoxia which may promote drug resistance and metastasis (Tran, et al. 2002). Given this dilemma, it was thought that this form of combination therapy may actually benefit from temporal controlled release, a feat that can well be mediated by using nanoparticles as drug delivery vehicles. On this premise, Sengupta, et al. (2005) developed a novel multi-functional nanoparticle formulation that, upon localization in the tumor mass, first releases the antiangiogenic drug combretastatin-A4 to shut down tumor vasculature, followed by the sustained release of the cytotoxic agent doxorubicin, already localized within the tumor mass, thereby avoiding the aforementioned problems associated with chemotherapeutic delivery after vascular shutdown (Sengupta, et al. 2005). By this mechanism, survival and tumor growth delay of mice bearing either Lewis Lung carcinoma or B16/F10 melanoma models drastically improved compared with simultaneous nanoparticle administration of the combination therapy lacking temporally controlled release.

Alternately, another treatment target in cancer that could greatly benefit from a therapeutic approach that utilizes multifunctional nanoparticles to carry drug combinations is in the treatment of tumors that present with multidrug resistance. Combination chemotherapy has been extensively used in the clinic to treat cancers that develop resistance, and it is of interest to note that the original use of combination chemotherapy derived by Frei, Freireich and Holland was intended to circumvent the establishment of drug resistance in ALL. However, treatment with multiple cytotoxic chemotherapeutic agents lacks in benefit, since these potently toxic drugs can provoke
detrimental adverse effects in patients, not to mention the fact that the occurrence of multidrug resistance rules out hope for much of combination chemotherapy (Harris AL, et al. 1992). Examples whereby such a strategy has been successfully employed have been detailed previously (Section VI above).

Nanoparticles are being developed as effective delivery vehicles due to their passive tumor targeting properties, which lead to the ability to enhance efficacy and reduce side effects of chemotherapeutic drugs. In addition, this unique capacity of nanoparticles to preferentially accumulate in and around the tumor mass also grants a platform for improved tumor diagnostics, hereby laying the foundation for the development of multi-functional nanoparticle systems in cancer therapy. Through inclusion of metals such as iron oxide, gold, gadolinium, and the various semiconductors that make up the body of quantum dots, nanoparticles can be formulated that combine tumor targeting, tumor imaging, and tumor therapy, both by chemical and physical means, all in one system.

A future outlook for this field promises even further improvement in combining therapy and imaging. Although iron oxide nanoparticles and gold nanoshells have been shown to combine imaging with hyperthermia treatment, or imaging with drug delivery, a cohesive formulation remains to be developed that can effectively image, deliver drugs and localize hyperthermia treatment to the tumor, whilst retaining the long circulating and tumor localization properties of the nanoparticle. Similarly, gadolinium nanoparticles are proving to be highly useful in combining cancer imaging with cancer therapy by NCT, and although these nanoparticles bear a potential to deliver a targeted therapeutic load efficiently, successful in-vivo proof of tumor imaging and tumor
treatment, both by NCT and drug delivery, rests unproven to this day (Oyewumi, et al. 2002). No different are the quantum dots which, although proven able to carry tumor targeting ligands and small molecule drugs without losing the tumor imaging strength (Lemarchand, et al. 2003), combining this multi-functional quantum dot with its newly found use in photodynamic therapy is as of yet unchartered territory (Bakalova, et al. 2004). An area that also remains unexplored is the synthesis of polymeric materials with intrinsic imaging (e.g., using fluorescent monomers) and drug delivery properties (e.g., polymer drugs).

As the development of cancer-directed polymeric nanoparticles evolves to include ever more functions, concerns arise about bioavailability, biocompatibility and the loss of other properties such as imaging strength and drug release. However, thus far nanoparticle formulations have already evolved from single function, through dual function, to multifunction all the while retaining the desired properties that make nanoparticles so useful in cancer therapy. Therefore the potential exists to successfully improve current nanoparticle formulations, gaining one step closer to the ultimate cancer therapy system.
CHAPTER 2: EVALUATION OF THE THERAPEUTIC EFFICACY OF COMBINATION PACLITAXEL AND CERAMIDE TO OVERCOME MDR IN IN-VITRO MODELS OF HUMAN BREAST AND OVARIAN CANCERS

I. INTRODUCTION

The purpose of this study was to overcome the cellular mechanisms of MDR through a therapeutic strategy that would alleviate the obstruction to apoptotic signaling thereby re-sensitizing the MDR cancer cells to chemotherapy. This novel therapeutic strategy co-administers the pro-apoptotic chemotherapeutic drug PTX with the apoptosis modulator CER, encapsulated within PEO-PCL nanoparticles. In this manner, it is hypothesized that administration of exogenous CER overwhelms the GCS metabolism enzyme, thereby rendering un-metabolized CER available in the cell to carry through the apoptotic signal resulting from PTX exposure. Wildtype (drug-sensitive) and MDR-1 positive human ovarian cancer cells (SKOV3) and human breast cancer cells (MCF7) were used to test therapeutic efficacy of the nanoparticle combination therapy given the relevance of MDR in patients with breast and ovarian cancer (Goldstein LJ, et al. 1989).

II. MATERIALS AND METHODS

1. Preparation and characterization of PEO-PCL nanoparticles

Poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles were prepared by controlled solvent displacement method using an acetone-water system. Poly(ε-caprolactone), with a number-average molecular weight of 14.8 kDa, (Polysciences, Warrington, PA) with addition of 20% w/w poly(ethylene oxide)-poly(propylene oxide) triblock copolymer (Pluronic® F- 108 NF grade) (BASF, Mount
Olive, NJ) was dissolved in 2 mL acetone (Fisher Scientific, Fair Lawn, NJ) and briefly heated at 55°C to facilitate dissolution. The nanoparticles were loaded individually with either paclitaxel (PTX) (ICN, Aurora, OH), N-hexanoyl-D-erythro-sphingosine (C6-ceramide) (CER) (Avanti Polar Lipids, Alabaster, AL), tamoxifen (TMX) (ICN, Aurora, OH) or verapamil HCl (VPM) (Sigma-Aldrich, St. Louis, MO) by dissolving these into the polymer-acetone mix at 10% w/w for PTX, or 20% w/w for CER, TMX and VPM. The nanoparticles were formed by gently adding the polymer-drug solution into 10V filtered water under rapid magnetic stirring, maintained until the organic solvent evaporated off. The nanoparticles were collected by centrifugation at >10,000 rpm and washed with filtered water before lyophilization. Post and prior to lyophilization, nanoparticle samples were collected and analyzed for size and surface charge on a Brookhaven ZetaPlus® particle analyzer (Brookhaven Instruments, Holtsville, NY). In addition, lyophilized nanoparticle samples were mounted on an aluminum sample mount, sputter coated with gold-palladium, and visualized by scanning electron microscopy (SEM) on a Hitachi S-4800 instrument, at 13,000x magnification under an accelerating voltage of 3kV.

Drug release was simulated by resuspending the lyophilized nanoparticles into PBS with 0.1% Tween®-80 at either pH 7.4, to simulate physiological conditions, and at pH 6.5, to simulate the tumor environment, and incubating the particles at 37°C for up to 5 days. At daily intervals, samples of release medium were collected, and the exact volume of release buffer taken was replaced to maintain sink conditions. PTX release was measured by reverse-phase high performance liquid chromatography assay on a C18 column with 50:50 acetonitrile:20 mM sodium dodecyl sulfate (SDS)-sodium phosphate
buffer as the release medium. CER release was measured by incorporating 1% w/w NBD-CER into the nanoparticles and monitoring NBD-CER fluorescence on a plate reader at 485/530 nm excitation/emission.

2. Cell culture and treatment

Wild-type (drug sensitive) SKOV3 and SKOV3<sub>TR</sub> (MDR-1 positive) human ovarian carcinoma cells (kindly provided by Dr. Michael Seiden, Massachusetts General Hospital, Boston, MA) were maintained in RPMI-1640 (Mediatech Inc., Herndon, VA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Mediatech Inc, Herndon, VA) and 1% penicillin/streptomycin (Cambrex, Walkersville, MD), incubated in a humidified air chamber at 37°C with 5% CO<sub>2</sub>. The SKOV3<sub>TR</sub> subculture was selected and maintained for MDR by the addition of 0.2 µM paclitaxel in the culture medium. AML-12 murine hepatocytes were obtained from ATCC (Manassas, VA) and maintained in DMEM (Mediatech Inc., Herndon, VA) similarly supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. For experiments, cells were harvested by brief incubation with 4 mL Trypsin-EDTA (Mediatech Inc., Herndon, VA) at room-temperature, collected with 8 mL supplemented medium, and centrifuged at 2000 rpm for 5 minutes. The cell pellet was then re-suspended in 5 mL of supplemented medium upon which viable cells were visualized by Trypan blue exclusion and counted using a conventional haemacytometer. Cells were subsequently plated at 5,000 cells per well in 96-well plates (Corning Inc., Corning, NY). Following overnight incubation, allowing the cells to adhere, cells were subjected to treatment with various doses of the investigational compounds as free (un-encapsulated) drugs or encapsulated in PEO-PCL nanoparticles, diluted in supplemented medium. Free drugs were prepared as stock
solutions, where PTX stock was obtained from the 6 mg/mL solution Onxol® (IVAX Pharmaceuticals, Miami, FL), CER and TMX stocks were prepared at 20 mM and 10 mM respectively in dimethyl sulfoxide (DMSO) (ICN, Aurora, OH), and VPM stock was prepared at 20 mM in filtered water. All stocks were subsequently diluted to working concentrations directly in serum supplemented medium. Stock solutions for PTX, CER, TAM, and VPM encapsulated in PEO-PCL nanoparticles were prepared at the same concentrations as their corresponding free drug counterparts by re-suspending the nanoparticles in filtered water and further diluting them directly in serum supplemented RPMI-1640. The pan-capase inhibitor ZVAD.fMK was obtained from Axxora (San Diego, CA). In all studies, treatment with vehicles was included for control purposes. Treatment with serum supplemented medium was used as a negative control (0% cell death) and treatment with 50 μg/mL poly(ethyleneimine) (mol. wt. 10 kDa) was used as a positive control (100% cell death). Treatment proceeded undisturbed for 6 days, after which cell viability was measured by the MTS assay. MTS reagent was prepared by dissolving 38 mg of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI) and 2 mg phenazine ethosulfate (PES) (ICN, Aurora, OH) in 20 mL Dulbecco’s PBS (pH 6.0). After the 6-day treatment period, cell medium was aspirated and replaced with 1:10 diluted MTS reagent in supplemented medium. Cells were incubated for at least 4 hours at 37°C, after which plates were read at 490 nm (Biotek Synergy HT plate-reader, software: KC4), where the quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the remaining number of living cells.
3. **Protein extraction and visualization**

To visualize basal P-glycoprotein and GCS expression from SKOV3 and SKOV3\textsubscript{TR} cells, and to visualize caspase-3 activity from drug treated SKOV3\textsubscript{TR} cells, protein was extracted and subjected to western blotting. A crude protein extract was prepared by lysing the cells in freshly prepared lysis buffer (0.01M Tris pH 7.4, 2 \( \mu \)M EDTA pH 8.0, 0.15M NaCl, 0.876% Brij\textsuperscript{®} 97, 0.125% Tween\textsuperscript{®} 20) for 5 min. at 4\( ^{\circ} \)C, followed by centrifugation at 13,000 rpm for 20 min at 4\( ^{\circ} \)C. The supernatant was collected and protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Samples were stored at –80\( ^{\circ} \)C until further use.

For SDS-PAGE, 25 \( \mu \)g and 100 \( \mu \)g extracted protein was loaded onto a pre-cast 4-15\% SDS-PAGE gradient gel (BioRad, Hercules, CA) for visualization of P-glycoprotein/\( \beta \)-actin and GCS/caspase 3/\( \beta \)-actin respectively, with protein samples diluted 1:1 in Laemmeli buffer (BioRad, Hercules, CA), and separated in Tris/Glycine/SDS running buffer (Invitrogen, Carlsbad, CA) at 150 V for 1 hour. Following this, the protein was transferred to nitrocellulose membrane (BioRad, Hercules, CA) in Tris/Glycine/SDS transfer buffer (Invitrogen, Carlsbad, CA) supplemented with 10\% (v/v) methanol, at 100 V for 1 hour at 4\( ^{\circ} \)C. The protein-immobilized membranes were subsequently blocked in 5\% NFD milk in TBS/0.05\% Tween\textsuperscript{®}-20 (for anti-P-glycoprotein and anti-GCS) or 5\% bovine serum albumin in PBS/0.05\% Tween\textsuperscript{®}-20 (for anti-caspase-3 and anti-\( \beta \)-actin) for 2 hours prior to an overnight incubation at 4\( ^{\circ} \)C with either the anti-P-glycoprotein monoclonal antibody C219 (Signet Labs, Dedham, MA), diluted 1:500 in blocking buffer, the anti-glucosylceramide synthase polyclonal antibody
Fluorescence-labeled nanoparticle trafficking

To visualize the path of PTX and C6-CER loaded nanoparticle trafficking within the cell, rhodamine-conjugated PTX (Boston Scientific Corporation, Natick, MA) and NBD-conjugated C6-CER (Molecular Probes, Eugene, OR) were independently loaded into PEO-PCL nanoparticles. Nanoparticles were prepared as previously described with the modification that the dye-conjugated drugs were loaded at 0.1% (w/w). SKOV3 and SKOV3TR cells were allowed to adhere on flame-sterilized glass coverslips within 6-well plates seeded at $5 \times 10^5$ cells/well/2 mL supplemented RPMI. The cells were subsequently incubated with a 0.1 μM solution of rhodamine-PTX and NBD-C6-CER fluorescence-labeled nanoparticles for 6 hours. Cells were washed and fixed onto slides as previously
described. Images were obtained on an Olympus BX61W1 fluorescence microscope utilizing 546/590 and 485/530 filters for rhodamine and NBD signals, respectively. All treatments were duplicated.

5. Statistical analysis

For the cytotoxicity experiments, n=8 measurements per treatment were used. Statistical analysis was performed by two tailed, equal variance student t-test. Statistical significance was accepted at p<0.05.

III. RESULTS

Studies on the SKOV3 and MCF7 lines and their MDR subcultures SKOV3_{TR} and MCF7_{TR} revealed that the MDR cells abundantly express GCS as well as the classical MDR marker P-glycoprotein, in contrast to the drug sensitive SKOV3 and MCF7 cells (Figure 6). This pattern of protein expression verifies the MDR phenotype. Concomitantly, dose-response studies against PTX revealed chemoresistance of both the SKOV3_{TR} and MCF7_{TR} lines compared to their drug-sensitive parent cultures as seen by the right-shifted dose-response curves (Figure 7). The experimental IC_{50} for the SKOV3 cells was set at approximately 0.008 µM, while the IC_{50} for the MDR subculture (SKOV3_{TR}) was set over 100-fold higher at 1.08 µM. Similarly, for the MCF7 line the IC_{50} was set at 0.10 µM, while the MCF7_{TR} IC_{50} it was set 10-fold higher at 0.98 µM. Various reports have suggested the use of synthetic CER with a 6-carbon chain length, C6-CER, as the most potent agent. To verify the therapeutic use of C6-CER over other CER analogs, a variety of CER analogs differing in carbon chain lengths (C2, C6, C10, C14, C18, and C24) were tested side by side on the SKOV3_{TR} cell line at equal doses of
Figure 6 – Western blot analysis of basal P-glycoprotein (P-gp) and glucosylceramide synthase (GCS) in wild-type ovarian (SKOV3), MDR ovarian (SKOV3TR), wild-type breast (MCF7), and MDR breast (MCF7TR) cancer cells. Beta-actin serves as an internal control for protein count.
Figure 7 – Paclitaxel (PTX) dose-response studies in a) human ovarian cancer cells (SKOV3) and b) human breast cancer cells (MCF7). Closed circles depict drug-responsiveness of wild-type (drug sensitive) cells and open circles depict drug-responsiveness of MDR cells.
15 µM. Results revealed that, coinciding with other scientific reports, the C6-CER analog had the most cytotoxic potential (at 52.4 ± 3.3% cell death), and was thus deemed the most appropriate therapeutic (Figure 8). It is important to note that the CER analogs were delivered to the cells packaged within PEO-PCL nanoparticles at 20% (w/w) to standardize internalization of the analogs with differing carbon chain lengths into the cells. However, equivalent studies were performed in parallel whereby the CER analogs were delivered to the cells as free drug, revealing that even then the C6 analog had the most cytotoxic potential (results not shown). Dose-response studies were carried out on all four cell lines to determine a therapeutic dose for C6-CER. Since the intention of the CER co-therapy is to act synergistically with PTX, rather than additionally to PTX, a therapeutic dose of CER was sought that in itself does not provoke cell death, but is of sufficient potency to mediate apoptotic signaling. The results in Figure 9 illustrate that a 10 µM concentration of CER deems the most appropriate therapeutic concentration for this use since it resides on the barrier between being inert and cytotoxic for the majority of carcinoma cell lines (SKOV3TR, MCF7, and MCF7TR). However, for the SKOV3 cells line, the 10 µM dose of CER caused 40% cell death, thus for this cell line, it cannot be sure whether therapeutic efficacy results from a synergistic or additive effect of CER with PTX.

Since CER, as a mediator of apoptotic signaling, can act as a cytotoxic agent at high enough doses, it is important to ensure that the therapeutic concentration does not show potential to cause adverse effects upon non-specific target accumulation. Cytotoxic potential of CER is likely due to the highly proliferative nature of malignant cells, nevertheless, potential cytotoxicity to normal, non-proliferating cells must not be ruled
Figure 8 – Cytotoxicity of various ceramide (CER) analogs with differing carbon chain lengths at a dose of 15 µM administered to SKOV3TR cells within PEO-PCL nanoparticles. ** indicates a statistical significance (p<0.001) in percent cell death between C6-CER and other CER analogs (n=8 samples/group)
Figure 9 – Dose-response relationship of ceramide (CER) to both wild-type (MCF7) and MDR (MCF7_{TR}) human breast cancer cells as well as wild-type (SKOV3) and MDR (SKOV3_{TR}) human ovarian cancer cells (n=8 samples/treatment/cell type)
out. Since it is a well known fact that liver is a common site non-specific accumulation and adverse toxicity, a murine hepatocyte cell line (AML-12) was chosen as a model to examine the potency of CER to non-malignant, non-proliferating cells. The results in Figure 10 demonstrate that CER induces relatively less cell-kill (79.4 ± 2.5% viability) to the hepatocytes at the therapeutic dose administered (10 µM); however, this is only seen in the free drug treatment and can be largely attributed to toxicity of the DMSO vehicle (86.5 ± 8.9 % viability). CER delivered in PEO-PCL nanoparticles did not show any toxicity to the cells at this dose (96.8 ± 3.6% viability) similar to the cytotoxicity of the blank nanoparticles (108.0 ± 7.0% viability). Similar results where obtained at the 100 µM dose, where a slight cytotoxicity of the drug could potentially be attributed to cell-kill by the vehicle. Therefore, the data suggests that the 10 µM therapeutic concentration of CER should not adversely affect liver hepatocytes upon non-target accumulation. Due to poor solubility and vehicle toxicity, doses above 100 µM could not be administered to the cells to determine an IC₅₀ for CER to these cells.

To determine whether the proposed PTX + CER co-therapy indeed possessed the ability to overcome MDR, both the SKOV3 and SKOV3_TR ovarian carcinoma cells, and the MCF7 and MCF7_TR breast carcinoma cells were subjected to treatments with either PTX alone or the co-therapy without nanoparticle encapsulation. Since PTX is a cell-cycle specific chemotherapeutic drug, treatments were allowed to proceed undisturbed for 6 days to ensure that all cells initiated mitosis. However, to minimize uncontrolled growth of the cell population in control samples due to the extended treatment duration, all cells were seeded near confluency.
Figure 10 – Ceramide (CER) cytotoxicity to AML-12 hepatocytes. Open circles depict drug administration in PEO-PCL nanoparticles, while closed circles depict administration of free drug. Similarly, solid lines depict cytotoxicity of CER, while dashed lines depict cytotoxicity of vehicle controls (dimethylsulfoxide free drug, and blank nanoparticles for nanoparticle administration of drug) (n=8 samples/treatment).
Results of these *in-vitro* studies revealed that a PTX + CER combination therapy indeed has the potential to chemosensitize MDR cancer cells. Figure 11 illustrates that the combination of CER with PTX completely eradicates both the SKOV3<sub>TR</sub> and MCF7<sub>TR</sub> cells when treated with a dose of PTX around their IC<sub>50</sub> (1 and 10 µM, respectively). Hereby, in the SKOV3<sub>TR</sub> population, a dose of 1 µM PTX alone merely results in 34.4 ± 2.2% cell death, while the same dose of PTX combined with 10 µM CER kills 97.3 ± 0.5% of the cell population (Figure 11a). The same results are seen with the MCF7<sub>TR</sub> cells around their IC<sub>50</sub> dose. Figure 11b illustrates that while 10 µM PTX only kills 73.3 ± 3.5% of the population, 10 µM PTX + CER improves chemosensitivity resulting in 103.5 ± 0.64% cell death. Consequently, these results are either comparable (in SKOV3<sub>TR</sub>) or an improvement (in MCF7<sub>TR</sub>) upon the MDR modulation efficacy seen with the first generation MDR modulator verapimil (VPM), which acts as an inhibitor of the P-glycoprotein efflux pump. It was of interest to examine whether the PTX + CER combination therapy, in itself, had the power to completely revert the MDR phenotype and chemosensitize the MDR cells to a level that mimicked the PTX response of the wild-type SKOV3 and MCF7 cells. Since the IC<sub>50</sub> values for the chemosensitive SKOV3 and MCF7 cell lines were around 0.01 µM and 0.1 µM PTX respectively, their MDR subcultures were treated with these doses in combination with CER. Results show that while the combination-therapy significantly increased chemosensitivity in the MCF7<sub>TR</sub> cells at the lower PTX dose, from 0 ± 14.38% cell death with PTX alone to 54.5 ± 2.8% cell death from PTX + CER (Figure 11b), it did not have the same power to chemosensitize the SKOV3<sub>TR</sub> cells at their low dose of 0.01 µM PTX (Figure 11a). This variation in, and lack of, power is likely due to adjunct
Figure 11 – In-vitro efficacy of the paclitaxel (PTX) + ceramide (CER) combination therapy to modulate MDR in a) SKOV3_{TR} and b) MCF7_{TR} cells, administered as free drug. Efficacy of the ceramide combination therapy is compared with efficacy of a paclitaxel + verapamil (VPM) combination therapy, where VPM modulates MDR by inhibiting P-glycoprotein. ** indicates a statistically significant difference (p<0.001) between a combination therapy and PTX alone at a given dose (n=8 samples/group/cell type).
mechanisms of MDR within the cells that gave rise to the MDR phenotype, such as drug efflux by P-glycoprotein, which facilitates removal of PTX from the cell, a phenomenon that is particularly noticeable at low doses of drug.

An interesting observation occurred when the combination treatment was tested on the wild-type SKOV3 and MCF7 cells. Unlike prior generations of MDR modulation strategies, which aimed to chemosensitize only MDR cancers, the CER combination therapy appeared to effectively increase cell-kill efficacy of drug sensitive cells as well (Figure 12), thereby chemosensitizing them further. The results in Figure 12 demonstrate that the PTX + CER combination increased cell death of the SKOV3 population from 27.6 ± 4.5% to 65.4 ± 3.8%, at a 0.01 µM dose of PTX, and also increased cell death similarly in the MCF7 population from 26.2 ± 6.1% to 40.4 ± 2.8%, at a 0.1 µM dose of PTX. These results suggest that the PTX + CER combination therapy can increase therapeutic efficacy against non-MDR cancers as well.

Although the use of nanoparticle drug delivery systems for cancer therapeutics stems from the notion that therapeutic efficacy is greatly increased due to a preferential accumulation of the therapeutic dose at the tumor site, it was of interest to examine the therapeutic efficacy of the PTX + CER combination therapy encapsulated within polymeric nanoparticles in-vitro, given the recent reports that nanoparticle delivery has been suggested to chemosensitize MDR cancers simply by bypassing the P-glycoprotein efflux mechanism. For this purpose, PTX and CER were individually encapsulated into PEO-PCL nanoparticles at 10% (w/w) and 20% (w/w), respectively. The resultant particles were spherical and of uniform size (211.6 ± 1.8 nm), as seen by scanning
Figure 12 – In-vitro efficacy of the paclitaxel (PTX) + ceramide (CER) therapeutic strategy on wild-type (drug sensitive) SKOV3 (ovarian) and MCF7 (breast) cancer cells. ** indicates a statistically significant difference (p<0.001) between a combination therapy and PTX alone at a given dose (n=8 samples/group/cell type).
electron microscopy (Figure 13a), and drug loading efficiency into these nanoparticles was 100% for PTX at 10% (w/w), and 70% for CER at 20% (w/w). Release studies revealed a complete release of CER within 3 days, while only about half the PTX load was released by day 5 (Figure 13b), allowing adequate time for nanoparticle accumulation at the tumor site prior to release of the majority of the load. Release of both PTX and CER did not appear to be affected by pH, where similar release was seen at pH 7.4 (physiological pH) as well as at pH 6.5 (tumor environment). The particles bear a negative zeta-potential (-31.1 mV), due to the presence of a carboxyl group (Lemarchand, et al. 2003). Although a negative surface charge can challenge nanoparticle endocytosis into cells, visual trafficking of these particles by incorporation of rhodamine-labeled PTX or NBD-labeled CER nevertheless revealed that these particles are efficiently taken up into both the drug sensitive (SKOV3) and the MDR (SKOV3TR) cells (Figure 14), whereby the particles appear to migrate from the periphery of the cell after 1 hour inwards, where they fully distribute throughout the cytoplasm within 6 hours. The granular appearance of both the NBD and rhodamine fluorescence suggests that the particles reside in endocytic vesicles.

To compare efficacy of the PTX + CER combination therapy administered as free drugs vs. encapsulation within PEO-PCL nanoparticles, both the SKOV3TR and MCF7TR cell lines were subjected to treatment whereby PTX doses were varied to determine a dose-response relationship, while the CER dose maintained at 10 µM for 6 days. The results from the dose-response studies with PEO-PCL nanoparticles, as shown in Figure 15, were displayed alongside the dose-response behavior of free (unencapsulated) PTX + CER and free PTX alone. While figure 11 revealed that at lower doses of PTX, the PTX
Figure 13 – Characterization of PEO-PCL nanoparticles by a) scanning electron microscopy (SEM) and b) drug release of paclitaxel (PTX – solid line) and ceramide (CER – dashed line) at pH 7.4 (closed circles) and pH 6.5 (open circles).
Figure 14 – Intracellular internalization and trafficking of PEO-PCL nanoparticles loaded with green fluorescent NBD-ceramide (NBD-CER) and red fluorescent rhodamine-paclitaxel (rhodamine-PTX) from 1 hour through 6 hours after administration in wild-type SKOV3 and MDR SKOV3 TR cells (200x magnification).
+ CER combination therapy lacked the ability to overcome MDR particularly in the SKOV3<sub>TR</sub> cells, this hurdle is overcome by delivering the combination therapy encapsulated within nanoparticles. Whereas the PTX/CER free drug combination therapy on the SKOV3<sub>TR</sub> cells did not increase chemosensitivity over PTX alone at a PTX dose of 0.01 µM, nanoparticle delivery of the combination therapy at this same PTX dose now significantly improved chemosensitivity, resulting in a drop in cell survival from complete survival of the population down to 64.0 ± 4.9% (Figure 15a). Similar results were also seen in the MCF7<sub>TR</sub> cells, where the PTX + CER nanoparticle therapy, at a 0.1 µM PTX dose, further chemosensitized the cells resulting in merely 20.2 ± 9.4% survival (Figure 15b). An interesting point to note is that at higher PTX doses (1 and 10 µM) in the SKOV3<sub>TR</sub> cells (Figure 15a), the cells suddenly exhibit a less cell death than the PTX + CER (free drug) treatment at these doses. This phenomenon can be potentially explained in the fact that nanoparticle uptake into cells is thought to be a saturable process, whereby the cells have reached their saturation limit at these higher doses of PTX + CER so that only a fraction of the dose encapsulated in nanoparticles is able to enter the cells for therapeutic action, a concept that is further supported in subsequent studies. Since diffusion of free drug into cells on the other hand is not saturable, this phenomenon is not observed in the free PTX + CER treatment.

While the results thus far have shown that a co-therapy of exogenous CER alongside PTX successfully modulates MDR, it was of interest to see if the same phenomenon occurs when GCS is blocked therein preventing endogenous CER from undergoing metabolism to glucosylceramide. The drug tamoxifen (TAM) has been reported to inhibit GCS, therefore it was speculated that a combination therapy of PTX
Figure 15 – Paclitaxel (PTX) dose-response on a) SKOV3\textsubscript{TR} (MDR ovarian cancer) cells and b) MCF7\textsubscript{TR} (MDR breast cancer) cells treated with PTX alone as free drug, PTX + ceramide (CER) as free drug, and PTX + CER in PEO-PCL nanoparticles (NP). CER dose was held constant at 10 \( \mu \text{M} \) alongside varying doses of PTX. ** indicates a statistical significance (\( p<0.001 \)) between PTX and PTX + CER. ## indicates a statistical significance (\( p<0.001 \)) between PTX + CER and PTX + CER NP. (n=8 samples/treatment/cell type)
with TAM would produce a similar chemosensitization profile as the PTX + CER therapy (Selzner M, et al. 2001; Sietsma H, et al. 2001; von Haefen C, et al. 2002; Radin NS 2003; Reynolds CP, et al. 2004). Figure 16b shows that this combination of PTX + TAM indeed also chemosensitized the SKOV3\textsubscript{TR} cell type to a similar degree as the PTX + CER co-treatment. And like the PTX + CER treatment, the PTX + TAM treatment was similarly enhanced by nanoparticle delivery. For example, while the PTX + TAM co-therapy administered as free drug at a 0.001 \( \mu \)M PTX dose did not produce any cell kill, the co-therapy delivered in nanoparticles at this dose resulted in slight cell kill (87.2 ± 3.8\% viability). Interestingly, like the PTX + CER nanoparticle therapy, the PTX + TAM nanoparticle therapy also exhibited saturation of cell internalization at the higher doses of PTX.

Unlike prior generations of MDR modulation strategies, therapeutically aimed at mechanisms particular to the MDR phenotype, modulation of the apoptotic signal could also enhance chemosensitization of drug sensitive cells, as verified in Figure 12. Similarly, figure 16c illustrates how the PTX + CER nanoparticle therapy greatly improves chemosensitization of the SKOV3 cells, as seen by a left-shift of the dose-response curve. Although the SKOV3 cells benefit from the addition of exogenous CER to induce cytotoxicity, it was not expected that they would respond to the PTX + TAM co-therapy, since the drug-sensitive cells do not suffer from an over-expression of GCS. And indeed the results verify that the PTX + TAM nanoparticle therapy did not enhance chemosensitivity in the SKOV3 cells. These results indicate not only the importance of GCS-mediated CER metabolism and apoptotic modulation as an important contributor to the MDR phenotype, but moreover, they reveal the success of an apoptosis modulation
Figure 16 – Paclitaxel (PTX) dose response of SKOV3 and SKOV3_TR cells with or without a co-therapy as free drug or encapsulated within PEO-PCL nanoparticles (NP). a) comparison of the PTX dose response on SKOV3 and SKOV3_TR and the effect of the PTX + (ceramide) CER therapy as free drug and in nanoparticles, b) comparison of the PTX dose response in SKOV3_TR cells to the PTX + CER therapy and the PTX + (tamoxifen) TAM therapy, and c) comparison of the PTX dose response in SKOV3 cells and the effect of the PTX + CER and the PTX + TAM therapies; ** indicates a statistically significant difference (p<0.001) between treatment with PTX alone and PTX + CER within the same cell type, ^^ indicates a statistically significant difference (p<0.001) between treatment with PTX alone and PTX + TAM within the same cell type, and ## indicates a statistically significant difference (p<0.001) between treatment with a co-therapy in solution and in nanoparticles (n= 8 samples/group).
strategy to not only revert MDR in cancer, but also chemosensitize non-MDR cancer types.

IV. DISCUSSION

The development of the MDR phenotype is a major hurdle for successful treatment of cancer, whereby patients with MDR tumor types are often left with few options but exceptionally high doses or combinations of chemotherapeutics (Bast RC, et al. 2000). PTX, a chemotherapeutic commonly used in the treatment of breast and ovarian tumors, is known to exert its anti-tumor effect by promoting programmed cell death (apoptosis) in response to its action as a mitotic spindle poison. Of interest was the recent observation that PTX cytotoxicity may provoke an intracellular accumulation of endogenous ceramide, a lipid that is known to function as a second messenger in the apoptotic signaling cascade (Charles AG, et al. 2001). Combining these facts with the latest observations that MDR cells have increased levels of GCS (the enzyme responsible for bioactivation of ceramide) and glucosylceramide (the apoptotically defunct metabolite of CER) led to the hypothesis that MDR may be reversed by co-administration of exogenous CER to reinstate the apoptotic signaling cascade and re-sensitize the cancer cells to PTX chemotherapy. To enhance overall efficacy of the therapy, the drugs were encapsulated and delivered to the cells within PEO-PCL nanoparticles.

The results of this study indicate that the combination of a PTX + CER therapy can indeed greatly improve chemo-sensitivity of both MDR breast and ovarian cancer cells, demonstrated when simply co-administering CER with PTX near the PTX IC$_{50}$ for
either cell type, nearly eradicated each MDR cell population. Furthermore, delivery of the combination therapy encapsulated within PEO-PCL nanoparticles greatly increased chemosensitivity to both MDR cancer lines as demonstrated by the drastic decrease in cell survival at doses 10-100 fold lower than the PTX IC$_{50}$. At these doses, the combination therapy in free drug form had little effect on MDR. It is hypothesized that this enhanced effect of nanoparticle delivery on MDR modulation results from modulation of a second cellular mechanism of MDR, namely P-glycoprotein mediated drug efflux, based on the findings that nanoparticle delivery helps to avoid P-glycoprotein mediated drug efflux (de Verdriere AC, et al. 1997; Koziara JM, et al. 2006; Lamprecht A, et al. 2006; Wong HL, et al. 2006). The mechanisms whereby this novel therapy exerts its effect on MDR cancer will be examined in the subsequent chapter.

The importance of MDR modulation by pharmacologically targeting GCS metabolism of CER, rather than exogenously administering CER, was further proven when co-administration of TAM alongside PTX produced similar chemosensitization effects as the CER combination therapy achieved. However, MDR modulation strategies that directly target cellular mechanisms of MDR, such as P-glycoprotein inhibition by VPM and GCS inhibition by TAM, did little to enhance chemosensitization of non-MDR (drug sensitive) cancer. However, apoptotic modulation by CER feedback proved not only effective to re-sensitize MDR cells to chemotherapy, but also showed promise in enhancing chemosensitivity of non-MDR cells. This effect would allow for the lowering of therapeutic doses of PTX to achieve similar effects in the clinic.

Thus, the results of this study portray a beneficial therapeutic strategy for reversing MDR in cancer, by a novel approach that modulates the apoptotic threshold,
which is elevated in MDR. Furthermore, unlike many prior therapeutic strategies to overcome MDR, this novel PTX/CER nanoparticle therapy also shows great potential for use in the treatment of non-MDR cancer types, where therapeutic efficacy of PTX is also enhanced.
CHAPTER 3: VERIFICATION OF THE HYPOTHESESIZED CELLULAR MECHANISM WHEREBY PACLITAXEL AND CERAMIDE CO-THERAPY OVERCOMES TUMOR MDR

I. INTRODUCTION

In the previous chapter, it was demonstrated that a combination therapy of a pro-apoptotic chemotherapeutic drug paclitaxel (PTX) and an apoptotic modulator ceramide (CER) effectively reversed chemo-resistance in multidrug resistant (MDR) breast and ovarian cancer lines. However, the efficacy of the combination therapy on both breast and ovarian MDR cancer types was greatly enhanced when the combination therapy was administered within polymeric PEO-PCL nanoparticles. Thus, it is of interest to verify the cellular mechanisms whereby (1) the combination therapy itself overcomes MDR and (2) delivery of the combination therapy in nanoparticles enhances MDR modulation of the cellular level.

Given the prior knowledge MDR can be modulated individually either by restoring pro-apoptotic ceramide levels within the cell (Kolesnick R 2002), or by bypassing drug efflux pumps of the ABC-transporter family (most notably P-glycoprotein) (Robert J, et al. 2003), it is hypothesized that the success of the PTX+CER combination therapy to overcome MDR is due to modulation of two cellular mechanisms of MDR: a restoration of apoptotic signaling due to the CER feedback to overwhelm the GCS metabolic machinery, and a bypass of P-glycoprotein drug efflux through protection and endocytosis of the drugs within nanoparticles, both of which principles were examined on the ovarian cancer cell line.
II. MATERIALS AND METHODS

1. Preparation and characterization of PEO-PCL nanoparticles

Drug loaded poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles were prepared by the methods described in chapter 2, with the addition of $^3$H-PTX (Moravek Biochemicals, Brea CA) and $^{14}$C-CER (American Radiolabeled Chemicals, St. Louis MO) at 1.5 µCi/mg unlabeled drug for intracellular drug accumulation studies, and 1% (w/w) rhodamine-123 (Invitrogen, Carlsbad, CA) for intracellular drug visualization.

2. Measurement of intracellular drug uptake

Cells were cultured and seeded according to the methods described in chapter 2. For experiments SKOV3 and SKOV3$_{TR}$ cells were seeded at 100,000 cells/well in 6-well plates, allowed to adhere overnight, and treated with nanoparticle or free drug treatments labeled with $^3$H-PTX and $^{14}$C-CER. Cells were treated with 1 µM PTX + 10 µM CER, 0.5 µM PTX + 5 µM CER, or 0.1 µM PTX + 1 µM CER for 6 hours, or with a constant dose of 1 µM PTX + 10 µM CER for varying time points up to 24 hours at 37°C in a humidified cell culture incubator. Following the treatment period, cells were washed twice with PBS, and lysed with 1 mL of lysis buffer after which the lysate was collected in scintillation vials. Each sample received 10 mL Scintisafe scintillation fluid (Fisher Scientific, Hampton, NH) per 1 mL lysis buffer, and was left to quench for 2 hours in the dark. Following this, counts per minute of the $^3$H and $^{14}$C signals were collected on a $\alpha/\beta$ scintillation counter. In parallel, to determine the total amount of protein in 100,000 cells
for each cell type, a crude protein extract was prepared by lysing the cells in freshly prepared lysis buffer for 5 min. at 4°C, followed by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was collected and protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The results are expressed as % of dose accumulated intracellularly per mg of total protein.

To visualize intracellular drug retention, SKOV3 and SKOV3TR cells were allowed to adhere on flame-sterilized glass coverslips within 6-well plates seeded at 5x10^5 cells/well/2mL supplemented RPMI. Cells were subsequently incubated with 10 μg rhodamine-123 administered as free compound or within nanoparticles, for 4 hours. The treatment was terminated by aspirating the medium, washing the cells thrice with PBS and mounting the coverslips, cell-side down, onto glass slides with 50 μL Fluoromount-G fixing medium (SBA, Birmingham, AL). Images were obtained on an Olympus BX61W1 fluorescence microscope using a 546/590 excitation/emission filter maintaining consistency of exposure time (400 ms) and other microscope settings. All treatments were run in triplicate.

3. **Analysis of apoptotic activity**

To measure the amount of cell death by apoptosis as a result of co-administration of C6-CER with PTX compared to PTX alone, apoptosis was measured using a commercial apoptosis assay that combined Yo-Pro-1®, propidium iodide (PI), and Hoechst-33342 stains to differentiate apoptotic and necrotic cells from viable cells by flow cytometry (Vybrant apoptosis assay kit #7, Molecular Probes, Eugene, OR). Green fluorescent Yo-Pro-1® stains apoptotic cells selectively, while dual stained cells with Yo-
Pro-1<sup>®</sup> and red fluorescent (PI) are selected as necrotic or dead, based on the potential of the two membrane impermeable dyes to enter the cell. Viable cells remain unstained by this method. Blue fluorescent Hoeschst-33342 is used as an internal control for cell count by staining the nucleus of all cells. SKOV3 and SKOV3<sub>TR</sub> cells were collected and counted as previously described, and subsequently plated in 96-well optical quality plates (Nalge-Nunc Int., Rochester NY) at a density of 2x10<sup>4</sup> cells/well/0.2 mL supplemented RPMI-1640, followed by incubation at 37°C with 5% CO<sub>2</sub>, allowing the cells to adhere overnight. The following day, both the drug sensitive and the drug resistant cell populations were subjected to the following treatments: control (RPMI-1640), 10 nM PTX (low dose), 1 µM PTX (high dose), 10 µM C6-ceramide, 10 nM PTX with 10 µM C6-CER, or 1 µM PTX with 10 µM C6-CER, in triplicate. All treatments were diluted in supplemented RPMI-1640. Treatment was allowed to proceed undisturbed for 12 and 24 hours, followed by in-situ cytometric analysis of live cells using the iCys<sup>®</sup> microplate cytometry platform (Compucyte Corp. Cambridge, MA) that combines laser scanning cytometry (LSC) with fluorescent microscopy. Culture media was removed, and each sample well was replaced with 200 µL sterile PBS pH 7.4 containing 0.02 µL of each dye (Yo-Pro-1, PI, and Hoechst-33342) to stain for apoptotic activity. The samples were left to incubate at 37°C in a humidified air chamber (5% CO<sub>2</sub>) for 20-30 minutes, immediately followed by cytometric analysis. The iCys<sup>®</sup> platform allows for simultaneous excitation and absorption of the three dyes for quantitative cell sorting and fluorescent microscopy in one scan. Yo-Pro<sup>®</sup> and PI were excited at 488 nm by an argon laser and absorbed at 515-545 nm and 600-635 nm respectively, while Hoechst was excited at 405 nm by a diode laser and absorbed at 445-485 nm. Each sample scan was
repeated 4 times, all treatments were run in triplicate, and the entire set up and analysis was repeated once more at a later date.

4. Protein extraction and visualization

To visualize basal caspase-3 activity in SKOV3 TR cells following drug treatment, protein was extracted and subjected to western blotting according to the methods described in chapter 2. The protein-immobilized membranes were blocked in 5% BSA in PBS/0.05% Tween-20 for 2 hours prior to an overnight incubation at 4°C with anti-caspase-3 monoclonal antibody (Cell Signaling Tech., Danvers, MA) diluted 1:2000 in blocking buffer, followed by incubation with an HRP-conjugated goat anti-rabbit secondary antibody (Cell Signaling Tech., Danvers, MA) at 1:5,000 dilution in 5% milk-TBST for 1 hour at room temperature.

5. Statistical analysis

For the cytotoxicity experiments, n=8 measurements per treatment, while for the apoptosis assay, n=6 measurements per treatment were used. Statistical analysis was performed by two tailed, equal variance student t-test. Statistical significance was accepted at p<0.05.

III. RESULTS

Figure 15 has previously shown that while the PTX + CER combination therapy in itself significantly enhanced chemosensitization on both MDR ovarian cancer cells (SKOV3 TR) and MDR breast cancer cells (MCF7 TR), delivery of the combination therapy within PEO-PCL nanoparticles significantly enhanced chemosensitization in both cell
lines at lower doses of PTX. It has been shown in our group that drug delivery mediated by these nanoparticles results in an enhanced intracellular drug accumulation profile, thus through tracing radio-labeled derivatives of both PTX and CER as free drug vs. nanoparticles it could be determined whether nanoparticle delivery of the combination drug load achieved greater chemosensitization of MDR cancer by simply enhancing intracellular drug accumulation, or if it indeed was further attributable to an avoidance of P-glycoprotein mediated drug efflux. Figure 17 illustrates that both intracellular levels of $^3$H-PTX and $^{14}$C-CER were increased in a time-dependent manner when the therapeutic was delivered to the cells within PEO-PCL nanoparticles. While at timepoints below 8 hours delivery of the therapeutics as free drugs resulted in an increase in intracellular drug accumulation over nanoparticle delivery of the dose (1 µM PTX + 10 µM CER), later timepoints this phenomenon was reversed to cause a significantly greater accumulation of both drugs intracellularly resulting from nanoparticle delivery in both drug sensitive and MDR SKOV3 cancer cells. However, this result would suggest that the enhancement of chemosensitivity by nanoparticle delivery of the drugs is merely due to an across-the-board enhancement of intracellular drug delivery, rather than a specific bypass of P-glycoprotein efflux. Figure 15, however, revealed that nanoparticle-enhanced chemosensitization of MDR cells occurred at much lower doses of PTX, thus it is important to observe intracellular drug accumulation profiles in a dose-dependent manner as well. Figure 18 illustrates intracellular drug accumulation in a dose-dependent manner at the 4 hour time point, when accumulation of free drug of the higher dose prevailed over nanoparticle delivery as demonstrated in Figure 17. As illustrated in Figure 18, at the lower doses of therapeutics (0.1 µM PTX and 1 µM CER) the
Figure 17 – Intracellular accumulation of a) radio-labeled paclitaxel (³H-PTX) and b) radio-labeled ceramide (¹⁴C-CER) of a 1 µM PTX + 10 µM CER dose over time in SKOV3 and SKOV3TR cells following administration as free drug (fd) or encapsulated within PEO-PCL nanoparticles (NP). * and ** indicate a statistical significance (p<0.05 and p<0.001 respectively) between NP and fd administration at a given timepoint in the same cell line (n=3-6 samples/treatment/cell type).
Figure 18 - Intracellular accumulation of a) radio-labeled paclitaxel (\(^3\)H-PTX) and b) radio-labeled ceramide (\(^{14}\)C-CER) of varying doses of PTX + CER after 4 hours in SKOV3 and SKOV3\(_{TR}\) cells following administration as free drug) or encapsulated within PEO-PCL nanoparticles (NP). * indicates a statistical significance (p<0.05) and between NP and fd administration at a given dose in the same cell line (n=3-6 samples/treatment/cell type).
difference in intracellular uptake or retention was most significant. While there was no difference in intracellular uptake of PTX to the SKOV3 cells, lacking P-glycoprotein between free drug and nanoparticle delivery (3.43 ± 1.35 % of dose for free drug and 3.71 ± 0.08 % of dose for nanoparticles), free drug administration to the SKOV3TR cells resulted in a significantly lower amount of intracellular PTX accumulation (1.70 ± 0.06% of dose). However, as expected, intracellular PTX levels increased dramatically in the SKOV3TR cells when the drugs were delivered in nanoparticles (4.34 ± 1.39% of dose), suggesting again that the enhanced chemosensitivity seen with nanoparticle delivery is indeed due to the evasion of P-glycoprotein efflux. Interestingly, CER also seemed to exhibit pronounced intracellular levels when administered in nanoparticles at a low dose compared to free drug administration (6.16 ± 2.43% and 2.61 ± 0.11% of dose respectively), however this behavior is seen at a dose of CER 10-fold lower than therapeutically administered and is therefore not relevant. It is not known whether CER is also a substrate for P-glycoprotein efflux, but the interesting observation of such an increase in intracellular accumulation specifically in the P-glycoprotein positive SKOV3TR cells renders the option possible. As doses of both PTX and CER are increased, the phenomenon of increased drug accumulation/retention in the MDR cells levels off and interestingly reverts by the higher doses of therapeutics. For example, with the 0.5 μM PTX and 5 μM CER dose, the difference between free drug and nanoparticle delivery is no longer detectible, while by the 1 μM PTX and 10 μM CER dose, intracellular levels from nanoparticle delivery are in fact significantly lower than those from free drug administration. This observation is in concert with the efficacy data reported earlier, and appears to verify the explanation that since nanoparticle delivery
undergoes endocytosis, nanoparticle accumulation in the cells is a saturable process. Therefore the observation that nanoparticle delivery merely enhances chemosensitization of the MDR cells at lower doses of PTX is fitting, due to saturation of the nanoparticle uptake (endocytosis) machinery.

Although the intracellular drug accumulation studies supported the idea that nanoparticle drug delivery may help the drug-load evade P-glycoprotein efflux, in this manner overcoming an additional mechanism of MDR, further proof was needed to support the idea. Since the fluorophore rhodamine-123 (R-123) is a known substrate for the P-glycoprotein transporter (Wang Y, et al. 2006), visualization of intracellular retention of this fluorophore following delivery as free compound or encapsulated within PEO-PCL nanoparticles could lead to a final understanding of the hypothesized evasion of drug efflux seen with nanoparticle delivery. The results in Figure 19 show that while R-123 is efficiently retained in the SKOV3 cells, lacking P-glycoprotein, 4 hours after administration of the compound either unencapsulated or in nanoparticles, the fluorophore is no longer detectable at this time-point within the SKOV3TR cells when it is administered as free compound. Strikingly though, the R-123 that was encapsulated within the nanoparticles, was retained to a greater extent in the SKOV3TR cells, suggesting again that intracellular nanoparticle delivery may indeed help avoid P-glycoprotein efflux of the drugs.

In addition to bypassing P-glycoprotein efflux, it was important to confirm the hypothesis that feedback of exogenous CER to MDR cells restores the blocked apoptotic signal resulting from endogenous CER metabolism to a defunct apoptotic mediator, gluCER. To verify that this experimental therapy indeed overcomes MDR by mending
Figure 19 – Intracellular retention of the red fluorescent compound rhodamine-123 (fluorescein) 4 hours after administration to SKOV3 and SKOV3_{TR} cells as free drug or encapsulated within PEO-PCL nanoparticles (NP). Images were taken at 400x magnification and an exposure time of 400 ms. DIC = differential interference contrast.
alterations in the apoptotic signaling cascade, apoptotic activity was measured 24 hours following treatment initiation by microplate cytometry and confirmed by simultaneous fluorescent microscopy, maintaining the same conditions set forth in the cytotoxicity studies. The SKOV3\textsubscript{TR} cells were used as a model to investigate this query. Figure 20a indicates that SKOV3\textsubscript{TR} cells exposed to the PTX/CER co-treatment, at a dose around the SKOV3\textsubscript{TR} IC\textsubscript{50} for PTX (1 \(\mu\)M PTX + 10 \(\mu\)M CER), displayed an increased amount of apoptotic staining, where the green fluorescent dye YO-PRO-1\textsuperscript{®} stains apoptotic cells and the red fluorescent propidium iodide stains necrotic cells (and dead cells), based on their ability to permeate the cell membrane. Given the recent observations that CER is involved in apoptotic cell death mechanisms that produce a necrosis-like morphology (Maurer BJ, et al. 1999; Kim WH, et al. 2005; Thon L, et al. 2005), apoptotic activity was identified in cells that stained both green fluorescent from YO-PRO-1 and red-fluorescent from propidium iodide, but with cytometry gating set to quantify only whole cells (thereby excluding dead cells and cell fragments). The quantitative results revealed that apoptotic activity in the MDR cells in response to the PTX + CER treatment is indeed doubled over PTX treatment alone (29.0 \(\pm\) 3.1 \% apoptosis with PTX/CER vs. 16.0 \(\pm\) 2\% with PTX alone) (Figure 20b). Analysis of the activity of the downstream effector caspase, caspase-3, by Western blotting after a 24 hour treatment period with 1 \(\mu\)M PTX and 10 \(\mu\)M CER as well as a 100-fold lower dose 0.01 \(\mu\)M + 10 \(\mu\)M CER revealed that PTX/CER co-treatment caused the greatest amount of cleavage of full-length caspase-3 (37 kDa) to its 19 and 17 kDa fragments (Figure 21), verifying that the PTX/CER co-treatment indeed restores apoptotic signaling in the MDR cells. To determine whether this restored apoptotic activity is via caspase-dependent mechanisms, or via the recently
Figure 20 – Apoptotic activity in SKOV3_TR cells 24 hours after treatment with 1 µM paclitaxel (PTX) + 10 µM ceramide (CER), 1 µM PTX, 10 µM CER, or control (no treatment).  
a) Microscopy images of SKOV3_TR cells stained for apoptotic activity with green fluorescent YO-PRO-1™ and red fluorescent propidium iodide, and counterstained with blue fluorescent Hoechst-33342 for cell count.  
b) Quantitation of % apoptotic cells by microplate cytometry for SKOV3_TR cells stained with YO-PRO-1™ and propidium iodide.  * indicates statistical significance (p<0.05) between PTX + CER and PTX, CER, or control treatments (n=6 samples/group).
Figure 21 – Western blot analysis of full-length procaspase-3 and caspase-3 cleavage products, indicating apoptotic activity, in SKOV3 TR cells treated with paclitaxel (PTX) + ceramide (CER) vs. treatment with PTX, CER, or control (no treatment)
hypothesized caspase-independent mechanisms that CER appears to be involved with, the MDR cells were again subjected to the PTX/CER treatment, this time with or without the pan-caspase inhibitor ZVAD.fMK. Cell survival analysis after the 6 day treatment revealed that ZVAD.fMK indeed inhibited a fraction of cell death resultant from the PTX/CER co-therapy, where inhibition was greater when the chemotherapeutic stress was less (76.6 ± 8.9% cell survival with ZVAD.fMK vs. 37.4 ± 9.7% cell survival without ZVAD.fMK at a dose of 0.01 µM PTX + 10 µM CER) (Figure 22). However, caspase blockade inhibited cell death merely partially, rather than fully, suggesting an involvement of both caspase-dependent and caspase-independent mechanisms of apoptosis in the restoration of apoptotic signal by this novel therapy.

Indeed, the results have demonstrated that, while the PTX/CER combination therapy in itself showed great potential to overcome MDR by chemosensitizing both MDR SKOV3TR and MCF7TR cell lines, via the proposed mechanism of modulating the defects in apoptotic signaling, a nanoparticle-based therapy, delivering the combination therapy, revealed a greater potential at the cellular level to overcome MDR even further by modulating a second MDR mechanism as well, mainly P-glycoprotein-mediated drug efflux.

IV. DISCUSSION

Prior MDR modulation strategies have largely aimed at inhibiting P-glycoprotein drug efflux, since the overexpression of P-glycoprotein, and related proteins, is most commonly the cause for the MDR phenotype (Szakacs G, et al. 2006). However, clinical failure of these therapeutics, albeit through poor target responsiveness, high systemic
Figure 22 - Percent cell survival with and without co-administration of the pan-caspase inhibitor ZVAD.fMK at doses of 0.01 µM paclitaxel (PTX) + 10 µM ceramide (CER) and 1 µM PTX + 10 µM CER. ** indicates a statistically significant difference (p<0.001) between ZVAD supplemented treatments and non-supplemented treatments at the same dose (n=8 samples/group).
toxicity, or pharmacokinetic interactions, has fueled the search for novel therapeutic strategies that can successfully overcome MDR by targeting alternate cellular mechanisms that give rise to the MDR phenotype (Robert J, et al. 2003). The modulation of apoptotic signaling has emerged as an important mechanism for MDR development (Pommier Y, et al. 2004), whereby CER synthesis and metabolism in particular has been shown to play an crucial role to elevate the apoptotic threshold (Kolesnick RN, et al. 1998; Lucci A, et al. 1998).

Recently, though, reports have emerged that CER metabolism by GCS may be intricately linked to P-glycoprotein expression (Shabbits JA, et al. 2002; Wang H, et al. 2002; Gouaze V, et al. 2004; Gouaze V, et al. 2005; Turzanski J, et al. 2005). In these findings it has not only been reported that GCS overexpression in MDR cells is coupled to P-glycoprotein expression, but that activity of both proteins may be co-regulated, as seen by a drastic decrease in function of P-glycoprotein when GCS is blocked and vice versa (Shabbits JA, et al. 2002; Wang H, et al. 2002; Gouaze V, et al. 2005). This phenomenon can be seen as a threat to successful MDR cancer therapy, since two cellular mechanisms must be modulated to truly overcome the MDR phenotype and revert chemoresistance. However, a future understanding of the mechanisms whereby these proteins are co-regulated may allow for the development of a therapeutic that simultaneously modulates both.

Nevertheless, given these findings, a multifunctional therapeutic strategy that simultaneously overcomes multiple mechanisms of MDR would be beneficial. Although the PTX + CER therapeutic strategy presented here was simply designed to modulate apoptotic signaling in MDR cancer cells, results from the study presented in chapter 2
revealed that delivery of this combination therapy in polymeric nanoparticles significantly enhanced efficacy against MDR cancer. Based the findings by de Verdiere et al. (1997), Lamprecht et al. (2006), Koziara, et al. (2006), and Wong et al. it was speculated that nanoparticle delivery of the combination therapy may help the drugs bypass P-glycoprotein mediated efflux, thereby enhancing the efficacy over the combination therapy alone.

Two-way analysis of apoptotic signaling, both by a dye inclusion assay that detects membrane permeability characteristic of apoptosis and by western blot analysis of downstream effector caspase-3 cleavage, supports the hypothesis that exogenous CER administration restores the defects in apoptotic signaling of these MDR cancer cells. While treatment with PTX or CER alone did not increase the percentage of cells undergoing apoptotic signaling in the MDR cancer cells, the combination therapy of these two drugs doubled the amount of apoptotic signaling that resulted at the same dose and timepoint. However, at most, only 30% of the population displayed apoptotic activity 24 hours following the PTX + CER combination therapy, while it was found that the same dose of drugs resulted in 100% cell death to these SKOV3_{TR} cells after 6 days. This may be explained by the fact that PTX exerts toxicity in a cell-cycle specific manner, and that by 24 hours not all the cells in the population had undergone mitosis to sense PTX poisoning. Synchronization the cell cycles in the SKOV3_{TR} population with agents such as mimosine or aphidicolin my help push apoptotic activity in the population to 100% in a similar study.

In addition to the verification of enhanced apoptotic activity by the combination therapy, the role nanoparticle delivery in bypass P-glycoprotein efflux was examined by
quantitative and qualitative studies that monitor intracellular drug accumulation and/or retention. Quantitation of intracellular $^3$H-PTX and $^{14}$C-CER accumulation at varying timepoints and varying dosages revealed that nanoparticle delivery indeed enhanced the percent of dose that internalized into the cells. However, this phenomenon occurred in both MDR SKOV3<sub>TR</sub> as well as drug-sensitive SKOV3 cells at timepoints beyond 8 hours. Since SKOV3 cells lack P-glycoprotein, an enhancement of intracellular accumulation even in these cells could suggest that nanoparticles simply enhance intracellular drug delivery independently of P-glycoprotein efflux. However, studies at lower doses of drug (0.1 µM PTX and 1 µM CER) revealed that free drug accumulation in the SKOV3<sub>TR</sub> cells was not only 2-fold lower than in the SKOV3 cells, but that nanoparticle delivery of the drugs to SKOV3<sub>TR</sub> cells increased the amount of intracellular drug accumulation to the same levels seen in the drug-sensitive SKOV3 cells. These findings support the fact that enhanced efficacy seen with nanoparticle delivery was most notable at low doses of PTX. Moreover, nanoparticle delivery at these lower doses did not enhance drug accumulation in the SKOV3 cells over accumulation of free drug, suggesting that this phenomenon is particular to the MDR cells and therefore potentially linked to P-glycoprotein drug efflux. However, given the recent knowledge that CER modulation can also inhibit P-glycoprotein (Gouaze V, et al. 2005), it was unsure whether the effects seen with nanoparticle drug delivery were due to a bypass of the P-glycoprotein machinery, or whether CER administration simply blocked P-glycoprotein. Additional studies that administered the fluorophore rhodamine-123 in lieu of PTX and CER to monitor retention of this compound in the presence of P-glycoprotein verified that nanoparticles indeed aide to bypass P-glycoprotein mediated efflux. Thus it can be
concluded that an avoidance of P-glycoprotein mediated drug efflux by nanoparticle delivery is more likely the cause for enhanced MDR modulation, rather than a two-prong effect of CER to both enhance apoptosis and inhibit P-glycoprotein.
I. INTRODUCTION

A combination therapy of the pro-apoptotic chemotherapeutic drug, paclitaxel (PTX) with an apoptosis modulating drug, ceramide (CER), encapsulated within PEO-surface modified PCL nanoparticles has been shown to overcome multidrug resistance (MDR) in in-vitro models of breast and ovarian cancer (van Vlerken, et al. 2007). This therapy exerts its effect by modulating two cellular mechanisms of MDR, as revealed in the previous two chapters. However, in this application, PTX and CER were loaded separately into nanoparticles rather than as a combination therapy. Dosing a combination therapy separately in this way does not ensure that the optimal effective dosing ratio accumulates at the target site at the same time upon each administration, thereby potentially reducing therapeutic efficacy of the combination therapy. In order to overcome this obstacle and encapsulate both PTX and CER into one nanoparticle, but maintain control over drug loading and release rates for each of the two drugs, a polymer blend nanoparticle was developed based on the principles originated by Little et al. of blending pH-responsive PbAE with slow degrading, non-pH responsive PCL or PLGA (Little S, et al. 2004)

II. MATERIALS AND METHODS

1. Nanoparticle manufacturing and characterization
Polymer-blend nanoparticles were manufactured by blending PCL (Polysciences, Warrington, PA) or PLGA (50:50) (Birmingham Polymers, Pelham, AL) with PbAE (kindly provided by Drs. Robert Langer, Mass. Institute of Technology, Boston, MA, and Steven Little, University of Pittsburgh, Pittsburgh, PA) at ratios of 10%:90%, 20%:80%, 30%:70%, 40%:60%, 50%:50%, 60%:40%, 70%:30%, 80%:20%, and 90%:10% respectively. PCL or PLGA was dissolved in acetone together with 20% (w/w) Pluronic F-108 and CER, while PbAE was dissolved in ethanol together with PTX. Both preparations were heated at 37°C to facilitate dissolution, after which they were joined and instantaneously added to 10 volumes water at pH 8.0 under rapid magnetic stirring at a rate of 2 mL/min. Polymer-drug complexes were dissolved in their respective organic phases at 10mg of total nanoparticle mass/ 0.5 mL organic solvent, using equal amounts of acetone and ethanol. Following overnight stirring to allow for evaporation of the organic solvents, nanoparticles were collected by centrifugation at 10,000 rpm for 40 min at 4°C, washed with dH water at pH 8.0, and either stored wet at 4°C or lyophilized for storage. Batches of unwashed, washed, and lyophilized nanoparticles were measured for size by dynamic light scattering and surface charge by zeta-potential analysis on a Brookhaven ZetaPlus particle analyzer (Brookhaven Instruments, Holtsville, NY). In addition, nanoparticle samples were airdried on glass coverslips and mounted on an aluminum sample mount, sputter coated with gold-palladium, and visualized by scanning electron microscopy (SEM) on a Hitachi S-4800 instrument, under an accelerating voltage of 3kV. In parallel, unstained wet nanoparticles were imaged on a JEOL JEM-1000 general purpose transmission electron microscope at under an accelerating voltage of 65 kV.
Lyophilized polymer-blend and unblended nanoparticles with and without Pluronic F-108 surface modification were analyzed for surface chemistry by X-ray Photoelectron Spectroscopy (XPS/ESCA) by Dr. Lara Gamble through the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO) at the University of Washington (Seattle, WA). Spectra were obtained on a Surface Science Instruments X-probe spectrometer using a spot size of 800 µm, a 150 eV pass energy for composition spectra and a 50 eV pass energy for high resolution C1 scans, and a take off angle of 55°.

2. **In-vitro drug release simulation**

*In-vitro* drug release of 70% PCL:30% PbAE, 70% PLGA:30% PbAE, 80% PCL:20% PbAE, and 80% PLGA:20% PbAE nanoparticles loaded at 2.5% (w/w) PTX and 10% (w/w) CER was simulated by re-suspending 10 mg of lyophilized nanoparticles into 5 mL PBS with 0.1% Tween-80® at pH 7.4, to simulate physiological conditions and incubating the particles at 37°C. At 0.5, 1, 2, 4, and 6 hours, samples of release medium were collected, and the exact volume of release buffer taken was replaced to maintain sink conditions. At the 6 hour time point, the pH of the release medium was dropped to 6.5 to simulate the tumor environment by the addition of a pre-determined amount of 1N HCl. Following this, drug release was maintained in a similar release buffer containing 0.1% Tween-80 but at pH 6.5 for the remainder of the study. At 6.5, 7, 8, 12, and 24 hours, samples of release medium were again collected, and the exact volume of release buffer taken was replaced to maintain sink conditions. PTX release was measured by reverse-phase high performance liquid chromatography (HPLC) using a C₁₈ column with 50:50 acetonitrile:20mM SDS-sodium phosphate buffer as the mobile phase. CER release was measured by incorporating 1% w/w NBD-CER into the nanoparticles and
monitoring NBD-CER fluorescene on a plate reader at 485/530 nm excitation/emission. Drug release simulation for each nanoparticle type was run in triplicate.

3. **Fluorescence - labeled nanoparticle trafficking**

To visualize the path of 70% PLGA: 30% PbAE polymer-blend nanoparticle internalization and trafficking within the cell, NBD-conjugated C6-CER (Molecular Probes, Eugene, OR) was loaded into nanoparticles. PTX + CER loaded nanoparticles were prepared as previously described with the modification that the NBD-conjugated CER was added into the PLGA fraction at 0.1% (w/w). SKOV3, SKOV3TR, MCF7, and MCF7TR cells were cultured as described in chapter 2, and allowed to adhere on flame-sterilized glass coverslips within 6-well plates seeded at 5x10⁵ cells/well/2 mL supplemented RPMI. The cells were subsequently incubated with a 1 µM PTX + 8.6 µM CER dose of NBD-labeled nanoparticles for 1, 2, and 4 hours. After treatment completed, cells were washed thrice with PBS and fixed onto slides with Fluoromount G. Images were obtained on an Olympus BX61W1 fluorescence microscope utilizing a 485/530 filters for the NBD signal.

4. **Polymer-blend film manufacturing and analysis**

To visualize the interaction of PCL or PLGA with PbAE as well as drug compartmentalization within the polymer-blends, polymers were dissolved and blended similar to nanoparticle preparation, however, rather than precipitating the polymer-drug complexes in water, the blends were cast into Petri-dishes containing sterilized glass coverslips and left overnight for the organic solvent to evaporate. This process caused the polymers to settle into films on the surface of the Petri-dish and the coverslips, where
present. To visualize drug incorporation, PTX and CER were incorporated into the polymer preparations at 2.5% (w/w) for PTX and 10% (w/w) CER with the inclusion of rhodamine-labeled PTX and NBD-labeled CER were incorporated into the polymer-blends at 0.1%/mg of drug. Coverslips containing the polymer films were visualized at 200x magnification under brightfield and epifluorescent microscopy using 546/590 and 485/530 filters for rhodamine and NBD signals respectively. Polymer films were maintained in PBS pH 7.4 to simulate physiological conditions, and in PBS pH 6.5 to simulate behavior in the tumor environment.

5. Statistical Analysis

For the cytotoxicity experiments, n=8 measurements per treatment, and for the drug release studies n=3 repeats per nanoparticle type were used. Statistical analysis was performed by two tailed, equal variance student t-test. Statistical significance was accepted at p<0.05.

III. RESULTS

Following an extensive evaluation of the efficacy of the nanoparticle PTX + CER combination therapy to treat MDR cancers, it was observed that an important and interesting relationship existed within the kinetics of dosing PTX and CER. The results in Figure 23 demonstrate that cell kill efficacy increased significantly when CER was administered with a delay of several hours following PTX administration, for example, in MCF7TR cells increasing from 39.1% cell death when the drugs were co-administered to 52.9% cell death when CER was administered 6 hours following CER (p<0.05) (Figure 23b). Interestingly, this increase was not observed when PTX was administered with a 6
Figure 23 – Cell-kill efficacy of kinetic dosing between paclitaxel (PTX) and ceramide (CER) in a) SKOV3<sub>TR</sub> and b) MCF7<sub>TR</sub> cells when PTX is administered at time (t)=0 hours with CER administration at t=6 hours, or when CER is administered at t=0 hours with PTX administration at t=6 hours (n=8 samples/treatment/cell type).
hour delay following CER (38.8% cell death) in this cell line. Similar results were seen in the SKOV3TR line, where dosing CER 6 hours following PTX increased cell kill efficacy significantly (6.0% cell death vs. 8.7% cell death when the drugs were co-administered), while the opposite relation did nothing to improve efficacy. To incorporate this dose-kinetic relationship into the formulation, a nanoparticle system was designed that could simultaneously carry both the PTX and CER therapeutics, but release each in a controlled manner within the tumor cells. In order to develop these multifunctional nanoparticles, the pH-responsive polymer PbAE was blended together with a hydrophobic polymer (either PLGA or PCL), with the intention that the polymers would be immiscible, causing PbAE to form pH-responsive pockets within the PCL or PLGA matrix (Figure 24), an idea adapted from previous work done by Langer’s group at MIT (Little S, et al. 2004). Figure 25 illustrates how the nanoparticles are manufactured by controlled solvent displacement. PCL or PLGA was dissolved in acetone together with 20% (w/w) Pluronic F-108 and 10% (w/w) CER, while PbAE was dissolved in ethanol together with 2.5% (w/w) PTX (Figure 25a). Both preparations were heated at 37°C to facilitate dissolution, after which they were joined and instantaneously added to 10 volumes water at pH 8.0 at a rate of 2 mL/min under rapid magnetic stirring to produce nanoparticles by hydrophobic forces between the polymers and drugs in the aqueous environment (Figure 25b). Overnight stirring allowed for the organic solvents to evaporate, causing the particles to condense and harden (Figure 25c), resulting in the final nanoparticle product. Polymer-drug complexes were dissolved in their respective organic phases at 10mg of total nanoparticle mass/ 0.5 mL organic solvent, using equal amounts of acetone and ethanol. The premise was for PTX to be
Figure 24 – Illustration of polymer-blend nanoparticle design, whereby poly(beta-amino ester) (PbAE) is used to form pH-sensitive pockets within a nanoparticle matrix composed of either poly(epsilon-caprolactone) (PCL) or poly(lactic co-glycolic acid) (PLGA). In this nanoparticle, paclitaxel is localized within the PbAE pH-responsive pockets to allow for a rapid release upon internalization into the tumor environment of pH 6.5, while ceramide is localized within the remainder of the nanoparticle matrix to allow for a delayed and slow release after paclitaxel.
Figure 25 – Illustration of the manufacturing process to assemble polymer-blend nanoparticles loaded with paclitaxel (PTX) and ceramide (CER). a) poly(epsilon-caprolactone) (PCL) or poly(lactic co-glycolic acid) (PLGA) is dissolved in acetone with 20% (w/w) pluronic F108 and 10% (w/w) CER, while poly(beta-amino ester) (PbAE) is dissolved in ethanol with 2.5% (w/w) PTX. Once dissolved the separate preparations are blended and nanoprecipitated by b) adding the mix to 10 volumes of deionized water at pH 8.0 under rapid magnetic stirring. c) Following prolonged stirring the organic solvent is evaporated causing the nanoparticles to condense and harden.
encapsulated within PbAE regions in the nanoparticle, while CER would be encapsulated within the PCL or PLGA nanoparticle matrix, so that upon internalization of the nanoparticle into the acidic environment of the tumor (~pH 6.5) an immediate release of PTX would result from dissolution of PbAE at pH <6.5, while CER would follow a delayed, sustained release from the hydrophobic matrix. Furthermore, the particles were surface modified with PEO to promote long-circulating properties and passive tumor targeting by the EPR effect.

It was found that the most stable particles resulted when blending 80% (w/w) PCL or PLGA with 20% (w/w) PbAE, and 70% (w/w) PCL or PLGA with 30% (w/w) PbAE, loaded at 2.5% (w/w) PTX and 10% (w/w) CER. The loading ratio of 2.5% PTX:10% CER assures that the dosing ratio between the two drugs, pre-set in in-vitro studies, remains, i.e., for a 1 µM PTX dose, the particles simultaneously deliver an 8.6 µM CER dose (as close as possible to the 10 µM CER concentration set in initial studies). Table 1 records the size and surface charge (ζ-potential) characterization of these blended particles. Of these formulations it was found that all the particles, except the 80% PLGA/20% PbAE blend, retained their average size around or below 200 nm, a parameter that is optimal for tumor targeting via the EPR effect. Scanning electron microscopy and transmission electron microscopy images (Figure 26) depicts representative batches of 70% PLGA:30% PbAE and 70% PCL:30% PbAE nanoparticles, the most appropriate candidate blend according to size and stability data, which shows that either blend produces spherical particles, although the PLGA/PbAE blend particles appear smoother than the PCL/PbAE blend, which presents with a staggered and perhaps brittle surface. Elemental composition on the surface of the nanoparticle, could help gain
<table>
<thead>
<tr>
<th></th>
<th>Size (nm)</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>100% (PEO) -PCL</strong></td>
<td>211.6 ± 1.8</td>
<td>-31.1 ± 1.5</td>
</tr>
<tr>
<td><strong>100% (PEO) -PLGA</strong></td>
<td>141.6 ± 0.9</td>
<td>-22.66 ± 7.63</td>
</tr>
<tr>
<td><strong>(PEO) -70% PCL/30% PBAE 2.5% PTX, 10% CER</strong></td>
<td>177.7 ± 1.9</td>
<td>-29.32 ± 0.67</td>
</tr>
<tr>
<td><strong>(PEO) - 80% PCL/20% PBAE 2.5% PTX, 10% CER</strong></td>
<td>162.1 ± 1.4</td>
<td>-16.93 ± 3.92</td>
</tr>
<tr>
<td><strong>(PEO) - 70% PLGA/30% PBAE 2.5% PTX, 10% CER</strong></td>
<td>208.2 ± 5.7</td>
<td>-26.89 ± 5.36</td>
</tr>
<tr>
<td><strong>(PEO) - 80% PLGA/20% PBAE 2.5% PTX, 10% CER</strong></td>
<td>439.4 ± 25.3</td>
<td>-6.02 ± 4.01</td>
</tr>
</tbody>
</table>
Figure 26 – Low magnification and high magnification scanning electron microscopy (SEM) images, and high magnification transmission electron microscopy (TEM) images of 70% PLGA/30% PbAE and 70% PCL/30% PbAE nanoparticles.
insight into whether the PLGA/PbAE and/or PCL/PbAE polymer blends formed PbAE pockets internally in the nanoparticle. Since PCL, PLGA, and PbAE all have a distinct elemental composition, surface chemistry could reveal whether PbAE, internalized into the nanoparticle as designed, or whether it localized to the surface of the nanoparticle, or it blended evenly with its co-polymer to distribute evenly throughout. Table 2 summarizes the surface chemical composition by electron spectroscopy for chemical analysis (ESCA) of the various polymer-blend nanoparticles. The data suggests that PCL and PbAE blend evenly into each other, since the percent composition of carbon, oxygen and nitrogen on the surface for these blend particles falls in between the composition for pure PCL and pure PbAE. High resolution carbon peak (C1s) analysis revealed that the distribution of carbon functional groups, characteristic to each polymer, also fell in between the composition for the pure polymers. In contrast, blending PLGA with PbAE revealed an elemental surface composition that rather closely resembled the composition of pure PLGA, with merely faint traces of PbAE on the surface. These results reveal that, unless the PbAE failed to complex into the particle entirely, the PbAE was successfully pocketed inside the PLGA matrix, producing a formulation that had pH-responsive centers within the hydrophobic matrix. Drug release profiles from the nanoparticle formulations can then conclusively tell whether the PCL/PbAE or PLGA/PbAE formulations were developed as designed, whereby PTX should be released upon a drop in pH from 7.4 to 6.5, while CER follows with a delayed and slow release pattern. Figure 27 shows drug release behavior from the four formulations, 70% PCL/30% PbAE, 80% PCL/20% PbAE, 70% PLGA/30% PbAE, and 80% PLGA/20% PbAE. Interestingly, each formulation exhibited a unique release profile, whereby they all shared
### Table 2 – Electron spectroscopy for chemical analysis (ESCA)

<table>
<thead>
<tr>
<th></th>
<th>Atomic composition (%)</th>
<th>High resolution C, peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>100% PCL</td>
<td>74.7 ± 0.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>100% PBAE</td>
<td>73.6 ± 0.7</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>100% PLGA</td>
<td>58.5 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>70% PCL/ 30% PBAE</td>
<td>78.4 ± 0.8</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>80% PCL/ 20% PBAE</td>
<td>78.0 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>70% PLGA/ 30% PBAE</td>
<td>61.5 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>80% PLGA/ 20% PBAE</td>
<td>62.8 ± 1.6</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

- 98 -
Figure 27 – Release profile of PTX (solid line) and CER (dashed line) from the polymer blend nanoparticles: 70% PCL/30% PBAE, 80% PCL/20% PBAE, 70% PLGA/30% PBAE, 80% PLGA/20% PBAE, at pH 7.4 for the first 6 hours, then spiked to pH 6.5 for the remainder of the release study. The arrow indicates a drop in pH from 7.4 to 6.5. (n=3 repeats/nanoparticle type).
the common trait of pH-responsiveness, suggesting that indeed all four formulations PbAE was successfully incorporated. The 70% PCL/30% PbAE particle released CER more rapidly than PTX, while the 80% PCL/20% PbAE particle released the two drugs at nearly equal rates. A drop in pH from 7.4 to 6.5 caused a surge in release of both PTX and CER from both PCL-based formulations, suggesting that the two drugs are evenly distributed throughout the matrix, thereby both exhibiting a pH-responsive release. Interestingly, the 80% PLGA/20% PbAE particle exhibited a release profile similar to the 70% PCL/30% PbAE formulation, where CER released prior to PTX, and both drugs exhibited a pH-responsive release. This behavior is of interest, since chemical surface analysis revealed that PLGA and PbAE did not blend evenly throughout the nanoparticle matrix, but rather internalized PbAE. Even though it seems that in this formulation the polymers did not blend evenly, it is likely that the drugs may have blended evenly, whereby PTX and CER localized to a portion in both PbAE and PLGA. Strikingly though, the 70% PLGA/30% PbAE particle exhibited a release profile that was not only unique from the other three formulations, but also corresponded to the release profile that the particle was designed to produce. From this nanoparticle, PTX was released more rapidly and exhibited a strong pH-responsive effect, whereby the remainder of the drug load was immediately released when the pH dropped to 6.5. CER, on the other hand, lagged in release whereby only 60% of its load was released by the time that PTX was completely unloaded. Moreover, CER release exhibited only a minimal pH responsive effect, suggesting that indeed most of the CER complexed into the PLGA matrix, whereas PTX then complexed preferentially into the pH-responsive PbAE matrix.
Aside from a characterization of size and shape of these nanoparticles, the more important characterization revolved around depicting the interior of the nanoparticles. Since the nanoparticles have been formulated to contain interior pH-sensitive PbAE pockets to compartmentalize the two drugs for temporal control of release, it is important to visualize this type of behavior. Although chemical surface composition and release data suggest that PbAE effectively separated from PLGA to internalize into the PLGA nanoparticles, whereas PbAE did not accomplish this in the PCL nanoparticle, it is important to visualize how these polymer-blends indeed interact. In order to visualize this, it was hypothesized that the polymer-blends would interact the same way when cast as a film as they would when formulated as nanoparticles, thereby allowing for visualization of polymer interaction and drug compartmentalization that cannot be achieved through imaging the nanoparticles. The formation of films has been used previously to characterize properties associated with polymer blending (Godbole, et al. 2003; Sarasam, et al. 2005; Garcia Cruz, et al. 2007). To develop films, both PLGA plus PbAE and PCL plus PbAE, were blended at a ratio of 70% to 30% and dissolved in organic solvent in the same manner that they were prepared for nanoparticle development. However, rather than nano-precipitating this mix in water, the polymer-blends were cast into Petri-dishes containing borosilicate glass coverslips, and left until the organic solvent evaporated completely. As the solvent evaporated, the polymers settled down as films adhered to the coverslips, which could be visualized by light-microscopy. As controls, 100% PCL, 100% PLGA, and 100% PbAE were similarly cast as polymer films (Figure 28 a,b,c). Light microscopy of these films revealed that the PLGA/PbAE blend formed an interesting morphology of raised bumps on a surface of a smooth material, as shown in
Figure 28d while the PCL/PbAE blend formed deposits of a crystalline-like state rather than a film (Figure 28e). The pure polymers, on the other hand, formed a very different morphology. Pure PCL formed crystalline deposits, similar to the ones formed in the PCL/PbAE blend (Figure 28a), while pure PLGA interestingly formed a smooth film (Figure 28b). Pure PbAE, on the other hand, formed a very rough film (Figure 28c). When these polymer films were submerged in PBS with a pH of 6.5, the smooth surface of the PLGA/PbAE blend rapidly began to degrade, while the raised bumps appeared unaffected (Figure 28i). A similar treatment to the PCL/PbAE blend did not produce such results though, but rather the crystalline deposits appeared to etch away evenly throughout (Figure 28j). The pure polymers, on the other hand, behaved as expected. Submerging the PCL film in PBS pH 6.5 did not effect the morphology of the crystalline deposits (Figure 28f), similar to the results seen in the PCL/PbAE blend (Figure 28j). Likewise, submerging the PLGA film in pH 6.5 also did not effect that film’s morphology (Figure 28g). However, submerging the PbAE film in pH 6.5 caused a rapid decomposition of the film, as seen by the formation of gaping holes in the film (Figure 28h). These results suggest that blending PLGA with PbAE causes the two polymers to separate from each other giving rise to distinct pH-responsive and pH-unresponsive regions. Blending PCL with PbAE, though, rather caused the polymers to blend evenly. These results are in unison with the chemical surface composition of the polymer-blend nanoparticles, and support the finding that the PLGA/PbAE blend in fact phase separates to form pH-sensitive regions within the nanoparticle, a feat that does not occur with the PCL/PbAE blend.
Figure 28– Polymer films of a) PCL, b) PLGA, c) PbAE, d) 70% PLGA:30% PbAE and e) 70% PCL:30% PbAE, at pH 7.4 mimicking the intact internal environment of the polymer-blend nanoparticles. The degradation pattern of the polymer-blend composition upon exposure to pH 6.5 conditions is depicted in f) PCL, g) PLGA, h) PbAE, i) 70% PLGA:30% PbAE and j) 70% PCL:30% PbAE.
Given that the intention for this polymer-blend nanoparticle was to compartmentalize the two drugs, ceramide and paclitaxel, within the nanoparticle to allow for temporal control of release of the two agents, an important step in characterization was to indeed visualize drug compartmentalization within the polymer blends. To observe this phenomenon, a red-fluorescent derivative of paclitaxel, rhodamine-PTX, and a green-fluorescent derivative of ceramide, NBD-CER, was incorporated into the polymer blend films, prepared as previously described. Epifluorescent microscopy of these films suggests that CER and PTX indeed compartmentalize in the PLGA/PbAE blend, as seen by the lack of co-localization of the red- and green-fluorescent signals under the merged image (Figure 29). Interestingly, NBD-CER appeared to concentrate itself within the raised bumps, while rhodamine-PTX appeared to concentrate itself in the same regions that etched away when the pH was dropped to 6.5, illustrated in Figure 28i. This result implies that CER would successfully localized mainly within the PLGA matrix, while PTX would localized mainly within the PbAE regions. As predicted by characterizations of drug release and polymer blend morphology and behavior, PTX and CER did not compartmentalize in the PCL/PbAE polymer blend, but rather distributed evenly throughout as seen by the co-localization of the red- and green-fluorescent signals under the merged image (Figure 29). To reverse the trend, the films were once again submerged in PBS at pH 6.5 to see if the red-fluorescent rhodamine-PTX would be preferentially released from either film to support the hypothesis that it had indeed compartmentalized within the pH-responsive PbAE. As illustrated by Figure 29, dropping the pH to 6.5 caused the red-fluorescent rhodamine-PTX to diffuse outward in the 70% PLGA/ 30% PbAE polymer blend. However, pH 6.5
Figure 29 – Drug compartmentalization in the 70% PCL/30% PbAE and 70% PLGA/30% PbAE polymer-blends modeled through the incorporation of red fluorescent rhodamine-paclitaxel (PTX) and green fluorescent NBD-ceramide (CER). Merging the red fluorescent and green fluorescent images indicates whether the rhodamine-PTX and NBD-CER are uniformly distributed in each polymer-blend film, or whether the two compounds compartmentalize into differing regions within the film. A drop to pH 6.5 simulates behavior of the polymer blend and drug release in the tumor environment. (200x magnification).
did not cause diffusion of the green-fluorescent NBD-CER, which mostly remained within the same regions as prior. Although it appears that the fluorescence intensity of rhodamine-PTX increased at pH 6.5 compared to prior in both the PCL/PbAE and PLGA/PbAE blends, this result is attributable to photobleaching of green-fluorescent NBD-CER, thereby causing the red-fluorescent rhodamine-PTX to appear more intense in relation. Nevertheless, these findings indicate compartmentalization of PTX with the PbAE regions and CER with the PLGA regions in the 70% PLGA/30% PbAE polymer blend. However, these results are merely indirect evidence of drug behavior within the polymer-blend films, since the incorporation of the fluorescent labels rhodamine and NBD alters the molecular structure of PTX and CER respectively, to potentially cause these fluorescent derivatives to associate differently with the polymers than PTX and CER might.

Of greatest importance is the retention of therapeutic efficacy of these polymer-blend nanoparticles to modulate MDR. Nanoparticles were loaded at 2.5% (w/w) PTX and 10% (w/w) CER, so that a 1 μM dose of PTX would correspondingly deliver an 8.6 μM dose of CER, as close as possible to the therapeutic dose of CER that was set previously at 10 μM. The results in Figure 30 illustrate that the polymer-blend nanoparticles indeed possessed the therapeutic potency to chemosensitize both the MDR MCF7_{TR} cells (Figure 30a) as well as the MDR SKOV3_{TR} cells (Figure 30b). The data reveals that while 1 μM PTX results in merely 13.0 ± 7.8% cell death in the MCF7_{TR} cells, treatment with the novel polymer-blend nanoparticles increases cell kill efficacy to 33.8 ± 4.8%, 40.5 ± 5.0%, and 39.2 ± 5.6% cell death from the 70% PCL/30% PbAE, 80% PCL/20% PbAE, and 70% PLGA/30% PbAE particles, respectively (Figure 30a).
Figure 30 – Cell kill efficacy of the paclitaxel (PTX) + ceramide (CER) therapy administered at a dose of 1 μM PTX + 8.6 μM CER encapsulated within the different polymer-blend nanoparticles (70% PCL/30% PbAE, 80% PCL/20% PbAE, 70% PLGA/30% PbAE, 20% PLGA/20% PbAE) in a) MCF7TR and b) SKOV3TR cells. Efficacy of the PTX + CER therapy in nanoparticles is compared to efficacy of 1 μM PTX administered as free drug. ** indicates a statistically significant difference (p<0.001) in cell kill between the PTX + CER therapy in nanoparticles and PTX alone (n=8 samples/group/cell type)
The 80% PLGA/20%PbAE particle did not appear to chemosensitize the MCF7<sub>TR</sub> cells, however, given the larger size of this latter formulation (>400 nm), these particles would in any case not be useful for a tumor-targeted therapy. Similar efficacy was seen in the SKOV3<sub>TR</sub> cells. While 1 µM PTX results in merely 64.9 ± 1.6% cell death in the SKOV3<sub>TR</sub> cells, treatment with the novel polymer-blend nanoparticles increases cell kill efficacy to 98.0 ± 0.5%, 97.9 ± 0.4%, 97.8 ± 0.8% and 90.6 ± 1.9% cell death from the 70% PCL/30% PbAE, 80% PCL/20% PbAE, 70% PLGA/30%PbAE, and 80% PLGA/20%PbAE particles, respectively (Figure 30b).

Based on the sizing, stability, release data, and efficacy results, the 70% PLGA/30% PbAE formulation, loaded at 2.5% (w/w) PTX and 10% (w/w) CER, was chosen as the optimal therapeutic formulation for treatment of MDR cancer. As a last measure, fluorescent nanoparticle trafficking studies were performed to ensure that the candidate nanoparticle (70% PLGA: 30% PbAE) effectively internalized into both the ovarian and breast cancer cell lines. It is important to eliminate the possibility that in-vitro efficacy of the nanoparticle formulation could be due to a release of drug outside the cell with subsequent drug diffusion into the cell, a situation that would likely greatly limit the success of this therapy in-vivo. Drug loaded 70% PLGA/30% PbAE nanoparticles were loaded with 0.1% NBD-CER tracer to visualize nanoparticle internalization and trafficking within the cells under epifluorescent microscopy. Figure 31 demonstrates that the nanoparticles internalized into all four cell types, MCF7, MCF7<sub>TR</sub>, SKOV3, and SKOV3<sub>TR</sub>, and migrated from the periphery of the cells to accumulate in the perinuclear region by four hours after administration in all cell types. Interestingly though, it appears that it took longer for the dose of nanoparticles to internalize into the MCF7 and MCF7<sub>TR</sub>
Figure 31 - Intracellular internalization and trafficking of 70% PLGA/30% PbAE nanoparticles loaded with green fluorescent NBD-ceramide (NBD-CER) from 1 hour through 4 hours after administration in MCF7, MCF7<sub>TR</sub>, SKOV3, and SKOV3<sub>TR</sub> cells. (200x magnification and 600 ms exposure time)
cells, since the fluorescence intensity increases in this cell line between 1 hour after administration and four hours. However, in the SKOV3 and SKOV3<sub>TR</sub> cells, fluorescence intensity remains the same between 1 hour and 4 hours after administration, suggesting that the dose internalized into the cells prior to 1 hour after administration.

IV. DISCUSSION

Polymeric nanoparticles have been widely used in controlled drug release applications since its inception nearly three decades ago (Uhrich KE, et al. 1999). The breadth of synthetic and natural polymers hereby allows for the development of nanoparticles with meticulous function, a feat that is quite extraordinary given that engineering of these particles occurs on a molecular scale. To advance controlled release applications, polymer blends are more frequently being used (Sarazin, et al. 2004; Mundargi, et al. 2007; Siepmann, et al. 2008).

A therapy that combined the chemotherapeutic drug PTX with an MDR modulating drug CER was shown to be effective to overcome MDR in both breast and ovarian cancer in previous chapters, however, further studies revealed that this combination therapy is most effective when the two drugs are administered with temporal spacing. It was found that a 10% increase in cell kill efficacy resulted when CER was administered 6 hours following PTX administration to the MCF7<sub>TR</sub> cells, for example. Interestingly, this increase was not seen when PTX was administered 6 hours following CER. Since PTX exerts toxicity in a cell cycle dependent manner, it is very likely that PTX resides in the cells for hours prior to initiating the apoptotic cascade. CER that is co-administered with PTX could be subject to metabolism by GCS in this span of time,
although the kinetics of GCS and the dose-dependent effect of CER on GCS activity in MDR cells remain largely unknown. However, based on these assumptions, it can be speculated that the enhancement that temporal spacing of the drugs has on efficacy may be due to the presence of a larger pool of unmetabolized CER to mediate the apoptotic signal. However, further studies would be required to determine this conclusively.

To incorporate this temporal relationship between PTX and CER into a nanoparticle formulation, a polymeric nanoparticle was designed by blending either poly(lactic co-glycolic acid) (PLGA) or poly(epsilon-caprolactone) (PCL) with poly(beta-amino ester) (PbAE) to produce a nanoparticle of PLGA or PCL that contained pH-responsive regions within its matrix. Within this nanoparticle, PTX was intended to localize within the pH-sensitive PbAE regions, while CER was intended to localize within the remainder of the nanoparticle matrix composed of either PLGA or PCL. The purpose for compartmentalization of the drugs was to control release of the two agents whereby PTX would exhibit a surge in release resulting from dissolution of the PbAE upon a drop in pH to 6.5, while CER would exhibit delayed and continuous release by diffusion and bulk erosion from the remainder of the nanoparticle matrix. Although it is speculated that the pH-dependent dissolution of the PbAE regions creates enough porosity in the nanoparticle for diffusion of PTX into the surrounding aqueous environment, the verification of this mechanism has not been examined. Similarly, since it is known that nanoparticles composed of PCL and PLGA undergo bulk erosion rather than surface erosion through hydrolysis (Uhrich KE, et al. 1999), suggesting porosity, it can be predicted that internal PbAE regions of the nanoparticle would have little trouble sensing pH changes in the environment.
Characterization of chemical surface composition revealed that blending PbAE with PLGA resulted in better internalization of PbAE than when it was blended with PCL. While it appeared from chemical surface composition and film analysis that PCL and PbAE blended evenly, the same data suggests that PLGA and PbAE separated from one another to produce distinct regions, which would aide in compartmentalization of the two drugs as intended. In fact, monitoring drug distribution in films of PCL/PbAE and PLGA/PbAE blends using fluorescent derivatives of PTX and CER revealed that the two drugs compartmentalized better in the PLGA/PbAE polymer blend, whereby it appeared that PTX largely localized within regions of PbAE and CER localized within regions of PLGA, although this is merely indirect evidence since the fluorescent derivatives PTX and CER differ in physiochemical properties from their parent compounds. It is suspected that this compartmentalization results not from the interaction of drugs with each respective polymer, but rather from the interaction between PLGA and PbAE. To manufacture these particles, CER and PTX are allowed to complex with PLGA (or PCL) and PbAE respectively prior to precipitating the preparations as nanoparticles in aqueous medium. It can be reasoned that at this stage hydrophobic interactions complex CER with PLGA or PCL and PTX with PbAE. However, since PCL and PbAE blend evenly throughout, so do PTX and CER, thereby failing to produce the nanoparticle as designed. However, since PLGA and PbAE separate from one another upon blending, it would allow for CER complexed with PLGA to compartmentalize into PLGA regions of the particle, while PTX complexed with PbAE would compartmentalize into PbAE regions within the nanoparticle. Conclusive evidence that the nanoparticle had in fact been developed as designed came from drug release studies. The 70% PLGA/30% PbAE
nanoparticle was the only formulation to release PTX with a surge upon a drop in pH to 6.5, while CER followed with a slower and sustained release that exhibited only minimal pH-responsiveness.

Aside from the work by Sengupta et al. (2005), where a combination therapy of doxorubicin and combretastatin-A4 was loaded into a single nanoparticle platform, this work describes the only other use of nanoparticles to deliver a combination therapy with temporal controlled release from a single nanoparticle. However, in the preparation designed by Sengupta et al. (2005) temporal controlled release was achieved by loading doxorubicin into a core polymeric nanoparticle, and combretastatin-A4 into a surrounding lipid bilayer shell, thereby compartmentalizing and controlling release of their combination therapy in a much different manner. This greatly differs from the polymer-blend nanoparticle presented in this work, which is original in its design. Furthermore, this work is the first to utilize nanoparticles to co-administer a combination therapy to MDR cancer.
CHAPTER 5: EVALUATION OF THE THERAPEUTIC EFFICACY AND SAFETY PROFILES OF PACLITAXEL AND CERAMIDE POLYMER-BLEND NANOPARTICLE THERAPY TO OVERCOME MDR IN IN-VIVO MODELS OF HUMAN BREAST AND OVARIAN CANCER

I. INTRODUCTION

Based on the principle that MDR has been shown to result from metabolism of the apoptotic mediator ceramide by the enzyme glucosylceramide synthase (GCS) to elevate the apoptotic threshold, a novel therapeutic strategy was developed to restore these defects through a combination therapy of exogenous ceramide (CER) and the chemotherapeutic drug paclitaxel (PTX). Based on preliminary evidence that the PTX + CER combination therapy is most effective against MDR breast and ovarian cancer, a polymer-blend nanoparticle was designed by blending 70% PLGA to 30% PbAE to temporally control release of the combination therapy for optimal therapeutic efficacy. It is hypothesized then that the exogenously administered CER reinstates the apoptotic signal to re-sensitize the cancer cells to the chemotherapeutic drug PTX, while nanoparticle delivery of the combination therapy enhances efficacy on a cellular level by increasing intracellular drug accumulation and optimizing the dose administered, and on a whole body level by increasing tumor targeting and retention of the drug.

Previous studies from our group have examined the efficacy of the PTX + CER combination therapy, separately encapsulated within PEO-PCL nanoparticles on and in-vivo model of MDR ovarian cancer. In this study, the nanoparticle combination therapy was 28% more effective against drug sensitive cancer and 40% more effective against MDR cancer than the free drug formulation of the combination therapy (Devalapally, et
al. 2007), indicating the advantage of nanoparticle delivery systems in tumor targeting and therapy.

To examine the therapeutic efficacy of the PTX + CER combination therapy encapsulated within 70% PLGA:30% PbAE nanoparticles, the therapy was administered \textit{in-vivo} to animals bearing orthotopic MDR human breast cancer and subcutaneous MDR human ovarian cancer. Efficacy was determined by monitoring tumor volume changes over time following a single dose administration. Furthermore, an extensive safety evaluation was conducted \textit{in-vivo} to assess any potential toxicity of the particles upon systemic administration.

II. MATERIALS AND METHODS

1. Preparation of silastic estradiol implants

Silastic implants containing 16 µg Estradiol each were manufactured according to a protocol obtained from Dr. Bo Rueda (Mass. General Hospital, Boston, MA). Silastic tubing (Dow Corning, Midland, MI) with an inner diameter of 1/16” and an outer diameter of 1/8” were cut in 11 mm sections and closed on one end with a 2mm rubber silicone plug (GE Silicone II, Huntersville, NC). Estradiol (Sigma-Aldrich, St. Louis, MO) and Cholesterol (Sigma-Aldrich, St. Louis, MO) were ground together at a ratio of 1:100 respectively with a mortar and pestle for 2 hours. Next, the silastic implants were filled with approximately 1.6 mg of the mixture each and closed on the remaining end
with another 2mm rubber silicone plug. Once dried, the implants were dusted off and sterilized under UV radiation overnight, and stored sterile until use.

2. Nanoparticle and free drug preparation

Polymer-blend nanoparticles were manufactured as described in chapter 4 at a ratio of 70% PLGA:30% PbAE, loaded with 2.5% (w/w) PTX and/or 10% (w/w) CER. Free drug treatment groups were prepared in Cremophore EL® (polyoxyethylated castor oil):ethanol (50:50) for both PTX and CER according to the recipe for the clinical formulation of PTX. PTX was dissolved at 6mg/mL with 527 mg of Cremophore EL® (BASF, Mount Olive, NJ) and 49.7% (v/v) dehydrated alcohol USP (Thermo Fisher Scientific, Waltham, MA), while CER was similarly made although at 20mg/mL.

3. Animal Care and Use

Female nu/nu (athymic) mice (Charles River Laboratories, Wilmington, MA) were housed in sterile cages with ad libitum access to sterile food and acidified water on a 12:12 light:dark cycle. All experiments were approved by Northeastern University’s Animal Care and Use Committee under protocol number 07-0204R.

Silastic implants were surgically inserted along the right flank of mice that would receive either MCF7 or MCF7TR tumor xenografts. To do so, mice were anesthetized by inhalation of a 40% isoflurane solution in USP quality mineral oil by the open drop method, and kept under anesthesia through a nose cone apparatus that administered a 30% isoflurane solution in mineral oil. A 5 mm insertion was made between the scapula,
and the subcutaneous implant was inserted longitudinally. The incision was then closed with 4-0 nylon sutures and animals were allowed to rest and recover for at least 48 hours.

To inoculate MCF7 or MCF7(TR) cells into the mammary fat pad for orthotopic tumor development, cultured cells were harvested and counted as described in chapter 2. For each mouse, $2 \times 10^6$ cells were resuspended into 100 µl of serum supplemented RPMI, to which 100 µl Matrigel HC (BD Biosciences, San Jose, CA) was mixed in. This mixture was injected subcutaneously into the mammary fat pad of lightly anesthetized animals using a 27G needle, after which animals were left to rest until tumors reached a palpable volume of 100 mm$^3$. Once tumors reached this size, mice were randomly assigned to one of eight treatment groups: control (no treatment), 20 mg/kg paclitaxel, 80 mg/kg ceramide, 20mg/kg + 80 mg/kg paclitaxel + ceramide, blank polymer-blend nanoparticles, 20 mg/kg paclitaxel in polymer-blend nanoparticles, 80 mg/kg ceramide in polymer-blend nanoparticles, and 20mg/kg + 80 mg/kg paclitaxel + ceramide in polymer-blend nanoparticles. Mice received a single dose IV administration of the treatment diluted in sterile saline for a total volume of 500 µL. Tumor volume was measured at the commencement of treatment and every other day following this up to 4 weeks out with metric calipers. Mice were weighed at the start of treatment and weekly thereafter, and similarly blood was collected by submandibular puncture at the start of treatment, and every 2 weeks thereafter. 5 µL blood was separated, treated with 20 µL 2% acetic acid and stained with µL crystal violet for white blood cell counting using a haemocytometer, while the remainder of the blood was centrifuged at 13,000 rpm for 10 min to collect plasma for future use. On day 28 of treatment, mice were sacrificed by carbon dioxide
inhalation, tumors harvested, weighed, flash frozen along with liver, and stored at -80°C for further use. Similarly, a small cohort of mice lacking estrogen priming was inoculated with SKOV3\textsubscript{TR} cells subcutaneously. Cultured SKOV3\textsubscript{TR} cells were harvested and counted as described in chapter 2 and re-suspended at 4×10\(^6\) cells per mouse into 100 μL RPMI + 100 μL Matrigel\textsuperscript{®}. SKOV3\textsubscript{TR} tumors were inoculated by injecting the prep subcutaneously into the right hind flank. Again, once tumors had reached a palpable volume of 100 mm\(^3\), mice were randomly assigned to one of 5 groups and subjected to a single dose IV treatment of control (no treatment), 20 mg/kg paclitaxel, 20mg/kg + 80 mg/kg paclitaxel + ceramide, 20 mg/kg paclitaxel in polymer-blend nanoparticles, and 20mg/kg + 80 mg/kg paclitaxel + ceramide in polymer-blend nanoparticles. Again, treatments proceeded for out to 4 weeks, and all intermittent and subsequent measurements that were taken from the MCF7 and MCF7\textsubscript{TR} tumor bearing mice were also applied here.

4. **Tumor TUNEL staining and immuno-histochemistry**

   Fresh frozen tumor tissues were cryosectioned at 14 micron-thick sections and mounted onto positively-charged glass slides (Superfrost Plus\textsuperscript{®}, Thermo Fisher Scientific, Waltham, MA) in sets of five. To stain for apoptotic activity within the tumor sections, one slide from each set was stained for the presence of fragmented DNA using a commercially available TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (Promega, Madison, WI) according to manufacturer’s protocol. Sections were visualized
under brightfield microscopy at 200x magnification to detect the presence of brown staining, indicative of apoptotic activity by DNA fragmentation.

Alternately one other slide from each set was each stained for the presence of either human P-glycoprotein, human glucosylceramide synthase, or mouse collagen IV by immuno-histochemistry. For these studies, the slides were first treated for antigen retrieval by boiling them in citrate buffer for 5 minutes. Following this, slides were incubated overnight in a 5% solution of BSA in PBS at 4°C. The following day, slides were incubated with a 1:100 dilution of either mouse anti-human P-glycoprotein monoclonal antibody (C219) (Signet Labs, Dedham, MA), rabbit anti-human glucosylceramide synthase polyclonal antibody (Exalpha Biochemicals, Maynard, MA), or rabbit anti-mouse collagen IV polyclonal antibody (Abcam, Cambridge, MA) diluted in 1% BSA-PBS for 1 hour at room-temperature. Following this, slides were washed, and incubated for 30 min at room temperature in a 1:200 dilution of HRP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich, St. Louis, MO) for P-glycoprotein detection, or HRP-conjugated goat anti-rabbit secondary antibody (Cell Signaling Tech., Danvers, MA) for GCS and collagen IV detection. The presence of antigen was then visualized with diaminobenzidine (DAB) staining, which is cleaved by horse-radish peroxidase to produce a brown stain. Slides were subsequently counterstained with hematoxylin and imaged under brightfield microscopy at 200 x magnification for P-glycoprotein and glucosylceramide synthase and 40X magnification for collagen IV.
5. **Estradiol ELISA**

ELISA was employed to quantitatively determine serum estradiol levels resulting from implantation of the silastic estradiol depots, and to determine whether serum estradiol levels remained steady during the drug treatment period. Plasma samples obtained from mice at day 1, day 14, and day 28 of treatment were diluted 1:5 with dI water assayed using a commercially available competitive ELISA for estradiol (Bio-Quant, Inc., San Diego, CA) according to manufactures protocol, whereby the presence of estradiol results in the decrease of color measured at 450 nm.

6. **Enzyme activity assays**

As a parameter of therapeutic safety, plasma samples collected at day 1, day 14, and/or day 28 of treatment were subjected to enzyme activity assays to measure the activity of serum alanine amino transferase (ALT) and lactate dehydrogenase (LDH). For LDH activity, plasma was diluted 1:5 and assayed for activity using a commercially available kit (Quantichrom™ LDH kit, Bioassay Systems, Hayward, CA) according to manufacturers protocol, whereby LDH activity is measured through the rate of development of a colorimetric output read at 565 nm. For ALT activity, 2 µl of plasma was assayed for activity using a commercially available kit (Roche Diagnostics, Basel Switzerland) according to manufacturer’s protocol, whereby ALT activity was measured through the rate of decrease of a colorimetric output read at 340 nm.
7. **Liver Tissue Histology**

As an alternate measure of therapeutic safety, fresh frozen livers, harvested from mice at the end of the treatment period, were cryosectioned at 14 micron sections and mounted onto positively-charged glass slides. Following this, liver sections were fixed in 10% buffered formalin and subjected to hematoxylin and eosin staining according to the following protocol: 1x 3 min hematoxylin, rinse in dI water, 1x 5 min tap water, 8 dips in acid ethanol (200 µL 12N HCL/100 mL dI water), 2x 1 min tap water, 1x 2 min dI water, 1 x 30 sec eosin, 3 x 5 min 95% ethanol, 3 x 5 min 100% ethanol. Slides were visualized by brightfield microscopy at 100x magnification centered on the central vein to look for any damage to the lobule structure.

8. **Statistical Analysis**

For animal efficacy and serum enzyme efficacy assays n= 3-5 repeats/group. For serum estradiol levels, n = 60 samples/group. Statistical analysis was performed by two tailed, equal variance student t-test. Statistical significance was accepted at p<0.05.

III. **RESULTS**

In order to evaluate *in-vivo* efficacy of the PTX + CER nanoparticle formulation, an *in-vivo* model of breast cancer was developed. Since breast cancer, as opposed to ovarian cancer, can be developed orthotopically in a rodent model, drug sensitive MCF7 and MDR MCF7\textsubscript{TR} tumors were preferred as a model to evaluate the efficacy of the novel therapeutic. To develop the *in-vivo* cancer model, MCF7 and MCF7\textsubscript{TR} cells were xenografted into the mammary fat pad of immune-compromised mice for tumor
development. However, similar to clinical forms of human breast cancer, the MCF7 cancer cell line is estrogen dependent for proliferation (Leonessa, et al. 1992). Since intact female mice do not produce high enough levels to support MCF7 tumor development, mice must be supplied with exogenous estrogen through the insertion of continuous release estrogen implants to achieve a steady state plasma estrogen level for at least 6 weeks. A preliminary investigation was carried out to evaluate estrogen-dependency on proliferation and survival of MCF7 and MCF7\textsubscript{TR} cells \textit{in-vitro} by stripping estrogen from growth medium and replacing it in a dose-dependent manner. It was found that stripping estrogen from growth medium resulted in a mere 10\% decrease in either survival or proliferation of both cell types in culture after 3 days (88.4 ± 6\% and 90.0 ± 3\% cell survival of MCF7 and MCF7\textsubscript{TR} cells respectively). However, in both cell lines, replacement with, as little as, 5 pg/mL of estradiol reverted this decrease to result in 100\% survival or proliferation of both cell types.

To prime the mice with sufficient estrogen to support tumor growth, silastic estradiol implants were prepared according to a protocol kindly provided by Dr. Bo Rueda and colleagues from Massachusetts General Hospital in Boston. Silastic implants were prepared by cutting commercially available silicone tubing (0.062” OD x 0.125” ID) into 11mm strips, plugged with silicone rubber and filled with a 1:100 mix of 17β-estradiol:cholesterol. These implants were inserted subcutaneously on the right dorsal side of intact female nu/nu mice, and after a 48 hour rest period either drug sensitive MCF7 or MDR MCF7\textsubscript{TR} tumor cells were mixed 1:1 with Matrigel HC\textsuperscript{®}, a solubilized basement membrane preparation that is extracted from EHS mouse sarcoma, and injected into the mammary fat pad at 2x10\textsuperscript{6} cells per mouse. The implants were designed to
release estradiol into the bloodstream to maintain a steady state level of 50 pg/mL for up to 6-8 weeks. **Table 3** depicts the mean serum estradiol levels for all mice between day 1 and day 28 of treatment. While the estradiol implants indeed maintained a steady-state level around 50 pg/mL around the start of treatment (2-3 weeks following implantation), this level sloughed off over the duration of treatment to settle at around 40 pg/mL by the end of the treatment period.

Upon establishment of a stable tumor model for both the wild-type (drug sensitive; MCF7) and the MDR (MCF7_{TR}) tumor types, therapeutic efficacy studies proceeded to determine the tumor response against a single dose treatment with PTX and CER encapsulated within the 70% PLGA/30% PbAE polymer-blend nanoparticles. Tumor bearing mice were randomly assigned to treatment with either control (untreated), PTX (free drug) at 20 mg/kg, CER (free drug) at 80 mg/kg, PTX + CER (free drug) at 20 mg/kg and 80 mg/kg respectively, blank nanoparticles (without drugs), PTX in polymer-blend nanoparticles at 20 mg/kg, CER in polymer-blend nanoparticles at 80 mg/kg, and PTX + CER in polymer-blend nanoparticles. Once the tumors had reached a palpable size of approximately 100 mm\(^3\), the mice were given a single dose IV injection of their assigned treatment, after which tumor volume was routinely measured with a metric vernier calipers until 28 days after the treatment initiated. **Figure 32** shows the therapeutic response as percent change in tumor volume over time for MCF7 tumors (**Figure 32a**) and MCF7_{TR} tumors (**Figure 32b**). The wild-type (drug sensitive) tumors exhibited a very different treatment response from the MDR tumors, as expected given the preservation of chemosensitivity in this tumor type. **Figure 32a** shows that, unlike in the MDR tumor type, treatment with PTX (free drug) has a significant effect on tumor
Table 3 – Average serum estradiol levels in pg/mL of all animals on day 1, day 14, and day 28 of treatment.

<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>std err</th>
<th>min</th>
<th>max</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 1</td>
<td>46.8</td>
<td>4.9</td>
<td>21.8</td>
<td>119.8</td>
</tr>
<tr>
<td>day 14</td>
<td>40.9</td>
<td>2.9</td>
<td>11.9</td>
<td>98.9</td>
</tr>
<tr>
<td>day 28</td>
<td>38.4</td>
<td>2.1</td>
<td>17.6</td>
<td>87.4</td>
</tr>
</tbody>
</table>
Figure 32– Tumor volume change ((Vt−Vo)/Vo x100%) over time following a single dose IV treatment of 20 mg/kg paclitaxel (PTX) and/or 80 mg/kg ceramide (CER) administered as free drug in a cremophore-ethanol formulation or encapsulated within polymer-blend nanoparticles (NP) in a) MCF7 and b) MCF7TR tumor bearing mice. (n=3-5 mice/treatment/tumor type)
volume reduction to shrink tumors by about 41% from their initial volume by 4 weeks after the treatment. Similarly, the PTX + CER group was also successful in regressing tumor growth following a single dose treatment, resulting in tumors that were suppressed by an average of 56% by week 4. However, although the drug treated tumors did show a significant reduction in tumor volume compared with the untreated control group, there was no significant enhancement of tumor regression in these mice when treated with the PTX + CER combination therapy vs. PTX alone. Similarly, while the PTX + CER nanoparticle therapy significantly reduced tumor volume compared with the untreated controls, no significant enhancement was observed with the nanoparticle treatment over treatment with the PTX or the PTX + CER combination therapy. PTX administered within polymer-blend nanoparticles to drug sensitive tumors also causes a significant tumor regression over time compared with the untreated control, and in fact, by week two after treatment initiation, tumor volume is reduced significantly more in response to the PTX nanoparticle treatment compared with PTX administered as free drug (for example, about 40 % vs. 20% volume reduction respectively, p<0.05). However, by week 3, this enhanced efficacy of the PTX nanoparticles was lost. The dose of CER given on its own also results in tumor regression in wild-type tumors, as seen by a significant decrease in tumor volume over time from control when administered either as free drug or within the polymer-blend nanoparticles. In earlier in-vitro studies (chapter 2) it was observed that CER has a far more potent effect on MCF7 cells than MCF7_{TR} cells (Figure 9), therefore this effect is not entirely out of the ordinary. What is striking is the effect that the blank nanoparticles have on tumor growth. It appears from the data that blank nanoparticles cause a significant reduction in tumor volume within the first two weeks after
administration. However, after this initial decrease in tumor volume, the tumors resume exponential growth to bounce back to their original starting volume by end of the 4 week monitoring period. While the final tumor volume of blank nanoparticle treated tumors is roughly 50% greater than drug treated tumors, this final tumor volume differs significantly from tumor growth in the untreated control mice.

The therapeutic results differed greatly for the MDR MCF7_{TR} tumors though (Figure 32b). Within this treatment set, the PTX + CER nanoparticle therapy shows the greatest trend towards tumor regression with a 36% drop in tumor volume from initial, although this result is merely significantly different from the control group, while it is not significant from the remaining drug treatment groups. Unlike the drug-sensitive MCF7 tumors though, the PTX (free drug) therapy did not cause a significant decrease in tumor volume from initial throughout the treatment period, again supporting the MDR phenotype of this tumor. PTX nanoparticles did show an initial drop in tumor volume down to a maximum of 29% reduction from initial by day 5 after treatment; however, this effect quickly disappeared thereafter. CER administered as free drug, interestingly, appeared more efficacious on the MDR tumors than CER nanoparticles were, although neither treatment caused a significant reduction in tumor volume from initial. And lastly, unlike the result seen in the MCF7 tumors, treatment with blank nanoparticles on the MCF7_{TR} tumors, did not alter tumor growth and differently from the untreated control tumors.

Alternately, when starting and final tumor volumes of individual mice are examined for all treatment groups, as depicted in Figure 33, the individual tumor volumes of mice in the control group of both tumor types displayed a trend toward increase from day 1 to
Figure 33 – Boxplot depicting differences in mean, maximum (max), and minimum (min) tumor volumes at day 1 (white boxes) and day 28 (gray boxes) of treatment following a single dose IV dose of 20 mg/kg paclitaxel (PTX) and/or 80 mg/kg ceramide (CER) administered as free drug or encapsulated within polymer-blend nanoparticles (NP) in a) MCF7 and b) MCF7_{TR} tumor bearing mice. Control = no treatment. (n=3-5 mice/treatment/tumor type).
day 28 after treatment, with a 148% increase for in mean tumor volume for MCF7 tumors and a 184% increase in mean tumor volume for MCF7\textsubscript{TR} tumors. Coinciding with the results depicted in Figure 32a, PTX administration either as free drug or nanoparticle, was highly effective on MCF7 tumors, both resulting in a 40% decrease in tumor volume from day 1 to day 28, although nanoparticle delivery did not enhance the efficacy over free drug administration. Similarly, results were seen with CER treatment, where free drug and nanoparticle administration caused an equal 50% decrease in tumor volume between day 1 and day 28. In the MDR tumors though, treatment with PTX or CER alone administered as free drug did not cause a significant decrease in tumor volume between day 1 and day 28 (Figure 33b). However, treatment with PTX in nanoparticles on the MCF7\textsubscript{TR} tumors did in fact cause a 20% decrease in tumor volume by day 28. Interestingly though, while the PTX + CER nanoparticle therapy did not cause a change in tumor volume in the MCF7 mice that was any different from the other drug treatment groups (Figure 33a), the PTX + CER nanoparticle therapy decreased tumor volume to the greatest extent in the MCF7\textsubscript{TR} tumors (31% mean tumor volume reduction between day 1 and day 28). These results coincide with the data depicted in Figure 32 to support the finding that while the PTX + CER nanoparticle therapy is no more efficacious on drug-sensitive MCF7 tumors than other treatments, the PTX + CER nanoparticle therapy is most effective against MDR MCF7\textsubscript{TR} tumors.

Final tumor weight, measured 28 days following treatment initiation, did reveal with significance that the PTX + CER nanoparticle therapy was the most efficacious for the MCF7\textsubscript{TR} tumor type (Figure 34b). At an average of $21 \pm 3$ mg the final tumor weight after treatment with PTX + CER nanoparticles is significantly lower than the final tumor
Figure 34 – final tumor mass (g) after 28 days of treatment with 20 mg/kg paclitaxel and/or 80 mg/kg ceramide administered as free drugs or as nanoparticles (NP) in a) MCF7 and b) MCF7TR tumor bearing mice. * indicates statistical significance (p<0.05) of the paclitaxel + ceramide NP treatment from paclitaxel, paclitaxel + ceramide, blank NP, paclitaxel NP, and ceramide NP. ^ indicates statistical significance (p<0.05) between the indicated treatment group and untreated control for each tumor type. (n=3-5 mice/treatment/tumor type).
weights of mice in not only the control group, but also of mice that received any of the other drug treatments (PTX, CER, PTX + CER, blank nanoparticles, PTX nanoparticles, and CER nanoparticles) (p<0.05, Figure 34b). Since the mice in all the treatment groups started out with, on average, equal initial tumor volumes at the start of treatment (Figure 33b), final tumor weight correlates well to treatment efficacy. These results suggest that, as hypothesized, the PTX + CER nanoparticle therapy has the greatest potential to overcome MDR in an orthotopic model of human MDR breast cancer. On the other hand, final tumor volumes did not differ significantly between the PTX + CER nanoparticle therapy and treatment with the individual drugs, administered as free drug or within nanoparticles, in the drug-sensitive MCF7 tumors (Figure 34a). Although the PTX + CER nanoparticle therapy significantly enhanced chemosensitization of drug-sensitive MCF7 cells in-vitro (Figure 12), the results from this study suggest that this therapy unfortunately does not enhance chemosensitization as greatly in in-vivo drug-sensitive tumors.

Complications arose in both the drug-sensitive and MDR tumor models, likely resulting from super-physiological levels of estrogen. Many of the mice during the study presented with swollen abdomens and hemorrhaging of the abdomen, eventually proving fatal to several of the mice. Due to these complications, the efficacy studies could not be carried out past day 28. Based on data from other groups, it is speculated that drug efficacy on these MCF7 and MCF7_{TR} human breast tumor models would benefit from a prolonged study carried out beyond 28 days. However, since it was not feasible to prolong the study given the toxicity observed from estrogen priming, an additional in-vivo model of human MDR ovarian cancer, xenografted from the SKOV3_{TR} cells used
previously in this project, was used to validate efficacy of this PTX + CER nanoparticle therapy to overcome MDR.

A smaller cohort of animals bearing subcutaneous SKOV3\textsubscript{TR} tumors were given the same single dose IV administration of either 20 mg/kg PTX (free drug), 20 mg/kg PTX + CER (free drug), 20 mg/kg PTX nanoparticles, or 20 mg/kg PTX + CER nanoparticles, aside an untreated control group. Again tumor volume was monitored for out to 28 days following treatment administration. Figure 35 depicts efficacy results on this MDR model. The results strikingly show that while neither the free drug PTX, PTX nanoparticles or free drug PTX + CER treatments alter tumor growth patterns compared with the untreated control group, the mice that received the PTX + CER nanoparticle treatment show significantly reduced tumor volumes and tumor growth from both control and other drug treatment groups as early as day 10 after drug administration through day 28. Although the PTX + CER treatment administered as free drug showed a delay in tumor growth early on by day 4 (30% tumor volume change from the PTX treated groups) this effect, however, was quickly lost thereafter. Similarly, the PTX + CER nanoparticle treatment quickly regressed tumor growth by day 4 to produce a 40% lower tumor volume change compared with the PTX treated groups, however, only in this treatment group was tumor growth delay significantly retained. Similar to the results seen in the MCF7\textsubscript{TR} MDR breast cancer model, again the PTX + CER nanoparticle treatment resulted in the smallest final tumor weight, at 31.2 ± 1.1 mg significantly lower than the final tumor weights of the other four treatment groups (Figure 36).

While the tumor efficacy data on both MCF7\textsubscript{TR} and SKOV3\textsubscript{R} MDR models appears to support the hypothesis that the PTX + CER combination therapy delivered within the
Figure 35 – Tumor volume change ((Vn-Vo)/Vo x100%) over time following single dose IV treatment of 20 mg/kg paclitaxel and/or 80 mg/kg ceramide administered as free drug or encapsulated within polymer-blend nanoparticles (NP) in SKOV3TR tumor bearing mice. * indicates statistical significance (p<0.05) of the paclitaxel + ceramide NP therapy from other treatment groups (n=4 mice/treatment)
Figure 36 – final tumor mass (g) after 28 days of treatment with 20 mg/kg paclitaxel with or without 80 mg/kg ceramide administered as free drug or as nanoparticles (NP) in SKOV3_tr tumor bearing mice. Control = no treatment. * indicates statistical significance (p<0.05) of the paclitaxel + ceramide NP treatment from other treatment groups (n=3-5 animals/group).
70% PLGA:30% PbAE nanoparticles significantly enhances the ability to eradicate MDR tumors, the question remains whether this therapy indeed accomplishes this feat by lowering the apoptotic threshold in MDR cancer cells. To determine apoptotic activity in response to the various treatment groups, tumors harvested on day 28 after treatment were cryopreserved, cryosectioned, and mounted onto slides at 14 micron sections. Slides were subsequently stained for apoptotic activity by TUNEL staining, which labels nicked-end DNA fragments, one of the hallmarks of apoptotic activity, resulting in the formation of a brown/purple stain where apoptotic activity is present. Figure 37 shows tumor sections from drug sensitive MCF7 tumors (Figure 37a) and from MDR MCF7TR tumors (Figure 37b). While TUNEL positive staining, indicating apoptotic activity, is present throughout many sections of drug sensitive tumors treated with the individual and combination drug therapy as either free drug, or encapsulated within nanoparticles, the most intense amount of TUNEL staining appeared in any of the tumor sections treated with the drugs in nanoparticles (PTX nanoparticles, CER nanoparticles, PTX + CER nanoparticles, and blank nanoparticles) (Figure 37a). This result though is not surprising since all the drug treatments, and particularly the nanoparticle drug treatments, were very efficacious on the drug sensitive tumors. Of greater interest though is the pattern of TUNEL staining on the MDR tumors (Figure 37b). While the free drug PTX and free drug PTX + CER treatments, as well as the PTX, CER, and blank nanoparticle treatments, showed minimal TUNEL staining in their sections apart from faint indications, the PTX + CER nanoparticle therapy caused a very intense TUNEL positive staining on all of its tumor sections (Figure 37b). This result appears to suggest that the combination drug therapy in the polymer-blend nanoparticles is able to restore apoptotic activity in the
<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>PTX</th>
<th>CER</th>
<th>PTX + CER</th>
<th>Blank NP</th>
<th>PTX NP</th>
<th>CER NP</th>
<th>PTX + CER NP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 37 – Tumor TUNEL staining to indicate apoptotic activity in a) MCF7 and b) MCF7_{TR} tumor sections, harvested at day 28 following a single dose IV treatment with 20 mg/kg paclitaxel (PTX) and/or 80 mg/kg ceramide (CER) administered as free drug or within polymer blend nanoparticles (NP). Tumors are sectioned in 12 \( \mu \)m sections and stained for exposed dUTP nick-end labels indicative of apoptotic activity in the tumor cells. Brown/purple staining indicate TUNEL positive regions in the tumor section.
MDR tumor cells to overcome MDR, since apoptotic activity is most overwhelming in the PTX + CER nanoparticle treated tumor sections, supporting the enhanced therapeutic efficacy of this treatment. Due to the complications associated with estrogen priming to the mice, several mice succumbed to mortality prior to day 28 of the study, when tumors were harvested for TUNEL analysis. Therefore, certain treatment groups have fewer tumor sections than other treatment groups.

To verify that MDR phenotype was retained once the cells were xenografted \textit{in-vivo} and maintained throughout the entire four week study, the expression of the MDR marker proteins P-glycoprotein and glucosylceramide synthase (GCS) was examined in the tumor sections of drug-sensitive MCF7 and MDR MCF7\textsubscript{TR} tumors by immunohistochemistry. Given the notion that any of the drug sensitive MCF7 tumors could have begun to develop the MDR phenotype either in response to the drug treatment or within the hypoxic environment of the tumor core it is of interest to analyze expression of these marker proteins by immunohistochemistry throughout the tumor sections. Tumors sections were labeled with a primary antibody against either P-glycoprotein or GCS followed by labeling with an HRP-conjugated secondary antibody. DAB treatment then caused the development of a strong brown pigment wherever the antigen, P-glycoprotein or GCS, was exposed. Tissue sections were then counterstained with hematoxylin to produce a blue hue in the cells of the tumor sections that were not stained brown already. \textbf{Figure 38} depicts tumor sections stained for the presence of P-glycoprotein in each of 2 representative tumor tissues treated with paclitaxel, paclitaxel + ceramide, paclitaxel + ceramide nanoparticles, or control (untreated), in both drug-sensitive MCF7 and MDR MCF7\textsubscript{TR} tumor types. While the MDR tissues
Figure 38 – Expression of the MDR marker protein P-glycoprotein (P-gp) by immunohistochemistry in tumor sections of wild-type (drug sensitive; DS) and MDR tumor bearing mice 28 days following treatment with control (untreated), paclitaxel (PTX), paclitaxel and ceramide (PTX + CER), or 70% PGLA:30% PbAE nanoparticles containing PTX and CER (PTX + CER NP). Representative tumor sections are shown from 2 mice of each group. Brown staining indicates presence of PgP. All tumor sections were subsequently counterstained with hematoxylin to stain the tissue blue. (magnification 100x)
overwhelmingly stain brown for the presence of P-glycoprotein, as predicted, the drug sensitive tissues also stain positive for the presence of P-glycoprotein, in both tumors that were untreated (control) as well as drug treated. Given some recent observations in our lab that MCF7 tumor cells exposed to hypoxic conditions rapidly developed expression of MDR marker proteins such as P-glycoprotein, it is possible that this P-glycoprotein expression in the drug-sensitive cells is in response to hypoxic conditions in the tumor core. Although, it is equally possible that after initial exposure to the drugs in the different treatment groups, the expression of P-glycoprotein was initiated in some of the cells in the MCF7 tumors. In any case, visually there does seem to be a greater degree of cells in each drug sensitive tumor tissue section that are stained blue, but lack the brown stain indicative of P-glycoprotein expression, whereas in the MDR tumor tissues all the sections are for the majority stained brown. However, any qualitative assessment of P-glycoprotein level and/or dispersion throughout the tumor mass in either the drug sensitive or MDR tumor types has not been performed.

More interestingly, though, is the expression profile of GCS. Figure 39 depicts that in the MCF7 TR MDR tumor tissues there is an abundance of GCS expression, as determined by the presence of brown staining. Although, unlike P-glycoprotein staining, which was overwhelmingly present in nearly all the MDR tumor sections, GCS staining is abundant only in a little over half of the sections depicted. Other MDR sections show only very little GCS staining or none at all. Perhaps the expression of GCS was diminished over time in these sections. However, what is of interest in Figure 39 is the complete lack of GCS staining in any of the drug-sensitive MCF7 tumor tissues. While it appears that P-glycoprotein expression can even be turned on in response to drug
Figure 39 – Expression of the MDR marker protein glucosylceramide synthase (GCS) by immunohistochemistry in tumor sections of wild-type (drug sensitive; DS) and MDR tumor bearing mice 28 days following treatment with control (untreated), paclitaxel (PTX), paclitaxel and ceramide (PTX + CER), or 70% PGLA:30% PbAE nanoparticles containing PTX and CER (PTX + CER NP). Representative tumor sections are shown from 2 mice of each group. Brown staining indicates presence of GCS. All tumor sections were subsequently counterstained with hematoxylin to stain the tissue blue. (magnification 100x)
treatment or environment, GCS expression appears to lack this short term inducibility, thereby seemingly only tied to established conditions of MDR. In any case, from these results it appears clear that MCF\textsubscript{TR} cells used to establish the MDR tumor xenografts have retained their MDR phenotype, while the wild-type (drug sensitive) MCF7 cells appear to develop the potential for the drug resistant phenotype over time and/or following treatment.

Since the tumors were xenografted with the aide of a Matrigel\textsuperscript{®} basement membrane plug, it was important to determine whether residual Matrigel\textsuperscript{®} caused any deviations in the makeup of tumors, particularly in the composition of tumor cell vs. tumor stroma, which could influence tumor volume inaccurately. Thus to detect the presence of tumor stroma, the tumor sections harvested at day 28 were stained for the presence of mouse collagen IV, an abundant factor that makes up the Matrigel\textsuperscript{®} composition as well as naturally occurring tumor stroma. For this purpose an antibody was used against mouse collagen IV that had no cross-reactivity with human collagen IV, to avoid the occurrence of false positives through the potential presence of human collagen IV from the tumor cells. As a control measure, cultured MCF7 cells were additionally stained for collagen IV to verify that mouse collagen IV is indeed not naturally present in the cells that were used for tumor formation (Figure 40). As a result, sections of the tumor where mouse collagen IV is in fact present stain brown, whereas unstained sections of the tumor were stained blue by a hematoxylin counterstain. Representative tumor images presented in Figure 40 indicate that at most faint traces of brown staining, indicating the presence of collagen IV, can be observed in the various tumor sections, and that the tumor sections for the most part display the blue color of the
Figure 40 – Expression of collagen IV by immunohistochemistry in tumor sections of wild-type (drug sensitive; DS) (top row) and MDR (bottom row) tumor bearing mice 28 days following treatment. Representative, randomly chosen, tumor sections are shown from 3 mice of each tumor type, alongside cultured cells (far left). Brown staining indicates presence of Collagen IV. All tumor sections were subsequently counterstained with hematoxylin to stain the tissue blue. (magnification 40x)
hematoxylin counterstain. This suggests that, if any, merely faint traces of residual Matrigel®, or natural tumor stroma, were present in the tumors at time of sacrifice, which is not likely to measure into tumor volume or final tumor weight to discredit efficacy results.

A key measure in the development of any new therapeutic is a safety and toxicity evaluation. To evaluate safety and thereby any potential toxicity of the PTX + CER polymer blend nanoparticle therapy, a measure of body weight changes, white blood cell count, and serum liver enzyme activity over time following treatment initiation was taken. Figure 41 demonstrates that neither of the treatment groups causes a significant drop in bodyweight up to four weeks following treatment initiation in either tumor model. It is observed that the Cremophore EL®-based free drug formulation causes slight decrease in body weight around week 3 and 4 in MCF7TR tumor bearing mice (Figure 41b) treated with PTX + CER or PTX administered as free drug respectively, however, this result is neither significant from control bodyweight, nor is it observed in mice bearing drug-sensitive MCF7 tumors.

Similarly, Figure 42 demonstrates that there is also a lack of significant changes in white blood cell count following any of the treatment groups over time. While in the MCF7 tumor model (Figure 42a) PTX, PTX + CER, and blank nanoparticles show an increase in white blood cell count, the increase in either of these groups does not change significantly from basal counts, and is not significantly different from the untreated control. Counteracting this though, in the MCF7TR tumor model (Figure 42b), neither PTX, PTX + CER, nor the blank nanoparticles show a trend towards elevation of white blood cell count, however, in this model now the PTX nanoparticle treatment shows a
Figure 41 – Average bodyweight changes (% change from basal) up to four weeks following a single dose IV administration of 20 mg/kg paclitaxel (PTX) and/or 80 mg/kg ceramide (CER) as free drug or in polymer-blend nanoparticles (NP) in mice bearing a) drug-sensitive MCF7 and b) MDR MCF7TR tumors.
Figure 42 – Average white blood cell (WBC) count at basal, and at two and four weeks following a single dose IV administration of 20 mg/kg paclitaxel (PTX) and/or 80 mg/kg ceramide (CER) in as free drug or in polymer-blend nanoparticles (NP) in mice bearing a) drug-sensitive MCF7 and b) MDR MCF7<sub>TR</sub> tumors.
slight elevation. Again, since this result is neither significantly elevated over basal, nor significantly different from the untreated control, it can be postulated that this also is not a relevant rise.

An alternate standard method to evaluate potential toxicity resulting from systemic administration is to monitor increases in serum enzyme activity of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT). Figure 43 shows the changes in serum ALT activity for mice bearing MCF7 (Figure 43a), MCF7\textsubscript{TR} (Figure 43b), and SKOV3\textsubscript{TR} (Figure 43c) tumors. Similarly Figure 44 shows the changes in serum LDH activity for mice bearing MCF7 (Figure 44a), MCF7\textsubscript{TR} (Figure 44b), and SKOV3\textsubscript{TR} (Figure 44c) tumors. While the results again indicate that there is no overall toxicity of the nanoparticles as measured by serum enzyme levels, isolated incidents whereby activity increases following treatment do occur. For example, in mice bearing MCF7 tumors, treatment with CER nanoparticles (Figure 44a) raises serum LDH activity from 186 U/L on day 1 to 338 U/L by day 28. Along with this result, CER nanoparticle administration raises serum ALT activity in mice bearing drug sensitive tumors from 5 U/L on day 1 to 8 U/L and 12 U/L by day 14 and 28 respectively. Thus, this appears to show that CER alone in nanoparticles does cause slight toxicity in terms of serum enzyme activity. However, since the CER nanoparticle treatment on MCF7\textsubscript{TR} tumor bearing mice does not cause any elevations of serum ALT (Figure 43b) or serum LDH (Figure 44b), it is not sure whether this slight toxicity result is factual or simply present in mice bearing the drug sensitive tumor type, albeit that the latter is a highly unlikely scenario. More importantly, no significant increases in serum LDH or ALT activity are seen in mice bearing any of the tumor types when treated with either blank nanoparticles, PTX
Figure 43 – Serum ALT levels in U/L before treatment (day 1), and day 14 and day 28 after treatment with 20 mg/kg paclitaxel (ptx) and/or 80 mg/kg ceramide (cer) administered as free drug or encapsulated in polymer-blend nanoparticles in a) MCF7, b) MCF7\textsubscript{TR}, and c) SKOV3\textsubscript{TR} tumor bearing mice.
Figure 43 – Serum LDH levels in U/L before treatment (day 1), and day 14 and day 28 after treatment with 20 mg/kg paclitaxel (ptx) and/or 80 mg/kg ceramide (cer) administered as free drug or encapsulated in polymer-blend nanoparticles in a) MCF7, b) MCF7TR, and c) SKOV3TR tumor bearing mice.
nanoparticles, and, most importantly, the experimental PTX + CER nanoparticles. Although an increase is seen in serum ALT activity (Figure 43b) at day 14 after administration of the PTX + CER nanoparticles to MCF7<sub>TR</sub> tumor bearing mice, this increase not only lacks in the MCF7 tumor bearing mice (Figure 43a) as well as the SKOV3<sub>TR</sub> tumor bearing mice (Figure 43c) from this treatment group, but the serum ALT activity in these mice returns back to basal by day 28, suggesting that this toxicity may be an artifact.

Lastly, to culminate the safety and toxicity evaluation, livers from MCF7 and MCF7<sub>TR</sub> tumor bearing mice were harvested at day 28 after treatment, cryosectioned, and subjected to hematoxylin & eosin staining to observe liver morphology. Figure 45 depicts representative images from mice bearing either MCF7 (Figure 45a) or MCF7<sub>TR</sub> (Figure 45b) breast tumors from each treatment group. Images center on the central vein with its surrounding hepatic lobule. Overall, neither of the images portrays any distinct damage to the hepatic lobule or macrophage infiltration. Together, the complete panel of toxicity data suggests that no adverse toxicity or liver damage results from systemic treatment with the PTX + CER nanoparticles, or any of the surrounding treatment groups, deeming them safe for in-vivo use.

IV. DISCUSSION

While many therapeutic strategies have been developed to modulate the cellular mechanisms whereby MDR arises (Kellen JA 2003; Szakacs G, et al. 2006), modulating the CER metabolic pathway has emerged as a promising therapeutic strategy to overcome MDR in cancer (Kolesnick R 2002). Based on these findings, a therapeutic strategy that
Figure 45 – Representative liver histology by hemotoxylin & eosin staining at day 28 after treatment initiation of mice bearing A) drug-sensitive MCF7 and B) MDR MCF7_{TR} tumors, from the following treatment groups: a,i) control, b,j) paclitaxel, c,k) ceramide, d,l) paclitaxel + ceramide, e,m) blank nanoparticles, f,n) paclitaxel nanoparticles, g,o) ceramide nanoparticles, h,p) paclitaxel + ceramide nanoparticles.
administers a combination therapy of PTX with CER, encapsulated within polymeric nanoparticles formulated for temporal controlled release of the two drugs was developed and characterized. Based on data presented in the previous chapters, it appeared that this therapy was highly efficient to overcome MDR in in-vitro models of breast and ovarian cancer. However, whether this therapy retained the potential to overcome MDR in in-vivo models of cancer, whereby drug targeting, drug metabolism and excretion, and non-target toxicity come into play. Thus to test efficacy following single dose systemic administration of the PTX + CER nanoparticle therapy, in-vivo models of drug-sensitive and MDR human breast cancer were developed by xenografting MCF7 or MCF7\textsubscript{TR} cells, respectively, into the mammary fat pad of female mice. The results demonstrate that the PTX + CER nanoparticle therapy appeared most efficacious against MDR MCF7\textsubscript{TR} tumors, since this therapy produced the greatest decrease in tumor volume over time and a significantly lower final tumor weight over other treatment groups. Although final tumor weight in this treatment group was significantly lower than the final tumor weight that resulted after treatment with PTX or CER individually, the combination therapy without nanoparticle encapsulation, or control groups (untreated control and blank nanoparticles), the lack of a significant difference in tumor volume change over time compared with other drug treatments raises the question whether this therapy is indeed efficacious to treat MDR cancer. Surprisingly, it was found that MCF7\textsubscript{TR} tumors treated with the PTX + CER nanoparticle therapy exhibited the greatest amount of apoptotic signaling, indicative that this therapy had the greatest potential to overcome MDR. This result is in agreement with the in-vitro results presented in chapter 2 and 3 whereby the PTX + CER therapy produced the greatest amount of apoptotic signaling in MDR cancer
cells, and in consequence also the greatest amount of efficacy. Since MCF7 and MCF7<sub>TR</sub> tumors, like many clinical forms of human breast cancer, are estrogen-dependent for proliferation, tumor-bearing mice were given exogenous estrogen through continuous release implants to support tumor growth. As a consequence though, many mice in the study succumbed to fatalities presumed to result from super-physiological levels of estrogen, which prevented the study from continuing past 3-4 weeks after treatment initiation. Furthermore, the data revealed that plasma estrogen levels sloughed off an average of 10 pg/mL within the first two weeks after treatment, thereby introducing the variable that tumor volume may have decreased in any of the mice resulting from a reduction in estrogen stimulation rather than as a consequence to the treatment it received. Thus, to conclusively determine efficacy of the PTX + CER nanoparticle therapy against MDR cancer, the therapy was additionally tested on mice bearing human MDR human ovarian cancer (SKOV3<sub>TR</sub>), a model that was also used in prior <i>in-vitro</i> studies presented in chapters 2 and 3. Since the SKOV<sub>TR</sub> model lacked the need for estrogen priming, it allowed for the evaluation of efficacy of the novel therapy without the inconsistencies associated with estrogen priming. This model, strikingly, revealed a drastic reduction in tumor growth over time and final tumor volume after treatment with PTX + CER in nanoparticles, compared with the results seen after PTX administration on its own and PTX + CER administration without the nanocarrier. This data supports that effectiveness of the PTX + CER nanoparticle therapy on MDR ovarian cancer, even though it remains inconclusive whether this therapy is indeed as effective on MDR breast cancer.

While prior <i>in-vitro</i> studies in chapter 2 revealed the ability of this PTX + CER nanoparticle therapy to enhance chemosensitization of drug-sensitive cancer as well,
thereby giving this therapy an extra advantage over other MDR modulating strategies, the in-vivo efficacy results refute this idea. Testing efficacy of the PTX + CER nanoparticle therapy on drug-sensitive MCF7 tumor bearing mice revealed that this therapy was no more efficacious than PTX or CER administered alone either as free drug or encapsulated within nanoparticles. While the doses of PTX and CER administered individually were not effective against either model of MDR cancer, it was expected that a 20 mg/kg dose of PTX and an 80 mg/kg dose of CER administered individually would in fact be effective to cause tumor growth delay in drug sensitive MCF7 tumors, since these doses closely approach clinical doses used for tumor eradication. Given the potency of the individual drugs against MCF7 tumors, it is possible that any possible enhancement of efficacy by the combination therapy did not stand out in this experiment. Any enhancement of efficacy by the PTX + CER nanoparticle therapy on drug-sensitive tumors would more likely exhibit itself at lower doses of PTX whereby the drugs individually would lack effectiveness.

Little et al. (2004) have reported slight cytotoxicity associated from PbAE administration to cells, thus to monitor any adverse toxicity from PbAE that is incorporated into the nanoparticle structure, blank nanoparticles were administered IV to both MCF7 and MCF7_{TR} tumor bearing mice. Curiously, while there was no toxicity associated with the blank nanoparticle treatment, or any of the other treatment groups including the PTX + CER nanoparticles, throughout the study, the blank nanoparticles caused a significant decrease in tumor growth of the MCF7 tumors compared to the untreated control group. While it is possible that upon degradation of the particle PbAE may have exerted some cytotoxicity to MCF7 tumor cells thereby inhibiting tumor
growth slightly, it can also be speculated that the nanoparticles itself could aggregate within the microvasculature to produce embolisms and thereby cut off the blood supply to the tumors. It is unknown at this point whether either scenario occurs and how this relates to overall efficacy of the PTX + CER nanoparticle treatment. However, since blank nanoparticles did not significantly affect tumor growth of the MCF7\textsubscript{TR} tumors, it is also possible that this result is merely an experimental artifact. It is unlikely though that the tumor growth delay associated with blank nanoparticles is resultant from a lack of estrogen stimulation to the tumors, since the growth delay manifested itself during the first two weeks after treatment initiation when plasma estrogen levels had not yet decreased.

Upon closer examination of the tumor response to drug treatment, it appears that a single dose administration of the PTX + CER nanoparticle therapy causes an unusually long suppression of tumor growth of particularly the MDR tumors, whereby tumor volume remains suppressed around its original volume up until 18 days following the single dose treatment in the SKOV3\textsubscript{TR} tumor model, and tumor volume actually decreases to 65% of its original volume in the MCF7\textsubscript{TR} tumor model 25 days following the single dose treatment. While chapter 6 will examine tumor drug retention more closely for this PTX + CER nanoparticle therapy, it is unlikely that is single dose treatment remains in the tumor or circulation to act for up to three weeks. However other studies conducted by our group showed very similar results. For example, a single 20mg/kg dose of paclitaxel in PbAE nanoparticles administered IV to SKOV3 tumor bearing mice suppressed tumor growth as profoundly, whereby 18 days after treatment initiation, tumors had increased a mere 20 mm\textsuperscript{3} in volume (Devalapally H, et al. 2006),
even though concentrations of PTX in the tumor were decreasing by 5 hours after 
adadministration (Shenoy D, et al. 2005b). Even though the drugs likely only retain in the body for minimal duration compared with the 4 week therapeutic monitoring period, the initial effect that the various treatment groups had on the tumor can determine their subsequent rate of growth for the weeks following this single treatment. However, the resulting rate of tumor growth following treatment can also be influenced by cell passage number, whereby cells that have been passaged many times can proliferate at a slower rate than young cells (Steel, et al. 1983). Although this effect does not influence the results between treatment groups in either tumor model of this study, since tumors were all developed from the same batch of cells, it can explain why tumor growth delay following a single dose drug treatment appears more profound in this study than it does in studies conducted by other groups. For example, a parallel study conducted in our group that tested efficacy of PTX + CER in PCL nanoparticles, found that a single dose administration of this therapy suppressed tumor growth of SKOV3 tumors for only approximately 14 days and SKOV3<sub>TR</sub> tumors for only approximately 9 days following dose administration (20 mg/kg PTX + 100 mg/kg CER), after which tumor growth resurged (Devalapally, et al. 2007). Even though the same SKOV3<sub>TR</sub> cells were used for that study as for the study presented in this work, senescence of the cells could have contributed to a slower tumor proliferation following treatment, which may give the illusion that the PTX + CER treatment has a more profound therapeutic efficacy than it in reality does. Likewise, the same effects that could have influenced tumor growth delay following a single dose treatment in the SKOV3<sub>TR</sub> model, could have also influenced that of the MCF7 and MCF7<sub>TR</sub> tumors, although the additional effects of a drop in plasma
estrogen around two weeks after treatment could have also contributed to excessively delayed tumor growth in the treatment groups.

While there are many therapeutic strategies, not only in development but also in clinical trials, aimed to modulate the various cellular mechanisms that give rise to MDR in cancer, few utilize the approach of combining the facets of nanoparticle drug delivery systems with MDR modulating therapies as presented in this work. This uniqueness gives the PTX + CER nanoparticle therapy a competitive edge over other MDR modulating therapies, although it is difficult to predict whether this therapy is more advantageous than the myriad of other MDR therapies unless they tested side by side on in-vivo models of MDR cancer. Nevertheless, the results presented here support the efficacy of this PTX + CER nanoparticle therapy on MDR breast and ovarian cancer, while the lack of toxicity makes it a suitable candidate for continued clinical evaluation. It is of interest to determine further whether this therapy has a similar therapeutic potential to other tumor types that present with the MDR, however, given the prevalence of MDR in breast and ovarian cancer, these results weigh heavily towards clinical potential clinical for this therapy.
CHAPTER 6: EVALUATION OF THE BIODISTRIBUTION AND PHARMACOKINETIC PROFILES OF PACLITAXEL AND CERAMIDE ADMINISTERED IN POLYMER-BLEND NANOPARTICLE IN-VIVO IN TUMOR-BEARING ANIMALS

I. INTRODUCTION

In the previous chapter (Chapter 5), the efficacy of the 70% PLGA: 30% PbAE nanoparticles were examined on in-vivo models of both breast and ovarian MDR cancer. It was concluded from these studies that the PTX + CER combination therapy encapsulated within these polymer-blend nanoparticles indeed effectively overcomes MDR to result in the greatest degree of tumor growth regression. Now, to examine the effectiveness of these nanoparticles to improve tumor accumulation and retention and plasma retention of the PTX + CER drug load, as a means towards enhanced efficacy, mice bearing drug-sensitive and MDR human breast tumor xenografts received radiolabeled tracers of PTX and CER to quantitatively measure pharmacokinetic profiles of the combination therapy administered within polymer blend nanoparticles as compared to the aqueous solution formulations. Furthermore, the effect of nanoparticle delivery of tissue biodistribution of the two drugs was also examined.

II. MATERIALS AND METHODS

1. Nanoparticle and free drug preparation

Polymer-blend nanoparticles were manufactured as described in chapter 4 at a ratio of 70%:30%, respectively, loaded with 2.5% (w/w) PTX and/or 10% (w/w) CER. In addition, the nanoparticles were co-loaded with 20 μCi $^3$H-PTX per mg of regular PTX
and 2.5 μCi $^{14}$C-CER per mg of regular CER. Furthermore 20 μg of rhodamine-PTX per mg of regular PTX was also co-loaded into the nanoparticles. Alongside, free drug formulations using chremophore-EL and ethanol were prepared as described in chapter 5, with the similar addition of 20 μCi $^{3}$H-PTX per mg of regular PTX, 2.5 μCi $^{14}$C-CER per mg of regular CER, and 20 μg of rhodamine-PTX per mg of regular PTX. Thus, hereby, for each dose administered, animals received radioactive tracers of 1 μCi $^{3}$H-PTX and 0.5 μCi $^{14}$C-CER, and a fluorescent tracer of 1 μg rhodamine-PTX.

2. Drug biodistribution and pharmacokinetic analyses

Silastic implants were surgically inserted to mice followed by the inoculation of MCF7 or MCF7<sub>TR</sub> tumors as described in the methods in chapter 5. Once tumors reached a palpable size of 100 mm$^3$ mice were randomly assigned to treatment with either PTX + CER in polymer blend nanoparticles or PTX + CER as free drug, subdivided into groups of four mice per timepoint for a total of four timepoints (30 min, 1 hour, 6 hours, 12 hours) per treatment group per tumor type. Mice received a single dose IV injection of nanoparticle or free drugs at 1 μCi PTX, and 0.5 μCi CER per mouse diluted in sterile saline at an injection volume of 500 μl. At the conclusion of the biodistribution time points, mice were euthanized by carbon dioxide inhalation. Blood was collected by cardiac puncture, after which in-vivo drug distribution was rapidly imaged by tracking the fluorescent output of rhodamine-PTX using a 565/690 filter set on a Kodak In Vivo Imaging Station FX. Following this, tumor, liver, spleen, kidney, lung, and heart were harvested and collected into pre-weighed test-tubes. 10% (w/v) tissue homogenate was prepared in dI water, and 100 μl of tissue homogenate or blood was dispensed into
scintillation vials. Each sample was then digested in 1 mL ScintiGest Tissue solubilizer (Thermo Fisher Scientific, Waltham, MA) at 55°C for 2 hours, followed by the addition of 200 µl 30% (v/v) hydrogen peroxide (Thermo Fisher Scientific, Waltham, MA). This prep was further incubated at 55°C for 30 min, after which each sample received 10 mL ScintiVerse scintillation fluid cocktail (Thermo Fisher Scientific, Waltham, MA). The samples were further left to quench in the dark for at least 2 hours, after which counts of $^3$H and $^{14}$C were measured on an α/β scintillation counter. Scintillation counting for the free drug and nanoparticle doses administered were performed simultaneously to minimize variability. The results were expressed as % of dose/g of tissue or % of dose/mL of blood. Tumor and blood pharmacokinetic results were analyzed according to a non-compartmental pharmacokinetic model to determine mean retention time (MRT), half-life (T½), volume of distribution (Vd), total body clearance (Cl), elimination rate constant (K), and area under the curve (AUC) for PTX and CER in tumor and blood.

3. Statistical Analysis

For drug biodistribution and pharmacokinetic profiling studies, n=4 mice/group/time point. Statistical analysis was performed by two tailed, equal variance student t-test. Statistical significance was accepted at p<0.05.

III. RESULTS

To achieve a direct comparison between PTX and CER drug biodistribution and pharmacokinetics when administered as a conventional Cremophore EL®-ethanol formulation versus encapsulation within the 70% PLGA/ 30% PbAE polymer-blend nanoparticles, both preparations were spiked with radio-labeled derivatives of PTX and
CER. Both $^3$H-PTX and $^{14}$C-CER were encapsulated within the nanoparticle and free drug formulations as described in the methods and materials in such a way that each animal received a tracer of 1 $\mu$Ci PTX and 0.5 $\mu$Ci CER of which the counts could be simultaneously read on an $\alpha/\beta$- scintillation counter. Furthermore, to qualitatively assess drug biodistribution, and particularly tumor localization, resulting from the dose formulation, both the free drug and nanoparticle forms of drug were additionally spiked with a red-fluorescent derivative of PTX (rhodamine-PTX), which could be visualized in-vivo using an in-vivo imaging module.

Qualitative data was obtained by standardizing $^3$H and $^{14}$C cpm from individual tissues or blood by the counts-per-minute (cpm) for the dose administered per g of tissue or mL of blood, to express tissue and blood drug levels over time as % of dose/g or mL. Figure 46 depicts the blood (Figure 46a) and tumor (Figure 46b) pharmacokinetic curves for both PTX and CER resulting from administration as free drug vs. nanoparticle delivery in mice bearing MCF7 tumors. As expected, the data shows that nanoparticle delivery prolongs plasma retention of PTX over free drug administration, indicated by a 2-fold increase in PTX from nanoparticle delivery over free drug (1.02 ± 0.25 vs. 0.46 ± 0.08 % dose/mL respectively, p<0.05). Similarly, tumor retention of PTX increases significantly with nanoparticle delivery of the drug, whereby at 12 hours out 6.5-fold more PTX resides in the tumor mass compared with the free drug administration (0.53 ± 0.08 vs. 0.08 ± 0.02 %/g of nanoparticle vs. free PTX, p<0.001). What is more striking is that while nanoparticles did not affect CER retention in the blood significantly, the nanoparticles cause significant CER accumulation at the tumor site, initially a 2-fold increase early on at 1 and 6 hours, elevating to a 4.5-fold increase by 12 hours.
Figure 46 – a) Blood and b) tumor pharmacokinetic profile of paclitaxel (PTX) and ceramide (CER) administered as free drug compared with polymer-blend nanoparticle (NP) administration over time in MCF7 tumor bearing mice. * and ** indicates statistical significance (p<0.05 and p<0.001, respectively) between NP and free drug treatment of each drug (n= 4 samples/group)
Figure 47 – Qualitative tissue biodistribution profile of rhodamine-PTX administered as free drug vs. nanoparticles (NP) at 1, 6, and 12 hours after administration in MCF7 tumor bearing mice. Arrow indicates the tumor site as located on photographic counter images.
Additionally, the maximal concentration of drug at the tumor site within this therapeutic window reaches a significantly higher peak with nanoparticle delivery over the free drug formulation (2.6 ± 0.4 vs. 1.42 ± 0.5); a phenomenon that perhaps explains the efficacy seen with CER treatment alone. Qualitative biodistribution images that traced red-fluorescent PTX (Figure 47) similarly revealed that while drug accumulation can be observed in tumors of mice subjected to free drug treatment 6 hours out, this signal is lost by 12 hours. However, the mice subjected to nanoparticle treatment retain their tumor accumulation signal beyond 6 hours to retain visibility even at 12 hours out. This result further suggests that the nanoparticles retain for a prolong period of time in the tumor mass. Similar results were seen in the blood and tumor pharmacokinetic profiles of PTX and CER when administered to MCF7<sub>TR</sub> tumor bearing mice (Figure 48). While nanoparticle delivery had no effect on blood retention of CER over free drug administration, PTX retention in the blood resulting from nanoparticle delivery elevated significantly 2- to 2.5-fold over that of free drug, for example to reach levels of 1.08 ± 0.16 vs. 0.43 ± 0.11 %/mL at 12 hours from nanoparticle vs. free drug administration respectively. And similar to the trend seen in the drug sensitive MCF7 tumor bearing mice, nanoparticles elevated not only PTX accumulation in the tumor mass 2-fold (0.15 ± 0.01 vs. 0.07 ± 0.00 at 12 hours, p<0.001), but particularly CER accumulation and maximal concentration (1.7 ± 0.13 vs. 0.75 ± 0.1 at 6 hours, p<0.001). Figure 49 depicts qualitative biodistribution images for the MCF7<sub>TR</sub> tumor bearing mice that once more revealed that while drug accumulation can be observed in tumors of mice administered free drug 6 hours out, this signal is lost by 12 hours, while the mice subjected to nanoparticle treatment retain their tumor accumulation signal at both 6 and 12 hours out.
Figure 48 – a) Blood and b) tumor pharmacokinetic profile of paclitaxel (PTX) and ceramide (CER) administered as free drug compared with polymer-blend nanoparticle (NP) administration over time in MCF7_{TR} tumor bearing mice. ** indicates statistical significance (p<0.001) between NP and free drug treatment of each drug (n= 4 samples/group)
Figure 49– Qualitative tissue biodistribution profile of rhodamine-PTX administered as free drug vs. nanoparticles (NP) at 1, 6, and 12 hours after administration in MCF7TR tumor bearing mice. Arrow indicates the tumor site as located on photographic counter images.
Analysis of tumor and blood pharmacokinetic data by non-compartmental pharmacokinetics revealed how nanoparticle drug delivery altered key pharmacokinetic parameters for both PTX and CER. **Table 4** and **Table 5** summarize the pharmacokinetic parameters of PTX and CER from free drug administration and nanoparticle delivery in blood (plasma) and tumor respectively. As suggested by the plots in **Figure 46** and **Figure 48**, nanoparticle delivery increased area-under-the-curve (AUC) in blood, for PTX but not for CER (Table 4). While mean retention time (MRT), the elimination constant (K), half-life (T½) and volume of distribution (Vd) appeared unchanged for either drug between free drug administration and nanoparticle delivery in the blood, nanoparticles did significantly lower the rate of total body clearance (Cl) for PTX, albeit not for CER, thereby supporting the idea that nanoparticles promote prolonged PTX retention in the blood. Oppositely, though, nanoparticles promoted a significantly increased AUC and decreased Cl for CER in the tumor tissue (Table 5), although at this site pharmacokinetics of PTX remained unchanged between nanoparticle vs. free drug delivery. Interestingly though, it can be concluded from this study that nanoparticle delivery does benefit both drugs, by increasing PTX levels and prolonging PTX retention in the circulation, and by increasing CER retention and decreasing CER clearance from the tumor site.

Lastly, to determine the effects of nanoparticle delivery of PTX and CER compared with administration of free PTX and CER on non-target distribution, drug distribution over time was monitored in liver, kidney, spleen, lung, and heart, as illustrated in **Figure 50** for MCF7 tumor bearing mice, and **Figure 51** for MCF7TR tumor bearing mice. From this data, it appears that while nanoparticle delivery does not significantly alter peak
Table 4 – Summary of plasma non-compartmental pharmacokinetic parameters of paclitaxel (PTX) and ceramide (CER) resulting from administration as free drug vs. delivery in polymer-blend nanoparticles. AUC = area under the curve; MRT = mean residence time; K = elimination rate constant; T½ = half-life; Cl = total body clearance; Vd = volume of distribution.

* and ** indicates statistical significance (p<0.05 and p<0.001, respectively) between free drug and nanoparticle dosage forms for each drug at the give pharmacokinetic parameter (n=3-4 repeats/group)

<table>
<thead>
<tr>
<th>Pharmacokinetic parameter</th>
<th>PTX</th>
<th>PTX nanoparticle</th>
<th>CER</th>
<th>CER nanoparticle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCF7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (%dose·h/mL)</td>
<td>6.9 ± 1.7</td>
<td>10.5 ± 2.1</td>
<td>2.5 ± 0.8</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>23.9 ± 10.7</td>
<td>25.1 ± 9.3</td>
<td>347.7 ± 107.5</td>
<td>1470 ± 483</td>
</tr>
<tr>
<td>K (h⁻¹)</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.003 ± 0.001</td>
<td>0.0009 ± 0.0006</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>16.6 ± 7.4</td>
<td>17.4 ± 6.4</td>
<td>240.9 ± 75.5</td>
<td>1019 ± 335.1</td>
</tr>
<tr>
<td>Clᵣ (% dose·h/(% dose/mL))</td>
<td>0.009 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>0.12 ± 0.04</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Vₐ (% dose/(% dose/mL))</td>
<td>0.22 ± 0.10</td>
<td>0.14 ± 0.07</td>
<td>36.6 ± 20.7</td>
<td>130.4 ± 48.8</td>
</tr>
</tbody>
</table>

| **MCF7t¹**:               |         |                  |         |                  |
| AUC (%dose·h/mL)          | 5.2 ± 0.9 | 10.2 ± 0.9 *    | 2.27 ± 0.3 | 2.9 ± 0.1        |
| MRT (h)                   | 115.4 ± 43.3 | 54.0 ± 19.6   | 166.7 ± 90.1 | 182.5 ± 25.1    |
| K (h⁻¹)                   | 0.007 ± 0.004 | 0.020 ± 0.007 | 0.007 ± 0.005 | 0.004 ± 0.001   |
| T½ (h)                    | 80.0 ± 30.0  | 37.4 ± 13.6    | 115.5 ± 62.5  | 126.5 ± 17.4    |
| Clᵣ (% dose·h/(% dose/mL))| 0.010 ± 0.001 | 0.005 ± 0.000 *| 0.09 ± 0.01   | 0.07 ± 0.00     |
| Vₐ (% dose/(% dose/mL))  | 1.4 ± 0.6   | 0.25 ± 0.06    | 17.8 ± 11.2    | 12.8 ± 1.8      |
Table 5 – Summary of tumor non-compartmental pharmacokinetic parameters of paclitaxel (PTX) and ceramide (CER) resulting from administration as free drug vs. delivery in polymer-blend nanoparticles. AUC = area under the curve; MRT = mean residence time; K = elimination rate constant; T½ = half-life; Cl\text{t} = total body clearance; V\text{d} = volume of distribution.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameter</th>
<th>PTX</th>
<th>PTX nanoparticle</th>
<th>CER</th>
<th>CER nanoparticle</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (%dose·h/g)</td>
<td>4.9 ± 2.3</td>
<td>6.0 ± 1.0</td>
<td>9.0 ± 0.9</td>
<td>22.5 ± 0.9**</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>114.7 ± 53.0</td>
<td>308.4 ± 119.0</td>
<td>39.0 ± 13.8</td>
<td>43.9 ± 6.7</td>
</tr>
<tr>
<td>K (h⁻¹)</td>
<td>0.011 ± 0.005</td>
<td>0.005 ± 0.002</td>
<td>0.04 ± 0.02</td>
<td>0.024 ± 0.004</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>79.5 ± 36.7</td>
<td>213.7 ± 82.5</td>
<td>27.0 ± 9.5</td>
<td>30.4 ± 4.7</td>
</tr>
<tr>
<td>Cl\text{t} (% dose·h/(% dose/g))</td>
<td>0.017 ± 0.006</td>
<td>0.009 ± 0.001</td>
<td>0.023 ± 0.003</td>
<td>0.008 ± 0.000*</td>
</tr>
<tr>
<td>V\text{d} (% dose/(% dose/g))</td>
<td>3.7 ± 1.6</td>
<td>2.9 ± 1.2</td>
<td>0.9 ± 0.3</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>MCF7R:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (%dose·h/g)</td>
<td>2.9 ± 0.6</td>
<td>3.2 ± 0.5</td>
<td>6.47 ± 0.5</td>
<td>11.0 ± 0.9**</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>81.7 ± 67.9</td>
<td>153.0 ± 117.8</td>
<td>5.9 ± 0.4</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>K (h⁻¹)</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.15 ± 0.00</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>56.6 ± 47.0</td>
<td>106.0 ± 81.6</td>
<td>4.1 ± 0.2</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>Cl\text{t} (% dose·h/(% dose/g))</td>
<td>0.019 ± 0.004</td>
<td>0.02 ± 0.002</td>
<td>0.031 ± 0.002</td>
<td>0.018 ± 0.001**</td>
</tr>
<tr>
<td>V\text{d} (% dose/(% dose/g))</td>
<td>2.19 ± 2</td>
<td>3.1 ± 2.5</td>
<td>0.19 ± 0.03</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

** indicates statistical significance (p<0.001) between free drug and nanoparticle dosage forms for each drug at the give pharmacokinetic parameter (n=3-4 repeats/group)
concentrations of PTX or CER that accumulate the non-target organs examined, nanoparticle delivery does appear to cause slower clearance of either drug from these non-target organs. While at 6 hours after administration, similar amounts of PTX and CER reside in the liver, kidney, spleen, lung and heart of mice bearing either drug sensitive or MDR tumor types, by 12 hours after administration, traces of both PTX and CER are nearly undetectable in the liver, kidney, spleen, lung and heart of these mice, suggesting clearance of the majority of the drug load. However, nanoparticle delivery of both PTX and CER retains to a significantly higher level in these tissue types by 12 hours after administration. Curiously, though, this effect is only observed in mice bearing the MCF7 tumor type (Figure 52), while no differences exist between nanoparticle and free drug retention at 12 hours for the MCF7_{TR} tumor bearing mice.

IV. DISCUSSION

It is widely know that nanoparticles are beneficial tumor targeting vehicles due to their passive targeting properties by the enhanced permeability and retention (EPR) effect, whereby the added advantage of stealth-shielding the particles with a poly(ethylene glycol/oxide) surface modification avoids uptakes reticulo-endothelial system, thereby improving circulation time of the nanoparticles (Gref, et al. 1994). The nanoparticle designed in this application was composed of a blend of poly(lactic co-glycolic acid) (PLGA) and poly(beta amino-ester) (PbAE) for temporal controlled release of PTX with CER to treat MDR cancer, whereby the nanoparticle was surfaced modified with poly(ethylene oxide) (PEO) to promote stealth-shielding and prolonged circulation of the combination drug therapy. Using radiolabeled derivatives of PTX and CER, namely \(^3\)H-PTX and \(^{14}\)C-CER, it was possible to quantitatively track drug distribution over time in-
Figure 50– Quantitative tissue biodistribution profile of a) paclitaxel and b) ceramide administered as free drug compared with encapsulation within polymer blend nanoparticles (NP) over at 30 min, 1 hour, 6 hours, and 12 hours following IV administration in MCF7 tumor bearing mice.
Figure 51– Quantitative tissue biodistribution profile of a) paclitaxel and b) ceramide administered as free drug compared with encapsulation within polymer blend nanoparticles (NP) over at 30 min, 1 hour, 6 hours, and 12 hours following IV administration in MCF7TR tumor bearing mice.
vivo resulting from administration as free drug vs. encapsulation within the polymer-blend nanoparticles.

Drug distribution was monitored in mice bearing either drug sensitive MCF7 or MDR MCF7_{TR} tumors, since differences between physiology of these two tumor types could potentially lead to differences in drug disposition and retention at the tumor site specifically. Surprisingly there was no difference in magnitude between the tumor accumulation at 6 hours after administration of free PTX, nanoparticle PTX, free CER, or nanoparticle CER between the drug-sensitive or MDR tumors. Beyond 6 hours, levels of PTX and CER delivered by nanoparticles remained unchanged in the MCF7 tumors, while levels of PTX and CER delivered as free drug began to decrease. Overall, the amount of tumor accumulation of both drugs administered in nanoparticles significantly exceeded that of free drug administration at all or some timepoints in these MCF7 tumors. This finding supports the hypothesis of enhanced tumor accumulation and retention of nanoparticle-delivered drugs by the EPR effect. However, the effects seen in the MDR MCF7_{TR} tumors were very different. While the magnitude of drug accumulation between the two tumor types was no different, intratumoral levels of PTX and CER from both free drug and nanoparticle administration tumors dropped drastically in the MCF7_{TR} after 6 hours to nearly entirely disappear from the tumor by 12 hours after administration, whereas drug levels remained steady during this period in the MCF7 tumors. This finding can suggest that drug efflux pumps like P-glycoprotein mediate removal of both PTX as well as CER from the MDR tumor after 6 hours, although since only free drug is susceptible to efflux this does indicate that the drugs must have been released from the nanoparticles by this timepoint. While the amount of nanoparticle-delivered CER that
reached the MCF7\textsubscript{TR} tumors exceeded the amount of CER administered as free drug by 2-fold, similar to the results seen in the MCF7 tumors, this increase was not seen in between PTX delivered as free drug and as nanoparticles. However, this result is in accordance with the prediction that the bulk of PTX releases immediately from this nanoparticle upon internalization into the tumor environment from a drop in pH, as established in chapter 4. Unfortunately, the presence of P-glycoprotein efflux pumps appears to quickly remove the released PTX from the tumor site. Nevertheless, the results still support the increased tumor accumulation of nanoparticle delivered drugs by the EPR effect in the MCF7\textsubscript{TR} tumors as well, although, retention of the drugs in the tumor site is hindered by the P-glycoprotein mediated efflux.

Blood pharmacokinetics of CER administered as free drug or nanoparticles was very similar in magnitude and profile between MCF7 and MCF7\textsubscript{TR} tumor bearing mice. Interestingly, though, in both animal models plasma CER does not display a profile characteristic of instantaneous input. Rather, CER blood pharmacokinetics displays profile that is more representative of oral absorption. Although an explanation for free CER to display this behavior would be that hydrophobicity of CER causes the drug to linger at the injection site, this does not apply to CER encapsulated in nanoparticles, which displays the same trend. It is unlikely that CER is prematurely released from the nanoparticle to linger at the injection site, since tumor pharmacokinetic results imply that CER is retained in the nanoparticle by 6 hours after administration. Since tumor and non-specific target accumulation of CER follows as similar profile as PTX, with peak accumulation at 1 hour after administration (with the exception of MCF7\textsubscript{TR} tumors, were peak accumulation occurred at 6 hours), it is again unlikely that peak CER plasma levels
take place prior to 30 minutes after administration. Unfortunately, the pharmacokinetics of CER and other sphingolipids following IV administration have not been evaluated to date by other groups for comparison, to derive conclusive findings regarding plasma CER pharmacokinetics. Thus, at this point it can merely be speculated that CER perhaps resides on the surface of the nanoparticle rather than internally to behave more like free CER in the circulation. This could cause CER of either formulation to linger at the injection site or sequester in more lipophilic compartments.

Blood pharmacokinetics of PTX on the other hand, behaved similar to the pharmacokinetic profile obtained in other studies, and displayed a profile characteristic of instantaneous input whereby peak concentration occurred at the time of injection, with a subsequent disappearance of PTX from the plasma over time. In both the MCF7 and MCF7\textsubscript{TR} tumor bearing mice, PTX administration as free drug peaked upon administration, with a gradual decrease over time to culminate at nearly undetectable levels by 12 hours after administration. Nanoparticle administration, on the other hand, caused a prolonged retention of PTX in the circulation of both MCF7 and MCF7\textsubscript{TR} tumor-bearing mice seen by the increased amount of drug present in the circulation at 6 hours after administration, compared with the amount of free drug present at this timepoint. Although it appears that PTX retention is more prominent in the MCF7\textsubscript{TR} tumor bearing mice over MCF7 tumor bearing mice, the magnitude of plasma drug level between these two animal models is very similar throughout all timepoints. These results are supportive of the known effects of PEO-surface modification on nanoparticles to produce prolonged circulation by stealth-shielding, and agree well with the blood and
tumor pharmacokinetic profile determined for PTX from free drug administration and nanoparticle delivery in SKOV3 tumor bearing mice (Devalapally H, et al. 2006).

Efficacy studies presented in chapter 5 demonstrate a profound effect of the PTX + CER nanoparticle therapy to suppress tumor growth for nearly four weeks following a single dose administration, an effect that is surprising given the removal of the majority of drug from the tumor site particularly in the MCF7TR tumors by 12 hours after administration. Although the latent tumor growth following treatment, as explained in chapter 5, can be attributed to various factors such as senescence of the cells and reduced plasma estrogen to limit proliferation of the tumor cells after the initial attack, further information to support the efficacy results can be deduced with this knowledge of tumor pharmacokinetics. Although in-vitro studies predicted that the PTX + CER nanoparticle therapy would be highly efficacious on MDR MCF7TR tumors, a significant enhancement of tumor growth delay over treatment with PTX alone was not conclusively seen. Given the PTX and CER pharmacokinetic behavior in MCF7TR tumors though, it can be reasoned that while the nanoparticles still appear to accumulate to a greater extent at the tumor site by the EPR effect, immediate release of PTX upon internalization in the tumor environment likely subjects PTX to efflux by P-glycoprotein, thereby quickly diminishing levels of PTX. Although CER accumulates to a substantial level in the tumor, particularly mediated by nanoparticle delivery, it may not have been sufficient to overcome MDR to the utmost extent without adequate retention of PTX in the MCF7TR tumor type. Of note though, based on this pharmacokinetic data it is found that the polymer-blend nanoparticles appear to release PTX and CER in order, as intended, at the tumor site, whereby PTX released with a surge upon internalization into the tumor.
environment followed by CER release after the nanoparticles internalized into the tumor environment at 6 hours. A similar relationship between efficacy and pharmacokinetics can be drawn for the drug-sensitive tumors as well. In this case, the elevated levels of CER over PTX at the tumor site early after administration can help explain the profound efficacy of CER alone, both as free drug and in nanoparticles, on tumor growth delay in the MCF7 tumor model. In light of these results, the significant inhibition of tumor growth resulted in SKOV3_{TR} tumors following single dose administration of the PTX + CER nanoparticle therapy can still be justified. As discussed in chapter 5, a tumor growth inhibition up to 3 weeks following single dose administration of therapy has been reported previously for SKOV3 tumors (Devalapally H, et al. 2006) regardless of a short tumor retention time. However, it is also understood that since SKOV_{TR} tumors likely present with a different physiology from MCF7_{TR} tumors, perhaps by exhibiting less P-glycoprotein activity to influence intracellular drug levels, the drug pharmacokinetic results in MCF7_{TR} tumor bearing mice are not necessarily applicable to SKOV3_{TR} tumor bearing mice.

An evaluation of non-specific target accumulation revealed that PTX administration in nanoparticles did not significantly alter non-target accumulation over that of free drug, with neither an increase nor a decrease in drug retention in the most perfused tissues. This result is identical to similar studies conducted for nanoparticle vs. free drug accumulation of PTX at non-specific sites (Zhang, et al. 2008), although other studies have reported a decreased accumulation in liver, spleen, and lung resulting from nanoparticle administration (Yang, et al. 2007) that were not seen here. On the other hand, although CER does not accumulate to any greater amount in non-target tissues
between free drug and nanoparticle administration, the nanoparticle dosage form does result in a prolonged retention of CER in all five non-target tissues examined, liver, kidney, spleen, lung, and heart, although likely not to any detrimental effects, since no toxicity was observed from any of the CER-associated treatment groups in chapter 5.
CONCLUDING REMARKS

The development of MDR in clinical cancer therapy presents a great threat to therapy and prognosis for the patient. While MDR can in principle develop in any tumor type, it is found that certain tumors types are far more prone towards the development of MDR than others. Among these, breast and ovarian cancers have been identified as prime candidates for the development of the MDR phenotype. Since MDR renders tumors poorly responsive to a variety of chemotherapeutic options, other therapeutic strategies are needed that target the cellular mechanisms that give rise to MDR in order to combat this disorder.

This work describes a novel therapeutic strategy against MDR cancer that combines a paclitaxel and ceramide combination therapy to modulate the apoptotic threshold of MDR cancer cells with the tumor targeting properties of nanoparticles. Moreover, this nanoparticle system has been designed for temporal control of release of the combination drug therapy, a platform that could be of further use with other therapeutic strategies that employ combination therapies, e.g., combination chemotherapy and anti-angiogenesis therapy.

This project has revealed that a polymeric nanoparticle intended for temporal release of a combination therapy has not only been effectively designed, but that this therapeutic system proves beneficial in the treatment of MDR breast and ovarian cancers to overcome this cancer phenotype. Although early in-vitro investigations suggested that this therapy can overcome MDR by both modulating the apoptotic threshold and drug efflux simultaneously, later in-vivo characterizations reveal that drug efflux may not be
modulated after all, due of the pH-dependent release of the therapeutics. Nevertheless, efficacy of this nanoparticle system, and lack of adverse toxicity, suggest potential clinical benefit of this therapeutic platform in the fight against MDR.
REFERENCES


