Sustained Intraperitoneal Chemotherapy via an Injectable Depot Delivery System for the Treatment of Ovarian Cancer

by

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

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Abstract

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Ovarian cancer has the highest mortality rate of all gynecological malignancies, due to inadequate treatment strategies and poor early diagnosis. Intraperitoneal (IP) chemotherapy administered on an intermittent schedule has been pursued for ovarian cancer treatment. However, local toxicities and complications associated with indwelling IP catheters required to deliver the chemotherapeutics have been documented. Furthermore, shortening or completely removing treatment-free periods between each chemotherapy cycle has shown improved efficacy compared to intermittent chemotherapy. The focus of this thesis was to develop and characterize a biocompatible and biodegradable IP injectable depot sustained drug delivery system as a new treatment strategy for ovarian cancer.

A polymer-lipid injectable formulation (PoLigel) was developed and used for sustained docetaxel (DTX) delivery. The PoLigel resulted in homogeneous DTX peritoneal distribution and sustained plasma levels in healthy mice, which was in contrast to Taxotere®, the clinically used formulation of DTX. Sustained plasma, tissue, tumor and ascites DTX concentrations were observed in mice bearing IP SKOV3 tumors or ID8 ascites over a 3 week period following IP administration of the PoLigel. The intratumoral distribution and tumor penetration of DTX in subcutaneous (SC) and IP SKOV3 tumors were characterized. DTX distributed more towards
the tumor core and diffused 1.5 fold further from blood vessels of the IP tumors compared to the SC tumors. The high efficacy observed in the IP SKOV3 and ID8 models and the SC SKOV3 model was attributed to favorable drug distribution at the whole-body, peritoneal and intratumoral levels in combination with local and systemic sustained drug exposure.

Sustained chemotherapy with DTX alone and in combination with a drug efflux transporter inhibitor was investigated in multidrug resistant (MDR) ovarian cancer. *In vitro*, combination delivery via the PoLigel resulted in more apoptosis, greater intracellular accumulation of DTX, and lower DTX efflux in MDR ovarian cancer cells. Sustained combination chemotherapy was more than twice as efficacious as intermittent Taxotere® treatment in MDR ovarian cancer. Significant anti-tumor efficacy was also observed in the MDR model following sustained DTX chemotherapy compared to intermittent Taxotere®. Overall, results presented here encourage the clinical investigation of IP sustained chemotherapy for ovarian cancer treatment.
Dedication

To Maman, Baba, Afshan, Roozbeh and Raquel who have unconditionally backed me and been the voice of reason throughout this journey.
Acknowledgments

I would like to thank my supervisor Dr. Christine Allen for her kindness, leadership, patience, and continuous support. She always believed in my abilities and pushed me to be the best I could be; for this I will always remain thankful to her. I am also grateful for being given the opportunity to have a leadership role in her laboratory. Without her mentorship this thesis would still be a distant dream.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration time curve</td>
</tr>
<tr>
<td>CRBP</td>
<td>Carboplatin</td>
</tr>
<tr>
<td>CEP</td>
<td>Cepharanthine</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
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<tr>
<td>DDS</td>
<td>Drug delivery systems / drug delivery system</td>
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<tr>
<td>DTX</td>
<td>Docetaxel</td>
</tr>
<tr>
<td>ePC</td>
<td>Egg phosphatidylcholines</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeation and retention</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Oncology</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GTMAC</td>
<td>Glycidyltrimethylammonium chloride</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance / multidrug resistant</td>
</tr>
<tr>
<td>LA</td>
<td>Lauric aldehyde</td>
</tr>
<tr>
<td>LCI</td>
<td>Lauric chloride</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>NBD-DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yI)</td>
</tr>
<tr>
<td>NDDS</td>
<td>Nano-sized drug delivery systems</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PoLigel</td>
<td>Polymer-lipid injectable gel</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>$^3$H</td>
<td>Tritium</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WSC</td>
<td>Water-soluble chitosan derivative</td>
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CHAPTER 1. Introduction


This chapter was written primarily by P. Zahedi. R. Yoganathan assisted with figures and tables.
1.1 Overview

Ovarian cancer is the fifth leading cause of cancer death in women and is associated with high morbidity and mortality rates as most patients are diagnosed with advanced stage disease [1]. In the 1970s platinum-based chemotherapy was introduced; the addition of taxanes followed in the late 80s, and more recently, the potential benefits of using intraperitoneal (IP) chemotherapy has been highlighted in several Phase III clinical trials [2]. Currently, first line therapy includes cytoreductive surgery followed by intravenous (iv) chemotherapy with carboplatin (CRBP) and paclitaxel (PTX). While most patients attain complete response following this standard treatment regimen, over 85% eventually relapse due to the emergence of multidrug resistance (MDR) [3]. As the peritoneal cavity is the site of disease both at diagnosis and relapse, IP chemotherapy has been explored. Although promising clinical results have been observed [4], the use of IP chemotherapy has remained limited due to local toxicities and complications associated with indwelling catheters. Improvements in the 5-year survival rates for women with ovarian cancer have been seen over the past few decades [5]. However, these gains have been rather modest, prompting the need to develop new treatment strategies.

Drug delivery systems (DDS) have been investigated to improve therapeutic outcomes in ovarian cancer. The primary goals have been to attain greater drug concentrations at disease sites, to bypass drug resistance and to minimize toxicities to healthy tissues. DDS can also address issues associated with anti-cancer agents such as poor aqueous solubility, low target-site specificity and rapid clearance [6], as well as circumvent problems linked to some pharmaceutical excipients used as formulation vehicles [7, 8]. The focus of this thesis was to develop and characterize a biocompatible and biodegradable IP injectable depot sustained DDS as a new therapy strategy for ovarian cancer. The following sections provide background information and outline the rationale for the studies presented in this thesis.
1.2 Ovarian Cancer

1.2.1 Disease

Ovarian cancer is malignant growth arising from different parts of the ovary. Patients typically present with non-specific symptoms at early stages, and due to the lack of effective screening methods, most are diagnosed with disseminated disease. In 2011 there were approximately 25,000 new cases of ovarian cancer in North America, resulting in about 15,000 deaths [9, 10]. Over 90% of ovarian cancers are believed to grow within the ovarian epithelium, while the remainder arise from germ or stromal cells [11]. Epithelial ovarian cancers represent the most lethal forms of the disease [11], and exhibit a variety of tumor morphologies and clinical symptoms. The common histological subtypes of epithelial ovarian cancers are serous, endometrioid, mucinous and clear cell [12]. The majority of epithelial ovarian cancers have been classified as serous, which can be further subdivided into high and low grade tumors [13]. High grade tumors are relatively chemosensitive while low grade tumors are slow growing and chemoresistant [14]. There are key differences in incidence, tumor behavior and clinical outcome between each subtype. High grade serous carcinoma tends to be aggressive disease that spreads throughout the peritoneal cavity, while endometrioid, mucinous and low grade serous cancers are commonly confined to the ovary [14, 15]. Tumor histology can also impact clinical response. For example, advanced-stage high grade serous tumors are initially responsive to chemotherapy, while clear cell tumors are resistant [12].

Risk factors that contribute to the development of ovarian cancer can be genetic or environmental. Although family history poses a significant risk towards ovarian cancer development, only about 5% of ovarian cancer patients are genetically predisposed to the disease [14]. Ovulation requires rupture and subsequent repair of the ovarian epithelium, and a repeated pattern of disruption and repair is thought to encourage abnormalities in the cells composing this epithelial lining [16]. Thus, phenomena associated with an increase in frequency of ovulation, such as nulliparity, late menopause, early menarche and advanced age are
associated with increased risk, whereas factors such as oral contraceptive use and pregnancy, which decrease the overall frequency of ovulation, are associated with reduced risk [17].

Several hypotheses have been proposed to explain the mechanism of epithelial ovarian cancer development. One hypothesis suggests that repeated damage and repair of the ovarian epithelium resulting from ovulation may encourage malignant transformation of epithelial cells [18]. Inflammation resulting from this and other processes occurring in the vicinity of the ovaries has also been implicated in disease development [16]. Various hormones are suggested to play a role in carcinogenesis, such as steroids produced post-ovulation, and androgens that rise in levels in postmenopausal women [19, 20]. Further, estrogen hormones are hypothesized to stimulate gonadotropin hormones, promoting the entrapment of epithelial cells in ovarian inclusion cysts, where malignancy can develop [21]. None of these theories, however, singularly explain all of the associated risk factors, therefore, it is likely that a combination of various parameters leads to this disease. Recent findings suggest that a large number of high grade serous ovarian cancers arise from the fallopian tube and metastasize to the ovary [22-24]. In support of this, it was observed that women with a genetic predisposition undergoing prophylactic surgery had no ovarian lesions but had premalignant lesions in the fallopian tubes [25], and these were histologically identical to serous epithelial ovarian cancer [26].

1.2.2 Diagnosis

Clinical symptoms of ovarian cancer are non-specific and include bloating, constipation, fatigue, nausea, pelvic or abdominal pain, urinary urgency or frequency, difficulty eating, and early satiety [27]. Current screening methods include pelvic examinations, measurement of serum CA-125 levels, and transvaginal pelvic ultrasonography [28]. Although serum CA-125 levels are elevated in more than 80% of patients with advanced epithelial ovarian cancer, this measurement alone is neither sufficiently sensitive nor specific enough to be diagnostic [29]. Elevated serum CA-125 levels may be associated with a number of other conditions, such as
pregnancy and pelvic inflammatory disease [29]. Furthermore, CA-125 levels are elevated in less than half of patients with early-stage ovarian cancer, underscoring the lack of sensitivity to diagnose at a curable stage [30]. CA-125 is, therefore, not a useful diagnostic serum biomarker. At present, surgery is needed to accurately diagnose and stage the disease.

Ovarian cancer is staged during debulking surgery, from stage I, where growth is limited to the ovaries, through to stage IV characterized by distant metastases to the liver or outside the peritoneal cavity [31]. Ovarian cancer can metastasize from the ovaries in different manners [32]. Firstly, by directly invading adjacent organs like the peritoneum, uterus, and rectum after bypassing the ovarian capsule [32]. Another way is for individual cancer cells to enter nearby lymph nodes, thereby spreading via the lymphatic system. This is a common process in advanced disease, but only occurs at a frequency of 20% in early stage disease [32]. Alternatively, cancer cells can seed from the primary tumor, circulate throughout the peritoneal cavity with the assistance of movement-promoting events such as peristalsis and respiration, and re-implant in various tissues in the peritoneal cavity [32]. At the time of diagnosis, almost 70% of cases present with distant metastasis [32]. When ovarian cancer is diagnosed at stages I or II, long-term (> 10 years) survival rates range from 80-95%; however, for over 75% of patients for whom diagnosis occurs at stages III or IV, long-term survival drops to below 30% [5].

1.2.3 Treatment

The standard first-line management of ovarian cancer consists of surgical staging, operative tumor debulking, followed by six cycles of iv chemotherapy with platinum (i.e. CRBP) and taxane (i.e. PTX) drugs administered every three weeks [5]. Cytoreductive surgery leads to increased patient survival due to several benefits, including: (1) the removal of resistant cancer cell populations can promote sensitivity to anti-cancer agents; (2) the removal of large tumor masses can result in a reduction in devascularized tumor beds, allowing greater drug delivery to
the remaining cancer cells; and, (3) the lower-volume disease that remains may require lower drug doses with increased chance of response [33]. The concept of cytoreductive surgery for ovarian cancer was first proposed in 1934 by Meigs [34]. In 1975 Griffiths demonstrated that patient survival depended on the extent of residual disease after primary surgery [35]. Studies by Hoskins et al. have demonstrated that the smaller the residual disease, the better the survival outcome, with patients left with no residual disease having the most favorable prognosis [36].

The first evidence supporting the role of chemotherapy in the management of ovarian cancer was reported for the alkylating agents cyclophosphamide and melphalan [2]. In the 1970s, the observation of improved patient progression following treatment with alkylating agents (i.e. cisplatin (CDDP)) led to the incorporation of this agent as a component of first-line therapy. In order to improve tolerability, other platinum agents were evaluated, and CRBP was found to be associated with fewer side effects than CDDP, in particular emesis, nephrotoxicity and neurotoxicity, and showed equivalent efficacy in several randomized trials [37-43]. Following the introduction of platinum based agents into standard chemotherapy, PTX incorporation became another landmark in the management of ovarian cancer. PTX combined with CDDP resulted in an improved outcome compared to one of the standard chemotherapy strategies used at that time (CDDP plus cyclophosphamide) in a randomized Phase III trial [41]. Studies have shown that toxicity can be lessened and modified by replacing CRBP for CDDP and docetaxel (DTX) for PTX, respectively [39, 42, 44]. However, studies to-date have yet to demonstrate a better iv chemotherapy approach to the platinum and taxane combination in terms of improving overall survival.

Over the past several years, Phase III trials and smaller randomized trials have been designed in an attempt to improve the clinical outcome beyond what is achievable with the current standard of care. One approach has been to double the duration of platinum treatment periods from 5-6 cycles to 10-12 cycles [45-47]. Another strategy has been to increase the
platinum dose intensity [48-50]. Other studies have looked at high-dose chemotherapy regimens [51-53]. Finally, the effects of adding a third anti-cancer agent to the platinum and taxane regimen have been explored [54-56]. These approaches have failed to reveal any significant impact on either progression-free or overall survival compared to standard of care. Furthermore, greater toxicity was observed when the number of dosing cycles was increased, the dosing concentrations were intensified or when a third agent was used.

1.3 Intraperitoneal Chemotherapy

IP chemotherapy has been explored due to promising clinical results in patients with optimally debulked advanced ovarian cancer [57-59]. The rationale for IP chemotherapy is to eliminate residual disease by achieving higher drug concentrations for longer periods directly in the peritoneal cavity while reducing systemic toxicities (Figure 1.1) [60]. The peritoneal cavity is the site of ovarian cancer presentation or recurrence, and tumors remain confined to the peritoneal cavity for most of their natural history [27]. Consequently, the delivery of anti-cancer agents directly into the peritoneal cavity is a logical approach. Drugs administered via the IP route can reach tumors through direct penetration from the peritoneal cavity and via systemic recirculation [61]. Anti-cancer agents including CRBP and PTX present several advantages when administered via the IP route, including a higher local concentration, prolonged tumor exposure, and reduced systemic toxicities [61]. The pharmacokinetic advantage, defined as the ratio of peritoneal area under the concentration time curve (AUC) to plasma AUC, has been reported to be 12, 18, 181 and 1000 following IP administration of CDDP [62], CRBP [63], DTX [64] and PTX [65], respectively, and this has also been observed with other anti-cancer agents [60].

To date, there have been three randomized controlled Phase III trials of first-line IP chemotherapy in conjunction with surgical cytoreduction [57-59]. These studies have become the basis for recommending IP chemotherapy as a standard treatment for selected patients with ovarian cancer. In the first trial conducted by Alberts et al. [57], patients with stage III ovarian
cancer and residual disease ≤ 2 cm received iv CDDP (100 mg/m²) and iv cyclophosphamide (600 mg/m²) (iv group) or IP CDDP (100 mg/m²) and iv cyclophosphamide (600 mg/m²) (IP group) on day one, every three weeks for six cycles. Overall survival was 41 months for the iv group and 49 months for the IP group and reported toxicities were lower in the IP group. The second study, by Markman et al. [59], randomized patients with stage III ovarian cancer and residual disease ≤ 1 cm to receive iv chemotherapy with PTX (135 mg/m² on day one) followed by CDDP (75 mg/m² on day two), administered every three weeks for six cycles. The IP group consisted of iv CRBP (AUC of nine) every four weeks for two cycles followed by IV PTX (135 mg/m² on day one) and IP CDDP (100 mg/m² on day two) every three weeks for six cycles. The IP group showed improvement in both progression-free survival (27.9 months vs. 22.2 months) and overall survival (63.2 months vs. 52.2 months). Toxicities including leukopenia were higher in the IP group and were thought to be caused by the high doses of CRBP and increased number of treatment cycles. The third trial, by Armstrong et al. [58] randomized patients with stage III ovarian cancer and residual disease ≤ 1 cm to receive iv PTX (135 mg/m² on day one) followed by iv CDDP (75 mg/m² on day two) every three weeks for six cycles. For the IP arm, patients received iv PTX (135 mg/m² on day one) followed by iv CDDP (100 mg/m² on day two) then IP PTX (60 mg/m² on day eight) every three weeks for six cycles. The IP group showed favorable outcomes in progression-free survival (23.8 months vs. 18.3 months) and overall survival (66.9 months vs. 49.5 months). The IP arm had more toxicities and only 42% of patients were able to complete the full six cycles of IP chemotherapy.

A follow-up study observed that the initially lower quality of life in patients receiving IP chemotherapy was improved to baseline after 1 year [66]. Overall, these studies have demonstrated that IP chemotherapy can lead to improved overall survival of 8-16 months in patients with optimally debulked advanced ovarian cancer. Based on the last Phase III trial by Armstrong et al., in 2006 the US National Cancer Institute issued a ‘Clinical Announcement’ informing patients, oncologists and the public, on the impact and benefits of IP chemotherapy
for advanced ovarian cancer therapy [67]. The most commonly used IP regimen in the clinical setting is derived from the Armstrong study [68].

Despite the survival benefit, the use of IP chemotherapy has been limited by various complications including local toxicities (e.g. intestinal toxicity, abdominal pain) and infection, bowel perforation and mechanical malfunctions due to prolonged use of an indwelling IP catheter [69, 70]. These problems have led to the inability of a majority of patients (up to 70%) to complete all treatment cycles [71] and the reluctance of oncologists to adopt IP chemotherapy [72]. DDS administered via the IP route may circumvent issues currently associated with IP chemotherapy administered via catheters, resulting in improvements in patient survival.
Figure 1.1 The pharmacologic advantage of IP chemotherapy compared to systemic (iv) chemotherapy for ovarian cancer treatment. The rationale for IP chemotherapy is to eliminate residual disease by inducing higher drug concentrations for longer periods directly in the peritoneal cavity while reducing systemic drug exposure. Lower systemic drug levels can potentially minimize typical toxicities seen when commercially available anti-cancer formulations are administered via the iv route at maximum tolerated dose (MTD), while potentially still providing therapeutically relevant systemic drug levels.
1.4 Taxanes

PTX (Taxol®, Bristol-Myers Squibb) and DTX (Taxotere®, Sanofi-Aventis), are two taxanes that are currently used for treatment of breast, lung and ovarian cancers [73] (Table 1.1). Taxanes are anti-mitotic agents that act through various mechanisms and have been shown to have anti-cancer activity in a variety of mouse and human cancer cell lines, in murine tumor models and human tumor xenograft models of cancer [74, 75]. PTX and DTX hinder mitosis by interfering with microtubule function. Microtubules play important roles in normal cellular functions, such as maintenance of shape, movement, signaling, division and mitosis, making microtubules highly attractive cancer targets [76]. PTX and DTX bind to β-tubulin (a microtubule subunit) and induce stabilization of microtubules, leading to cell death [77]. Taxanes also have anti-angiogenic properties and induce apoptotic cell death via phosphorylation of Bcl-2, an anti-apoptotic protein [73, 78]. DTX has been shown to be more effective than PTX in a number of preclinical and clinical investigations due to improved cellular uptake, longer intracellular retention, and increased potency of Bcl-2 phosphorylation and microtubule stabilization [79, 80]. Pre-clinical and clinical studies comparing iv dosing of Taxol® and Taxotere® have established DTX to be as active as PTX [44, 81]. DTX has been clinically shown to be active in ovarian cancer patients who relapsed after therapy with PTX and platinum regimen [82]. In a phase III study, DTX-CRBP combination resulted in equivalent efficacy in ovarian cancer patients as PTX-CRBP, with a more favorable toxicity profile [44]. While PTX-CRBP induced irreversible sensory neuropathy, DTX-CRBP caused neutropenia, an easily manageable event [44]. Since this study, various groups have shown low incidence of neurotoxicity with DTX treatment and high occurrence with PTX treatment [83-85]. It is thus plausible that DTX may replace PTX as a frontline therapy [86, 87].

Both taxanes are highly protein bound, and are eliminated via hepatic metabolism [73]. The high molecular weight of these drugs makes them ideal candidates for IP therapy, as bulky hydrophobic molecules [88] generally have higher retention times in the peritoneal cavity [79].
The $\text{AUC}_{\text{peritoneal}}/\text{AUC}_{\text{plasma}}$ value of PTX is several folds greater than DTX owing to its higher molecular weight and lower solubility. Due to the high lipophilicity of taxanes, the non-ionic surfactants Cremophor EL and Polysorbate 80 are used as formulation vehicles for PTX and DTX, respectively. However, both surfactants have been associated with hypersensitivity and neurotoxicity, and have been shown to interfere with anti-angiogenic activity, cellular uptake and distribution of both drugs [7, 89, 90].
**Table 1.1** Chemical structure and properties of DTX and PTX.

<table>
<thead>
<tr>
<th>Taxane</th>
<th>Structure</th>
<th>MW (g/mol)</th>
<th>Solubility (µg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\frac{AUC_{\text{peritoneal}}}{AUC_{\text{plasma}}}$&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX</td>
<td><img src="image" alt="DTX Structure" /></td>
<td>807</td>
<td>5-6</td>
<td>152-181</td>
</tr>
<tr>
<td>PTX</td>
<td><img src="image" alt="PTX Structure" /></td>
<td>854</td>
<td>0.3</td>
<td>1000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Solubility values are from Ali et al [88].

<sup>b</sup> AUC ratios were measured following IP administration of DTX [64] and PTX [65].
1.5  Reduction in Treatment-Free Intervals

Conventional chemotherapy consists of administering anti-cancer agents at maximum tolerable doses with prolonged breaks (usually 2-4 weeks) between successive cycles of therapy [91]. Consecutive treatment-free intervals have been shown to promote accelerated repopulation of the tumor by drug resistant cancer cells, which can eventually lead to untreatable disease [92, 93]. Various studies have investigated shortening treatment-free periods as a means to improve efficacy [93-98]. Furthermore, cell cycle specific drugs such as DTX [99] may benefit from shorter treatment breaks, as only a fraction of tumor cells are found at vulnerable cell phases at any given time [100]. Exposing cancer cells to drugs on a more frequent schedule can ensure the drug is present when more cells cycle through specific phases, potentially resulting in greater cell death.

Clinical trials have shown that shortening treatment-free intervals via weekly chemotherapy improves therapeutic efficacy in ovarian cancer for both PTX [101-114] and DTX [84, 115-117]. A Phase III clinical trial demonstrated that weekly administration of PTX results in a significant improvement in the median progression-free survival and overall survival at 3 years when compared to administration of PTX every three weeks [118]. Phase II clinical trials in other cancers have shown high response rates with a weekly DTX regimen in the first and second line settings [119]. A more frequent schedule of biweekly DTX has shown good tolerability and response rates in prostate and non-small cell lung cancer [120, 121]. More recently, a movement towards complete elimination of treatment-free periods has been achieved with implantable and injectable depot systems that provide sustained delivery of PTX or DTX over prolonged periods. Treatment with these systems has been shown to be more efficacious than intermittent delivery in several pre-clinical studies [94, 96, 122, 123].
1.6 Multidrug Resistance

One of the leading causes of chemotherapy failure in ovarian cancer is the development of MDR. MDR is a phenomenon by which cancer cells develop the ability to survive in the presence of structurally and functionally different anti-cancer agents [124]. The current chemotherapy dosing regimen of intermittent iv infusions results in onset of chemoresistant disease within two years post-treatment [125]. Possible reasons for the onset of MDR include (Figure 1.2): (1) cancer cell genetics and phenotype; (2) tumor microenvironment; and (3) inadequate drug exposure.

The mutation and/or amplification of genes encoding ATP-dependent drug efflux transporters have been widely documented as key contributors to MDR [126]. Drug efflux transporters, when overexpressed, lead to cellular resistance to a broad spectrum of hydrophobic anti-cancer agents [127]. Another cellular mechanism involved in MDR is the decreased uptake of water-soluble drugs such as platinum agents which require transporters to enter cells [126]. Furthermore, modifications in drug metabolism, alterations in cell cycle kinetics, enhanced DNA damage repair mechanisms and changes in apoptotic signaling pathways have also been identified as sources of cancer cell specific mechanisms of MDR [128, 129].

Drug resistance can also develop as a result of the tumor microenvironment properties. Some of these properties found within tumors include hypoxic regions, low extracellular pH, heterogeneous vascular density and blood flow, and increased interstitial fluid pressure [130-135]. These physiological properties have been shown to result in limited drug penetration, heterogeneous distribution of drug and regions with slowly proliferating cells that are less chemosensitive [136].

As well, inadequate drug exposure at tumor sites can lead to MDR. This may result from an insufficient therapeutic dose reaching the tumor, short drug residence time and/or drug metabolism and excretion [137]. Different approaches to overcome MDR have been pursued
including the use of drug efflux transporter inhibitors, synthesis of more active drug analogues and development of prodrugs [138]. Some investigators have explored DDS such as nanoparticles to improve the effectiveness of chemotherapy in resistant disease [139-143], while others have shown that extending drug exposure time using localized DDS can increase tumor responsiveness [94, 96-98, 123] (Figure 1.2).
Figure 1.2 Factors leading to MDR in ovarian cancer and strategies investigated to overcome them using DDS. One of the leading causes of treatment failure in ovarian cancer is the onset of resistant disease. Factors leading to MDR can be divided into three broad categories: cancer cell genetics and phenotype; tumor microenvironment and inadequate drug exposure. (A) Cancer cell genetics and phenotype changes leading to MDR can arise from mutation or amplification of genes encoding ATP-dependent drug efflux transporters modifications in drug metabolism, alterations in cell cycle, increase in DNA damage repair mechanisms and changes in the apoptotic signaling pathway. (B) Tumor microenvironment properties such as hypoxic regions, low extracellular pH, heterogeneous vascular density and blood flow and increased interstitial fluid pressure can cause MDR. (C) Inadequate drug distribution due to dose, pharmacokinetics, drug properties and drug--cell interactions can also lead to MDR. The DDS that can be utilized to overcome MDR include (D) nano-sized DDS or (E) implants, injectable...
depots and microspheres. (D) Due to their size, NDDS can passively accumulate in tumors (i.e., enhanced permeation and retention (EPR) effect), which can be enhanced by attachment of targeting moieties onto the surface of NDDS (i.e., active targeting). (E) Implants, injectable depots and microspheres used locally can by-pass MDR mechanisms via sustained prolonged drug delivery. Panels (A) [128] (B) and (C) [144] are adapted.
1.7 Advanced Drug Delivery Strategies for Treatment of Ovarian Cancer

To date, a number of DDS have been designed to improve the therapeutic index of cancer drugs for treatment of ovarian cancer (Figure 1.3). One reason DDS have been put forward is to improve drug solubility and circumvent issues associated with pharmaceutical excipients such as non-ionic surfactants. Furthermore, DDS have also been developed to bypass systemic and peritoneal transport barriers in order to achieve higher therapeutic drug concentrations at diseased sites. Nano-sized DDS (NDDS) are ideal for iv administration as they can take advantage of the EPR effect [145]. The EPR effect occurs due to leaky vasculature and impaired lymphatic clearance within tumors, which in turn leads to preferential NDDS extravasation and retention in tumors [146]. Once administered iv, NDDS can reduce systemic drug exposure, increase tumor accumulation and bypass MDR mechanisms [147]. On the other hand, IP administration is more suitable for microspheres, implants and injectable depots. These DDS have the potential to provide sustained drug release over extended periods in the peritoneal cavity. Prolonged drug exposure has been shown to increase tumor responsiveness to chemotherapeutics in various ovarian cancer models [94, 96-98, 123]. Sustained and localized delivery may also improve intratumoral drug distribution and penetration [148] and allow for circumvention of MDR mechanisms [97, 122, 149]. A number of drugs relying on advanced DDS have entered clinical development for use in the treatment of ovarian cancer, and one has reached the market as a second line therapy (Table 1.2). The following sections highlight DDS developed for ovarian cancer therapy and discuss the advantages and limitations associated with each (Table 1.3).
Figure 1.3 DDS that have been investigated for ovarian cancer therapy. Nanoparticles, micelles, liposomes, drug conjugates, microspheres, implants and injectable depots have been investigated as DDS for ovarian cancer therapy. The main advantages associated with using DDS over conventional chemotherapy include achieving higher therapeutic drug concentrations in tumors and ascites, decreased non-specific toxicities, increased drug exposure to cancer cells, circumvention of drug resistance and improvements in drug solubility.
Table 1.2 DDS that have entered clinical trials for ovarian cancer therapy.

<table>
<thead>
<tr>
<th>Name</th>
<th>Delivery system</th>
<th>Compound</th>
<th>Clinical stage</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOXIL®</td>
<td>Liposome</td>
<td>Doxorubicin</td>
<td>Approved</td>
<td>[150]</td>
</tr>
<tr>
<td>OPAXIO™</td>
<td>Drug conjugate</td>
<td>PTX</td>
<td>Phase III</td>
<td>[151]</td>
</tr>
<tr>
<td>CT-2106</td>
<td>Drug conjugate</td>
<td>Camptothecin</td>
<td>Phase II</td>
<td>[151]</td>
</tr>
<tr>
<td>Genexol-PM</td>
<td>Block copolymer</td>
<td>PTX</td>
<td>Phase II (recruiting)</td>
<td>[152]</td>
</tr>
<tr>
<td>Paclimer®</td>
<td>Microsphere</td>
<td>PTX</td>
<td>Failed in Phase I</td>
<td>[153]</td>
</tr>
</tbody>
</table>
Table 1.3 Advantages and limitations of DDS investigated for ovarian cancer therapy.

<table>
<thead>
<tr>
<th>System</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nano-sized</strong></td>
<td>• Passive targeting due to size</td>
<td>• Small size may lead to rapid clearance and limited half-life</td>
</tr>
<tr>
<td></td>
<td>• Active targeting</td>
<td>• Frequent dosing required</td>
</tr>
<tr>
<td></td>
<td>• Ease of administration</td>
<td>• May not be applicable for residual disease following cytoreductive surgery</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microspheres</strong></td>
<td>• Prolonged release profile</td>
<td>• Peritoneal adhesions</td>
</tr>
<tr>
<td></td>
<td>• Several formulations have reached market for other cancers</td>
<td>• Limited tumor penetration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>**Implants and injectable</td>
<td>• Localized delivery</td>
<td>• Invasive if implantable</td>
</tr>
<tr>
<td>depots**</td>
<td>• Sustained drug release</td>
<td>• Surgical expertise needed for implantation and removal if not biodegradable</td>
</tr>
<tr>
<td></td>
<td>• Lower systemic toxicity</td>
<td>• Limited tumor penetration</td>
</tr>
<tr>
<td></td>
<td>• Facilitates delivery of drugs with short half life</td>
<td>• Viscosity issues if injectable</td>
</tr>
<tr>
<td></td>
<td>• Increased bioavailability by decreasing 1st pass hepatic metabolism</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Enhanced effect of cell cycle specific drugs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Reduced dose dumping</td>
<td></td>
</tr>
</tbody>
</table>
1.7.1 Nano-sized Drug Delivery Systems

NDDS such as nanoparticles [154-162], block copolymer micelles [163-169], liposomes [170-173] and drugs conjugated to peptides [174, 175], small molecules [176] or polymers [177-185] have been investigated for ovarian cancer therapy. These systems can be defined as drug carriers that are in the "1-100 nm range in at least one dimension", a scale at which unique physical, chemical, and biological properties emerge that can be used to develop novel technologies and products [186]. Anti-cancer agents can be loaded in the NDDS by encapsulation or conjugation [187]. In terms of biocompatibility, material components such as block copolymers used to prepare NDDS are less toxic alternatives to solubilize free drug compared to excipients such as non-ionic surfactants [188]. After systemic administration, NDDS have been shown to increase drug accumulation at solid tumor sites via passive targeting which relies on the EPR effect. The NDDS have an extended circulation lifetime following iv administration in comparison to free drugs [189-191]. This can result in an increase in tumor accumulation via the EPR effect, and in turn, increase efficacy [192]. NDDS and their material components have been shown to overcome MDR mechanisms by directing endocytosis-mediated cellular internalization of drug [147, 193] and/or interacting directly with efflux pumps [194-196]. In an attempt to further increase the therapeutic index of drug-loaded NDDS for ovarian cancer, scientists have pursued active targeting by functionalizing the NDDS surface with moieties that recognize targets expressed on or near cancer cells [197-200]. Active targeting may also aid in bypassing MDR mechanisms, resulting in an increase in regional and cellular accumulation of drug [201]. Another approach used to overcome MDR using NDDS has been combination drug delivery, whereby drugs with different mechanisms of action are co-delivered [154, 155]. However, NDDS do not provide a means to increase the peritoneal residence time of the drug significantly. Following IP administration, NDDS are typically cleared rapidly from the peritoneal cavity through absorption into the lymphatic drainage [202-204]. As
the goal of IP administration is to increase the residence time of drug in the peritoneal cavity, NDDS may not be suitable for this route of delivery.

Of the limited number of DDS evaluated in clinical trials for the treatment of ovarian cancer, most have been nano-sized systems. Genexol-PM is a formulation developed by Samyang Corporation in Korea, that consists of poly(ethylene glycol)-b-poly(L-lactide) block copolymer micelles loaded with PTX. Genexol-PM has been shown to increase the tumor accumulation of drug and result in significant improvements in efficacy in comparison to Taxol® in a murine model of ovarian cancer [205]. A subsequent Phase I study of Genexol-PM in patients with advanced, refractory malignancies including lung, breast and ovarian showed Genexol-PM permits the administration of a higher dose of PTX compared to Taxol® without additional toxicity [206]. Currently, ovarian cancer patients are being recruited for a Phase II clinical trial to evaluate Genexol-PM in combination with CRBP for first line treatment. DOXIL®, a liposome formulation of doxorubicin has been approved for treatment of refractory Kaposi’s sarcoma, ovarian cancer, and recurrent breast cancer [150]. OPAXIO™ (Cell Therapeutics Inc.) is a polymer-drug conjugate system of PTX linked to polyglutamate. This polymer-drug conjugate is currently being evaluated as monthly maintenance therapy in a Phase III clinical trial in ovarian cancer patients who have achieved a complete response following standard first-line chemotherapy [151]. A similar formulation from Cell Therapeutics Inc. (camptothecin conjugated to polyglutamate) was investigated in a Phase II open label study as a single agent in patients with advanced metastatic ovarian cancer who had failed one prior platinum and taxane based regimen [151].

1.7.2 Microspheres

Microsphere DDS, typically between 1 and 1000 µm in size, have been explored for delivery of a range of cancer therapies. Indeed, a number of microsphere formulations composed of the biodegradable copolymer poly(D,L-lactide-co-glycolide) have been approved for prostate cancer
therapy and have been reviewed elsewhere [207]. Paclimer®, a poly(phosphoester) microsphere formulation of PTX, with a sustained in vitro release profile (1–2% per day) over a 90-day period [208], was investigated by Armstrong et al. in a Phase I clinical trial [153]. Patients with recurrent ovarian cancer were treated with Paclimer® every 8 weeks for 2 cycles via the IP route. It was reported that sustained plasma levels of PTX were achieved throughout the 8-week period. However, biocompatibility issues associated with the microspheres were observed, including visible residual polymer filaments 7 months post treatment. Beyond providing sustained plasma levels, microspheres may be used to increase the peritoneal residence time for drugs. Compared to NDDS, microspheres have been shown to have longer peritoneal retention following IP administration [204]. Consequently, microspheres have the potential to be utilized as a means to provide sustained plasma and peritoneal drug levels combined with localized drug exposure when delivered via the IP route. To date, only a limited number of preclinical studies have investigated microsphere formulations for IP chemotherapy in ovarian cancer models [204, 209, 210].

1.7.3 Implants and Injectable Depots

Implantable and injectable depot DDS have been investigated for localized and sustained delivery of anti-cancer agents [211]. Examples of such systems investigated in clinical trials include the injectable depot OncoGel™ for esophageal and brain cancers [212] and implantable Gliadel® wafers for brain cancer [213, 214]. IP implantable and injectable depot systems may be the most promising approach for treatment of ovarian cancers, given that the disease is located primarily within the peritoneal cavity during its natural history. Advantages associated with these DDS for ovarian cancer are higher drug concentrations at the disease site, extended drug exposure which may be particularly beneficial for cell cycle specific anti-cancer agents, and lower systemic toxicity. Yet, implantable and injectable depot DDS face a number of drawbacks that have limited their implementation in the clinical setting. One challenge is achieving
homogeneous distribution of drug in the peritoneal cavity following release from the delivery system. In addition, metastatic regions outside of the peritoneal cavity may not be reached with a localized approach. However, the sustained plasma levels provided by some implantable and injectable depot DDS may be effective at treating metastatic disease. The extent of tumor drug penetration may be low compared to iv chemotherapy as local drug exposure from the periphery of tumors may not provide sufficient intratumoral drug distribution. Yet if the DDS results in sustained plasma levels, albeit at low doses, the systemic circulation may provide an additional route for drug entry into tumor nodules. Difficulties in precise implantation in the peritoneal cavity may arise, requiring a high level of surgical expertise. Finally, the viscosity of injectable depots may lead to issues. It has been reported that low viscosity injectable systems may fail to provide a delayed drug release profile, while high viscosity injectables may be difficult to administer [215].

Yang et al. have incorporated DTX into an injectable thermosensitive mixed micelle gel [216]. The impact of site of administration (i.e. intratumoral, peritumoral and subcutaneous) of the gel on efficacy in a subcutaneous SKOV-3 murine model of ovarian cancer was investigated. Intratumoral administration resulted in the lowest tumor growth rate and was 4.2, 2.6 and 1.7 fold lower than that in the non-treated control, subcutaneous and peritumoral groups, respectively. Grant et al. designed an implantable chitosan-phospholipid film that provided sustained IP delivery of PTX for the treatment of ovarian cancer [217]. Implantation of the film in the peritoneal cavity of healthy mice revealed no signs of toxicities over a 4-week period [218]. Treatment with PTX-loaded chitosan-phospholipid film resulted in complete tumor inhibition in a murine xenograft model of human ovarian cancer, whereas only 47% inhibition was observed in animals given equivalent bolus IP doses of PTX [123]. Intermittent doses of PTX, as is conventionally administered, resulted in a pronounced induction of the mdr1 gene, which plays a significant role in MDR, while sustained delivery of PTX via the film did not result
in *mdr1* induction [149]. To avoid the need for surgical implantation, Grant et al. developed an injectable chitosan-phospholipid depot for sustained PTX delivery [219].

### 1.8 Rationale

At present the prognosis for women with ovarian cancer is poor and new approaches for treatment must be developed. In recent years, various delivery strategies to improve tumor responsiveness to chemotherapy have been proposed. Three pivotal Phase III clinical trials have highlighted the potential benefits of IP chemotherapy. Although survival benefits have been documented, implementation of IP chemotherapy has been hindered by various issues including local toxicities and catheter related problems. To date, several studies suggest that longer drug exposure times and shorter treatment-free periods can increase efficacy due to factors such as enhanced anti-angiogenic effects and decreased tumor repopulation. DDS may be able to circumvent issues facing conventional catheter based IP chemotherapy and allow for longer drug exposure periods.

Sustained IP delivery of PTX from implants placed inside the peritoneal cavity of mice bearing ovarian cancer has been shown to significantly improve efficacy compared to intermittent PTX delivery. However, due to the invasive nature of surgical implantation, an injectable formulation with similar functional attributes and improved ease of administration would be more desirable. Furthermore, DTX has been shown to be more effective than PTX in a number of studies due to improved cellular uptake and lower efflux, increased potency, lower incidence of neurotoxicity and increased efficacy in PTX-resistant disease.

The overall goal of this thesis was to develop and characterize an injectable delivery system that provides sustained release of DTX following IP administration for treatment of ovarian cancer. Furthermore, this thesis sought to understand the influence of DTX distribution following sustained IP delivery at the whole-body, peritoneal and intratumoral levels on the therapeutic effect of this treatment approach.
1.9 Hypothesis
Sustained IP DTX delivery via an injectable depot system will be efficacious in murine models of ovarian cancer owing to favorable peritoneal and intratumoral drug distribution and prolonged drug exposure.

1.10 Objectives

Objective 1: To develop and characterize an injectable depot formulation (PoLigel) for sustained IP delivery of DTX.

Objective 2: To compare the biodistribution of DTX in healthy mice delivered via the PoLigel to Taxotere®, the clinically approved formulation of DTX.

Objective 3: To evaluate the influence of ovarian cancer tumor burden and ascites volume on DTX biodistribution following IP administration of the PoLigel.

In addition to the above objectives, a fourth objective was pursued to assess the impact of sustained DTX chemotherapy on MDR ovarian cancer. The onset of MDR is one of the main causes of treatment failure and low survival rates in ovarian cancer patients. Inadequate drug exposure and treatment-free periods that follow each intermittent chemotherapy cycle select for cancer cells overexpressing drug efflux transporters, resulting in resistant disease. The following additional objective was set:

Objective 4: To examine the effect of sustained DTX delivery alone and in combination with a drug efflux transporter inhibitor on MDR ovarian cancer.
1.11 Overview of Thesis Chapters

Studies addressing the objectives set for this thesis are described in Chapters 2-5. The preparation, physicochemical characterization and in vivo performance of the injectable depot formulation used in this thesis for sustained and localized delivery of DTX following IP administration is outlined in Chapter 2. The formulation (PoLigel) was prepared by combining a water-soluble chitosan derivative with phospholipids and DTX solubilized in lauric aldehyde. In vitro stability of the PoLigel was examined by turbidity and pH measurements. The rheological properties of the PoLigel were assessed by viscosity measurements using steady shear tests. The molecular interactions and the organization of the materials within the PoLigel were evaluated by using Fourier transform infrared (FTIR) spectroscopy and confocal laser scanning microscopy, respectively. The in vitro release profile for DTX from the PoLigel was measured as a function of initial drug loading. DTX distribution in plasma and tissues following IP administration of the PoLigel was characterized in healthy female CD-1 mice. Finally, the therapeutic effect of the PoLigel at two different DTX doses was assessed in a SKOV3 murine model of ovarian cancer.

Chapters 3 and 4 report on the distribution of DTX following PoLigel administration in healthy mice and murine models of ovarian cancer, respectively. Studies described in Chapter 3 investigated the difference between the distribution of DTX in healthy mice following IP administration in PoLigel or Taxotere®. Chapter 4 examines the influence of ovarian cancer disease parameters (i.e. tumor burden and ascites fluid) on drug distribution at the whole body, peritoneal and intratumoral levels. Distribution and efficacy studies were carried out in SCID mice bearing IP solid tumors arising from SKOV3 cells and C57BL/6 mice with IP ascites fluid arising from ID8 cells. In addition, a subcutaneous (SC) SKOV3 model was used to determine whether the systemic drug levels that result from IP administration of the PoLigel influence antitumor efficacy. Immunostained IP and SC SKOV3 tumor sections were used to study cell death, intratumoral drug distribution and tumor penetration.
The motivation for studies described in Chapter 5 came from previous work showing upregulation of Pgp in ovarian cancer tumors following intermittent, but not sustained, PTX chemotherapy. This chapter explores the effects of sustained DTX delivery alone and in combination with a drug efflux transporter inhibitor on MDR ovarian cancer. This was done using the human ovarian cancer cell line HeyA8 and its DTX-resistant counterpart, HeyA8-MDR. In vitro cytotoxicity, combination index and apoptotic response of monotherapy and combination therapy were determined in both cell lines. The cellular uptake and efflux of DTX were also quantified. Finally, the efficacy of sustained monotherapy, combination therapy, intermittent DTX therapy and drug-free formulation was evaluated in both taxane-sensitive and taxane-resistant murine ovarian cancer models in SCID mice.

Chapter 6 presents overall conclusions, summary of findings and several proposed future research directions.
CHAPTER 2. Chitosan-Phospholipid Blend for Sustained and Localized Delivery of Docetaxel to the Peritoneal Cavity

Payam Zahedi, Raquel De Souza, Micheline Piquette-Miller, Christine Allen.

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All experiments and data analyses were carried out by P. Zahedi except for the anti-tumor efficacy study which was carried out by R. De Souza. Distribution studies were performed with technical assistance from R. De Souza.

This chapter was written primarily by P. Zahedi.
2.1 Abstract

Localized and sustained delivery of chemotherapeutics presents a “magic bullet” effect by providing high drug concentrations at the target site, extended drug exposure and reduced systemic toxicity. In the present study, an injectable chitosan-phospholipid (PoLigel) blend is put forth as a strategy to achieve sustained and localized delivery of DTX following IP administration. The stability of the blend was confirmed in vitro, by turbidity measurements and attributed to specific molecular interactions and the organization of the materials within the blend, as evidenced by FTIR analysis and confocal laser scanning microscopy, respectively. The chitosan and phospholipid were found to colocalize in regions surrounding a mean object area of 11.2 µm² with colocalization coefficients of 43% and 46% for the chitosan and phospholipid, respectively. The PoLigel blend afforded sustained drug release, as seen both in vitro (2.4 ± 0.7% DTX per day) and in vivo (4.4 ± 0.7% DTX per day). Constant concentrations of DTX were observed over a two-week period in plasma and relevant peritoneal tissues, with no signs of toxicity or inflammation, following IP administration of the blend in healthy CD-1 mice. At DTX doses of 28.8 mg/kg and 19.2 mg/kg, the blend showed significant tumor inhibition of 87 ± 9% and 74 ± 26%, respectively, in a murine xenograft model of human ovarian cancer. This localized delivery system has shown excellent potential for sustained IP treatment of cancers, such as ovarian, that reside in the peritoneal cavity.
2.2 Introduction

Traditionally chemotherapy has consisted of systemic, intermittent administration at the maximum tolerated dose, yet this can lead to acute and long-term toxicities [91, 220], inadequate dosing at the target site [128, 134, 221], and development of drug resistance [128, 222]. Localized delivery of lower doses of chemotherapy in a sustained manner presents a “magic bullet” effect by providing high drug concentrations at the target site, extended drug exposure which may be particularly beneficial for cell cycle specific drugs, and lower systemic toxicity [91, 223]. Implantable and injectable systems including polymer-based injectable pastes, gels, microspheres and nanospheres have been pursued for localized cancer therapy [215, 221, 224, 225]. However, the majority of these systems are derived from polyester-based polymers [204, 223, 226, 227], which are known to produce a foreign body response (i.e. fibrous encapsulation) and acidic by-products that can accelerate drug degradation [218, 228-230]. As a result, there has been an increased interest in the use of naturally occurring polymers such as protein-based polymers (e.g. collagen, gelatin) and polysaccharides (e.g. chitosan, agarose) to alleviate the issues associated with polyester systems [231, 232].

PTX and its semi-synthetic analogue DTX, two FDA approved taxanes, show considerable activity in the treatment of breast, lung and ovarian cancers [73]. PTX and DTX are highly lipophilic with reported water solubilities of 0.3 and 5-6 µg/ml, respectively [88]. To improve solubility, Cremophor EL and Polysorbate 80 are used as formulation vehicles for PTX and DTX, respectively. These surfactants have been associated with hypersensitivity and neurotoxicity, and have been shown to alter cellular uptake and influence drug distribution [7, 89, 90]. Alternative strategies have been explored for surfactant-free delivery of taxanes in order to circumvent these toxicity issues [7, 8]. As an example, our group developed an implantable chitosan-phospholipid film for localized, sustained delivery of PTX in the peritoneal cavity [217]. In vitro studies in an ovarian cancer cell line and in vivo studies in both healthy mice and a murine xenograft model of human ovarian cancer have confirmed that the chitosan-based films
are biocompatible, non-immunogenic, non-toxic, efficacious and provide a sustained release of PTX over periods of up to four months [96, 123, 149, 217, 218, 233, 234]. Due to the invasive nature of surgical implantation, an injectable formulation with similar functional attributes and improved ease of administration was pursued for PTX [219].

It has been shown that DTX is more effective than PTX in a number of preclinical and clinical investigations due to improved cellular uptake and lower efflux, increased potency, lower incidence of neurotoxicity and increased efficacy in PTX-resistant disease [79, 80, 235]. Furthermore, to date no localized and sustained delivery systems for DTX have been reported. In the present study, the physicochemical and rheological properties and in vivo performance of an injectable chitosan-phospholipid blend were evaluated as a formulation strategy for IP sustained delivery of DTX.

2.3 Materials and Methods

2.3.1 Materials

Chitosan (2.2 x 10^5 g/mol, 92% degree of deacetylation) was purchased from Marinard Biotech Inc. (Quebec City, Canada). DTX was purchased from Lianyungang Jari Pharmaceutical Co. (Jiangsu, China). Albumin, Egg phosphatidylcholine (ePC), glycidyltrimethylammonium chloride (GTMAC), lauric chloride (LCl), lauric aldehyde (LA) and lysozyme were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada). The fluorescent probes, Alexa Fluor® 633 and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzo diazol-4-yl) (NBD-DPPE) were purchased from Molecular Probes Inc. (Eugene, USA) and Avanti Polar Lipids Inc. (Alabaster, USA), respectively. SKOV3 human ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, USA). RPMI 1640 cell culture medium was purchased from Gibco (Grand Island, USA). Fetal bovine serum and penicillin-streptomycin were purchased from Invitrogen (Burlington, Canada). All other chemicals were reagent grade and used as received.
2.3.2 Preparation of Polymer-Lipid Formulations

A water-soluble chitosan (WSC) derivative was synthesized using a method reported previously [236]. Briefly, chitosan was suspended in a 0.5% v/v acetic acid solution, and GTMAC was added dropwise (GTMAC:chitosan 3:1 mol/mol). The reaction was stirred at 55°C for 18 h. Following the reaction, undissolved chitosan was removed by centrifugation of the reaction mixture at 5000 rpm (4835 x g) for 10 min at 25°C (Centrifuge 5804T, Eppendorf, Germany). Excess GTMAC was removed using methanol followed by precipitation of the WSC in acetone. This procedure was repeated in triplicate and the purified WSC was dried in a vacuum oven at 25°C with subsequent grinding of the product to obtain a fine powder. FTIR analysis and \(^1\)H NMR spectra were used to confirm the conjugation of GTMAC to chitosan (data not shown) [236]. The degree of substitution of GTMAC on the chitosan backbone was calculated to be 45% using an established titration method [237].

Chitosan-phospholipid (PoLigel) blends were prepared as outlined elsewhere [219]. In brief, WSC was dissolved in distilled deionized water to prepare a 4.2% (w/v) solution. ePC was solubilized in LA or LCl (ePC to LA or LCl ratio of 1:4 w/w) and then added to the WSC solution. The final material ratio of the WSC-LA-ePC (PoLigel-LA) or WSC-LCl-ePC (PoLigel-LCl) blends was 1:4:1 w/w/w. For preparation of drug loaded blends, DTX (10, 20 and 30 mg) dissolved in anhydrous ethanol was dried under nitrogen to form a thin layered film and then placed under vacuum for 24 h to remove any residual solvent. An ePC-LA solution was used to re-suspend the DTX film prior to mixing with WSC solution to achieve WSC-LA-ePC-DTX (PoLigel-LA-DTX) blends with final material ratios of 1:4:1:(DTX: 0.24, 0.48 and 0.71) w/w/w/w.

2.3.3 Characterization of PoLigel blend Stability and pH Profile

The stability and pH profile of PoLigel-LA, PoLigel-LA-DTX (1:4:1:0.71 w/w/w/w) and PoLigel-LCl blends were assessed in buffer solution containing lysozyme stored at 37°C over a two-week period, as outlined elsewhere with slight modifications [219]. 150 µL of each blend was
injected into a vial containing 15 mL 0.01 M PBS (pH 7.4) and 2 mg/mL lysozyme. At specific time points, an aliquot was removed for stability and pH measurements, and then returned to the vial containing the blend for subsequent analysis. For stability assessment, turbidity analysis was conducted using a UV spectrophotometer with $\lambda = 700$ nm (Cary 50 UV–vis spectrophotometer, Varian Inc., USA). pH was measured under constant stirring by a pH meter (SympHony SB20 pH meter, VWR Scientific, USA).

2.3.4 Fourier Transform Infrared Spectroscopy Analysis

FTIR spectra of the PoLigel-LA and PoLigel-LCl blends and their individual components were obtained using a universal ATR Spectrum-one spectrophotometer (Spectrum One FTIR, PerkinElmer, USA). The spectra were recorded from 4000 to 650 cm$^{-1}$ and analyzed using Spectrum V5.0.1 software (Perkin-Elmer, USA). All spectra were an average of 20 scans at a resolution of 2 cm$^{-1}$.

2.3.5 Confocal Microscopy Analysis

The PoLigel-LA blend was analyzed using an inverted two photon confocal laser scanning fluorescence microscope (LSM 510 META, Carl Zeiss MicrolImaging Inc., Germany) as outlined elsewhere [219]. In brief, the fluorescent probe Alexa Fluor® 633 ($\lambda_{\text{ex}} = 632$ nm, $\lambda_{\text{em}} = 647$ nm), which contains an amine reactive group, was conjugated to the WSC according to the manufacturer’s protocol and quantified by FTIR analysis (data not shown). To prepare the fluorescent labeled PoLigel-LA, 1 mol% of the fluorescent phospholipid NBD-DPPE ($\lambda_{\text{ex}} = 460$ nm, $\lambda_{\text{em}} = 534$ nm) was mixed with an ePC-LA solution. The ePC-LA fluorescent solution was then mixed with WSC solution containing 1% w/w of the Alexa Fluor® 633 conjugated WSC to prepare a final WSC-ePC-LA 1:4:1 w/w/w fluorescent blend. The fluorescent blend was cast onto a glass slide, covered with a glass cover slip and allowed to dry in the dark for 24 h prior to microscopy analysis. A colocalization map was generated from the images obtained.
Parameters including colocalization coefficients, mean object area, and mean gray value were obtained by Image-Pro Analyzer V6.3 (Media Cybernetics Inc, USA).

2.3.6 Rheological Measurements

The rheological properties of the PoLigel-LA, PoLigel-LA-DTX and PoLigel-LCl blends were characterized by a stress-controlled rheometer with a 4 cm cone and 2° angle plate geometry attachment at room temperature (AR-2000, TA Instruments, USA). The rheometer was calibrated and rotational mapping was performed according to instrument specifications. The viscosity was measured using a continuous ramping flow mode while increasing the shear stress from 1 to 500 Pa. The blends were stored for 3 h prior to mechanical testing. A 600 µL injection of each sample was placed on the rheometer plate for testing.

2.3.7 In vitro Drug Release

PoLigel-LA-DTX blends of different initial DTX loading levels (WSC-LA-ePC-DTX material ratios: 1:4:1:(0.24, 0.48 and 0.71) w/w/w/w) were injected into vials containing 15 ml of 0.01M PBS (pH 7.4) with 2 mg/mL lysozyme and 40 mg/mL albumin. The samples were incubated at 37°C and at specific time points 12 mL aliquots were removed for analysis by high-performance liquid chromatography (HPLC). The removed volume was replaced with 12 mL fresh 0.01M PBS (pH 7.4) containing 2 mg/mL lysozyme and 40 mg/mL albumin.

2.3.8 Biodistribution and Toxicity Assessment

In vivo studies were conducted in healthy female CD-1 mice (6-8 weeks old, 20-25 g) purchased from Charles River (St. Constant, Canada). All studies were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee and the Canadian Animal Care Council. Each mouse was injected IP with the PoLigel-LA-DTX (DTX dose: 28.8 mg/kg) blend in the lower left quadrant, with an injection depth of 1 cm using a 25 gauge needle. The 28.8
mg/kg dose was chosen as it corresponds to the clinically tested IP DTX dose given as Taxotere® in humans [64]. Samples were sterilized under UV-light (Sterilizer T209, Intercosmetics, Canada) for 3 h prior to injection. Mice were weighed and observed weekly for signs of distress (e.g. weight loss, paleness, inactivity). Control mice did not receive an injection. At specific time points, mice (n = 6) were anesthetized and sacrificed by exsanguinations via cardiac puncture. Plasma and various tissues were collected for HPLC analysis.

Animals were visually assessed daily throughout the study for signs of DTX toxicity, infection at injection site, peritonitis and weight loss. The peritoneal cavity was assessed post-mortem for signs of fibrous encapsulation and inflammation. Hepatotoxicity was assessed by alanine aminotransferase (ALT) activity in serum using an ALT Reagent kit (Thermo Electron Corporation, Australia) following the manufacturer’s protocol. Systemic inflammation was assessed by measuring levels of circulating Interleukin-6 (IL-6) using a mouse IL-6 ELISA kit according to manufacturer’s instructions (BD Biosciences, USA).

2.3.9 Efficacy Assessment

Efficacy studies were conducted in female CD-1 nu/nu mice (6-8 weeks old, 18-20 g) purchased from Charles River (St. Constant, Canada). All studies were conducted using sterile techniques and in accordance with the guidelines of the University of Toronto Animal Care Committee and the Canadian Animal Care Council. Animals were housed under sterile conditions in microisolator cages, fed standard chow diet with water ad libitum and maintained on an automatic 12 h light cycle at 22-24°C. SKOV3 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 mg/mL streptomycin). Cells were allowed to grow in a monolayer in a tissue culture flask incubated at 37°C, gassed with 5% CO₂ and held at 90% relative humidity. Mice were injected IP with 1 x 10⁷ SKOV3 cells suspended in 300 µL serum-free RPMI 1640 medium. Fourteen days post-inoculation, mice (n = 4 per group) were injected
IP with PoLigel-LA-DTX (DTX doses of 19.2 mg/kg or 28.8 mg/kg) blend or 20 µL sterile saline solution. Two doses were employed to examine the occurrence of a dose-dependent response. All injections were in the lower left quadrant, with an injection depth of 1 cm using a 25 gauge needle. Weight loss was monitored by regular body weight measurements, and the presence of all other endpoints was examined visually by animal care technicians and veterinarians. All animals were sacrificed 14 days after treatment initiation, and tumors were collected and weighed for tumor burden assessment.

2.3.10 High Performance Liquid Chromatography Analysis

An Agilent Series 1100 HPLC (Agilent Technologies, Canada) equipped with a Waters 4.6 mm x 250 mm column (XTerra® MS C18, 5 µm particle size) and Waters 3.9 x 20 mm guard column (XTerra® MS C18, 5 µm particle size), Waters Dual Absorbance Detector 2487 (Waters, USA) and ChemStation software (Agilent Technologies, Canada) was used for analysis. The wavelength of detection used for DTX was 227 nm. For plasma and tissue samples, a mobile phase of 60% 0.01 M PBS (pH 10) and 40% acetonitrile was used (DTX retention time of 40 min). For in vitro release samples a mobile phase of 52% H₂O and 48% acetonitrile was used (DTX retention time of 11 min). All samples were run at a flow rate of 1 mL/min with an injection volume of 20 µL per sample. An internal standard of PTX (10 µg/mL) was used for all HPLC analysis. For the extraction of DTX from in vitro release and plasma samples, 300 µL of sample was added to a vial containing PTX standard. The contents were vortexed for 5 min and then 5 mL tert-butyl methyl ester was added followed by 10 min of additional vortexing. The solution was then centrifuged (Centrifuge 5804R, Eppendorf, Germany) at 4000 rpm (3095 x g) for 15 min. The organic layer was transferred to a new vial followed by drying under nitrogen. The dried sample was then re-suspended in 300 µL of mobile phase and analyzed by HPLC. The extraction efficiency of DTX from in vitro release and plasma samples was greater than 90%. For the extraction of DTX from tissue samples, distilled deionized water was added at a ratio of
4 mL per gram of tissue. The sample was then homogenized (100 Sonic Dismembrator, Fisher Scientific, Canada) and added to a vial containing PTX standard. The DTX extraction procedure was similar to that described above. The extraction efficiency of DTX from tissues and blend formulation samples was greater than 80%. The limit of detection (LOD) was 5 ng/mL and 10 ng/mL for plasma and tissues, respectively. The limit of quantification (LOQ) was 10 ng/mL and 20 ng/mL for plasma and tissues, respectively.

2.3.11 Statistical Data Analysis

All results were obtained from data groups of n ≥ 3 and are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). A two-sample t-test was used to measure statistical significance between pairs of results. For statistical analyses among three or more groups, one-way analysis of variance (ANOVA) was used and subsequent multiple comparisons with Bonferroni correction was performed if statistical significance was detected by the ANOVA. A p-value < 0.05 was considered statistically significant.

2.4 Results and Discussion

2.4.1 Poligel Blend Characterization

In order to establish the interactions that stabilize the Poligel blends, FTIR spectra were collected. The spectra of each blend and their individual components were analyzed for characteristic peaks (Figure 2.1). In agreement with other reports, FTIR analysis confirmed favorable interactions between chitosan and ePC with a shift of the primary amine group of WSC from 1564 (Figure 2.1, line a) to around 1575 cm⁻¹ with the addition of ePC [217, 236]. Furthermore, the absence of a defined peak at 1800 cm⁻¹ (CClO group in LCl) in the Poligel-LCl blend spectra and at 1725 cm⁻¹ (CHO group LA) in the Poligel-LA blend spectra, as seen in Figure 2.1, indicates that LCl and LA groups interacted with the primary amine groups of WSC.
(peak at 1564 cm\(^{-1}\)) and/or groups within ePC (P=O at 1226 cm\(^{-1}\)). Previous work by Grant et al. outlines in detail these stabilizing interactions for various PoLigel-LCl blends [219], with similar interactions seen here for the PoLigel-LA blend.
Figure 2.1 FTIR spectra of WSC, ePC, LA, LCI and PoLigel-LA and PoLigel-LCI blends. Line a indicates the primary amine group of WSC (1564 cm⁻¹).
It has been shown that using C12-LCl as compared to C10, C14 and C16 LCls, results in the most stable injectable chitosan-phospholipid blend formulation; however, a low pH profile was observed \textit{in vitro} [219]. It has been reported in different studies that C12 alkyl chains grafted onto the chitosan backbone lead to formation of stable gels [238, 239]. Rinaudo et al. have shown from a series of alkylated chitosan derivatives that the C12 alkyl chain was the optimal chain length for forming a stable gel [240]. In addition, in a recent study by our group, De Souza et al. demonstrated superior biocompatibility of the PoLigel blend both \textit{in vitro} and \textit{in vivo} when C12-LCl was replaced by C12-LA [241]. To confirm the advantage of using C12-LA over C12-LCl for preparing the PoLigel blend, stability and pH testing were conducted. The effect of drug loading on stability and pH were also investigated for PoLigel-LA blend. The stability of the PoLigel-LA, PoLigel-LA-DTX and PoLigel-LCl blends was assessed by turbidity measurements in 0.01 M PBS (pH 7.4) containing 2 mg/mL lysozyme at 37°C over a two-week period (Figure 2.2A). It has been shown that chitosan is degraded by various enzymes including chitosanase, chitinases, cellulase, protease, lipase and lysozyme [242]. Lysozyme solutions have been used in various reports for studying the \textit{in vitro} biodegradation of chitosan-based systems [243-248]. The PoLigel-LCl blend disintegrated within 3 h following injection into the lysozyme solution as can be seen from the high absorbance values in Figure 2.2A. However, the PoLigel-LA and PoLigel-LA-DTX blends were stable over the two-week incubation period as indicated by negligible absorbance values (Figure 2.2A). A significant decrease in the pH of 0.01M PBS solution from 7.4 to approximately 2.75 within 3 h of incubation was seen for the PoLigel-LCl blend (Figure 2.2B). This low pH was maintained over the two-week observation period. The decrease in pH can be attributed to the formation of the acidic byproducts of LCl during the reaction with water and/or WSC as reported in our previous work [219], and to the low stability of the PoLigel-LCl formulation as confirmed by turbidity measurements. In contrast, the pH profiles of the PoLigel-LA and PoLigel-LA-DTX blends were maintained close to neutral pH over two weeks (p > 0.05).
Figure 2.2 *In vitro* characterization of the stability and pH profiles of PoLigel blends. (A) Turbidity measured as absorbance of 0.01 M PBS (pH 7.4) solution containing PoLigel blend, as a function of time. (B) pH values of 0.01M PBS (pH 7.4) solution containing PoLigel blend, as a function of time. The results represent mean ± SD (n = 3).
2.4.2 PoLigel-LA Blend Morphology

Confocal laser scanning microscopy was used to gain insight into the molecular organization of the materials within the PoLigel-LA blend. The red regions shown in Figure 2.3A represent the WSC component and the green regions in Figure 2.3B correspond to the phospholipid (ePC) component. Figure 2.3C shows the overlay of the phospholipid and WSC regions, indicating areas of colocalization between the WSC and ePC. The colocalization between the WSC and phospholipid is critical in stabilization of the blend as reported previously [219]. In order to quantify the extent to which WSC-ePC colocalized, a map was generated (Figure 2.3D). WSC-ePC was found to colocalize in regions surrounding a mean object area of 11.2 µm² (i.e. yellow regions). The mean gray value within the colocalization map (i.e. amount of bright pixels relative to the background) was 31%. The colocalization coefficients M1 and M2, which represent the contribution of the ePC (green fluorescent signal) and WSC (red fluorescent signal) to the colocalized areas, were 46% and 43% respectively. As summarized in Table 2.1, the PoLigel-LA blend showed higher mean object area and colocalization coefficients (M1 and M2) and comparable mean gray value to the PoLigel-LCl blend. As noted elsewhere, higher mean object area, colocalization coefficients and mean gray value are often indicative of enhanced stability [219].
Figure 2.3 Representative confocal laser scanning fluorescence microscopy images of the PoLigel-LA blend material components. (A) WSC regions, (B) ePC regions, (C) overlay of the WSC and ePC regions and (D) colocalization of WSC and ePC regions. The scale bar in each image represents 20 µm.
Table 2.1: Colocalization analysis of the WSC and ePC regions within the PoLigel-LA and PoLigel-LCL blends using confocal microscopy.

<table>
<thead>
<tr>
<th>Blend</th>
<th>Mean object area (µm²)ᵃ</th>
<th>M1 (ePC)ᵇ</th>
<th>M2 (WSC)ᵇ</th>
<th>Mean gray valueᶜ</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>PoLigel-LA</td>
<td>11.2</td>
<td>46</td>
<td>43</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>PoLigel-LCL</td>
<td>8.5</td>
<td>42</td>
<td>39</td>
<td>30</td>
<td>[219]</td>
</tr>
</tbody>
</table>

ᵃ Mean object areas represent the average area surrounded by colocalized WSC and ePC in the colocalization map (Figure 2.3D).

ᵇ M1 and M2 represent the percentage of ePC (green) and WSC (red) fluorescence signals in the colocalized area relative to the total ePC and WSC fluorescence signals, respectively (Figure 2.3C).

c Mean gray values represent the amount of bright pixels detected in the WSC-ePC colocalization map (Figure 2.3D).
2.4.3 PoLigel Blend Rheological Analysis

The rheological properties of the injectable blends were assessed by viscosity measurements using steady shear tests (Figure 2.4). It has been reported that low viscosity injectable systems may fail to provide a delayed drug release profile while high viscosity injectables may be difficult to administer [215]. It has been established that needles of 22 gauge and above are acceptable for injection of a drug formulation, otherwise, pumps and other systems need to be utilized [249]. The blends tested here were injectable through a 22 gauge needle. At low shear rate all the blends displayed Newtonian flow, and as the shear rate increased a shear-thinning (non-Newtonian) trend was observed. The PoLigel-LCl blend had a faster onset of shear-thinning than the PoLigel-LA blend (Figure 2.4). During rheological testing the PoLigel-LCl blend at high shear rates separated, however, this was not observed for the PoLigel-LA blend. This further illustrates the higher stability of the PoLigel-LA blend compared to the PoLigel-LCl blend. The effect of initial drug loading on rheology of the PoLigel-LA blend was also assessed. As shown in Figure 2.4, an increase in the drug loading level resulted in an increase in the viscosity of the formulation. Furthermore, the onset of shear-thinning was seen at higher shear rates as the drug loading level increased. Based on stability assessment, pH profile, colocalization data and rheological measurements, the PoLigel-LA blend showed superior stability properties in comparison to the PoLigel-LCl blend. As such, the PoLigel-LA blend was further characterized as a delivery system for DTX.
Figure 2.4 Viscosity as a function of shear stress for different PoLigel blends characterized by a stress-controlled rheometer with a 4 cm cone and 2° angle plate geometry attachment at room temperature.
2.4.4 In Vitro Drug Release

As shown in Figure 2.5, the in vitro release of DTX from the PoLigel-LA blend as a function of initial drug loading was evaluated in buffer containing lysozyme and albumin. As mentioned previously, lysozyme is known to degrade chitosan. We have previously shown that evaluation of in vitro drug release in PBS buffer (0.01 M pH 7.4) containing 2 mg/mL lysozyme accurately models drug release in ascites, a fluid found in high abundance in the peritoneal cavity of ovarian cancer patients [234]. Ascites (pH ~ 7.4 to 7.8) is composed of various proteins including serum albumin [250] and taxane drugs such as DTX have been shown to have high affinity for albumin [251]. For these reasons, lysozyme and albumin were added to the release media to better model physiological conditions.

A biphasic release profile was obtained for DTX over the two-week study period, characterized by a more rapid release phase during the initial 24 h and a sustained, slower drug release phase for the remainder of the 14-day period. The initial higher release phase can be attributed to drug associated with the surface of the blend. The sustained phase can be attributed to the DTX that has partitioned into the hydrophobic regions (i.e. ePC and LA) within the blend. During the initial 24 h period, 8-17% of total DTX loaded in the PoLigel-LA blends was released. Following the initial 24 h, a sustained release rate of 3.7 ± 1.9%, 3.4 ± 0.6% and 2.4 ± 0.7% of DTX per day was seen from the 10, 20 and 30 mg DTX loaded PoLigel-LA blends, respectively. The release rate of DTX from the PoLigel-LA blend was concentration-dependent, with a lower drug loading level resulting in more drug released within the first 24 h followed by a higher rate of sustained drug release during the second phase, in comparison to the formulations with higher drug loading levels. This may be attributed to the rheological properties of the formulations. As shown in Figure 2.4, the rheological properties increased by approximately two orders of magnitude when the drug loading level increased from 10 mg to 30 mg. In general, blend viscosity can increase when there are greater interactions between the different molecules that comprise the formulation, including molecular entanglements, physical
interactions (i.e. hydrophobic, van der Waals and hydrogen bonding) and cross-linking [219].
These types of interactions can consequently entrap the drug and therefore reduce the rate of drug release.
Figure 2.5 *In vitro* release of DTX from PoLigel-LA blends as a function of initial DTX loading level in 0.01M PBS (pH 7.4) with 2 mg/mL lysozyme and 40 mg/mL albumin at 37°C over a two-week period. The results represent mean ± SD (n = 3).
2.4.5 DTX Biodistribution and Toxicity Assessment

As shown in Figure 2.6A, constant plasma drug levels, with an average of 0.056 ± 0.009 ng/mL DTX, were detected over the two-week study period (4.4 ± 0.7% DTX per day). The sustained in vivo DTX levels were on par with the observed sustained in vitro release (2.4 ± 0.7% DTX per day). Tissue drug concentrations were also examined (Figure 2.6B). Levels were significantly higher (p < 0.05) in the peritoneal lining and intestines when compared to liver, spleen, kidneys and heart. The average drug accumulation in the various organs or tissues, over the two-week study period, was as follows: peritoneal lining (13 ± 3 µg/g), intestines (1.2 ± 0.4 µg/g), liver (0.52 ± 0.08 µg/g), spleen (0.46 ± 0.06 µg/g), kidneys (0.24 ± 0.06 µg/g) and heart (0.20 ± 0.02 µg/g). Constant drug levels were seen in all tissues over two weeks (p > 0.05) with the exception of the peritoneal lining on day 7 (p < 0.05).

DTX concentrations in the peritoneal lining and intestines were 234 and 22 times higher than concentrations found in plasma, respectively. Comparable to our findings, Marchettini et al. also detected highest drug concentrations within the abdominal wall and colon of male Sprague Dawley rats following bolus IP administration of Taxotere® [252]. This is explained by the fact that IP administration leads to tissue drug uptake not only via the systemic circulation, as would be the case after iv administration, but also directly from the peritoneal cavity [253]. Our localized delivery approach leads to high local drug concentrations and much lower systemic exposure which should theoretically result in reduced toxicity to healthy tissues such as the bone marrow and greater efficacy than iv chemotherapy [254]. High concentrations in the peritoneal region, especially in the peritoneal muscle, suggest the application of this formulation in the treatment of cancers originating in the peritoneal cavity and those presenting peritoneal dissemination, such as gastrointestinal, ovarian, colorectal, pancreatic and others [255]. In these cases, metastatic lesions form along the peritoneal lining. Damage to the lining due to surgical removal of the lesions is known to encourage further peritoneal metastasis; thus, the
high drug levels in the peritoneal lining seen in this study present an attractive therapeutic alternative to surgery [256].

Consistent with biocompatibility studies performed using the drug-free PoLigel-LA blend, loading the formulation with DTX does not alter its biocompatibility [241]. According to visual observation, the high drug levels found in the intestines did not lead to intestinal toxicity (i.e. no signs of paralytic ileus). In fact, clinical observation has noted the presence of a rich capillary network with high flow rates which protects the proliferative intestinal tissue upon IP chemotherapeutic delivery [257]. Throughout the study period no signs of toxicity were observed, as the animals did not display weight loss, signs of immobility, anorexia, dehydration, or peritonitis. At all time points, the formulation was found free of fibrous encapsulation, which would be indicative of a foreign body response. ALT levels in both control mice and those injected with the formulation (25 ± 8 U/L and 17 ± 4 U/L, respectively) remained well below 200 U/L, levels which are indicative of hepatotoxicity in mice [258-260]. As it is plausible that introduction of foreign material into the body can result in immune response and tissue injury, resulting in the activation of an inflammatory reaction [261, 262], we monitored circulating levels of IL-6, a systemic pro-inflammatory mediator. IL-6 plasma concentrations of 53 ± 9 and 27 ± 5 pg/mL were seen in treated and control animals, respectively (p < 0.05). Despite the slight increase in IL-6 values seen in treated mice, these levels are still within the normal reported range for healthy CD-1 mice and are greatly below levels reported to be indicative of significant inflammation [263]. For example, CD-1 mice with peritonitis typically have serum IL-6 levels in the range of 10-15 ng/mL [264]. Overall, these results indicate that PoLigel-LA-DTX blend does not induce significant local toxicity or systemic inflammation.
Figure 2.6 DTX distribution following IP administration of PoLigel-LA-DTX blend in healthy female CD-1 mice. (A) Plasma and (B) liver, spleen, kidney, heart, intestine and peritoneal layer DTX concentrations. No significant differences ($p > 0.05$) in plasma or tissue concentrations were seen between samples obtained from treated mice on days 1, 4, 7 and 14 with the exception of the peritoneal muscle on day 7. The results represent mean ± SD ($n = 6$).
2.4.6 Anti-tumor Efficacy

The ability of PoLigel-LA-DTX blend to inhibit tumor growth was assessed in a SKOV3 xenograft model of human ovarian adenocarcinoma. As shown in Figure 2.7, both doses of PoLigel-LA-DTX blends used demonstrated antitumor efficacy when compared to control animals \((p < 0.05)\). A tumor burden inhibition of \(87 \pm 9\%\) was achieved with the 28.8 mg/kg dose. A \(74 \pm 26\%\) tumor burden reduction was achieved at a dose of 19.2 mg/kg, a dose that is lower than the recommended IP dose for ovarian cancer patients in clinical trials [64]. These results show that even a lower dose of DTX delivered in a sustained and localized manner via the PoLigel-LA blend results in significant tumor inhibition. It should be noted that since the PoLigel-LA blend provides sustained release of DTX over a prolonged period of time in a localized environment, the therapeutically relevant dose for treatment of ovarian cancer may differ from that required for bolus IP administration of this agent.

It has been shown that frequent, low-dose chemotherapy, referred to as ‘metronomic’ chemotherapy, provides superior antitumor effects and clinical efficacy over the traditional MTD approach [265]. Previously developed chitosan-phospholipid film implants capable of continuous PTX delivery have demonstrated excellent efficacy against SKOV3 xenograft tumor models when compared to that achieved by intermittent MTD regimens [96, 123]. In these studies, greater efficacy of sustained therapy was attributed to a decrease in tumor cell repopulation and a lack of drug resistance gene up-regulation [149, 266]. Since DTX is a semi-synthetic analog of PTX, it is plausible that these similar mechanisms contribute to the high efficacy seen in this study; however, further studies are needed to explore possible mechanisms. The PoLigel-LA-DTX blend has the potential to provide low toxicity and high efficacy associated with continuous, low-dose chemotherapy, as shown by this pilot study of efficacy. Future studies will focus on comparing the efficacy of frequent low doses of DTX, provided by PoLigel-LA-DTX blend, to Taxotere® administered in a MTD regimen.
Figure 2.7 PoLigel-LA-DTX blend anti-tumor efficacy in SKOV3 ovarian cancer xenograft model. Human ovarian tumors were induced by SKOV3 cell inoculation in female CD-1 nu/nu mice. Treatment with PoLigel-LA-DTX blends (total DTX doses: 28.8 and 19.2 mg/kg) was initiated 14 days post SKOV3 inoculation, with 20 μL injected sterile saline solution as control. * represents significant difference between the treatment groups and control (p < 0.05). The results represent mean ± SD (n = 4).
2.5 Conclusions

To our knowledge, this is the first report of a delivery system that provides localized and sustained delivery of DTX following IP administration. The injectable blend was found to result in sustained plasma concentrations of DTX and constant levels of drug exposure in the peritoneal cavity of healthy mice over the two-week period, with no signs of toxicity or inflammation. Furthermore, the injectable blend showed significant tumor inhibition in a murine xenograft model of human ovarian adenocarcinoma. This localized delivery system has excellent potential for sustained IP treatment of cancers, such as ovarian, that reside in the peritoneal cavity and may be utilized for delivery of other anti-cancer drugs as well.

2.6 Acknowledgments

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CHAPTER 3. Docetaxel Distribution Following Intraperitoneal Administration in Mice

Payam Zahedi, Raquel De Souza, Micheline Piquette-Miller, Christine Allen.


All experiments and data analyses were carried out by P. Zahedi. Animal experiments were performed with technical assistance from R. De Souza.

This chapter was written primarily by P. Zahedi.


### 3.1 Abstract

IP chemotherapy with high molecular weight lipophilic anti-neoplastic agents such as the taxanes has shown promise in clinical trial evaluation for treatment of localized peritoneal cancers. We have previously developed an IP injectable hydrogel formulation (PoLigel) for sustained peritoneal delivery of DTX, and observed significant efficacy in murine models of ovarian cancer when compared to Taxotere®, the FDA approved formulation of DTX. In order to understand the relationship between drug distribution and efficacy, the current study compares the tissue distribution and pharmacokinetics of DTX administered IP in the PoLigel or Taxotere® formulations. The PoLigel was prepared by blending a water-soluble chitosan derivative, egg phosphatidylcholine and lauric aldehyde with DTX (drug to material ratio 1:8 w/w). DTX concentrations in plasma, heart, liver, spleen, stomach, intestine, kidney and peritoneal layer were measured over a five day period following IP administration of the PoLigel and Taxotere® formulations in CD-1 female mice. Three days after Taxotere® administration, no detectable levels of DTX were seen in plasma, while sustained DTX plasma levels of 0.06 µg/mL ± 0.01 per day were observed with PoLigel. At five days post Taxotere® administration, only intestine, stomach and peritoneal layer showed detectable DTX concentrations whereas all tissues and plasma showed sustained DTX levels in mice that received PoLigel. DTX concentrations that resulted from PoLigel administration were significantly higher in the peritoneal cavity and 200 fold higher than concentrations found in plasma. Overall, the PoLigel formulation increases tissue and plasma drug retention and provides sustained DTX levels compared to the clinically used Taxotere® formulation. The sustained DTX levels seen in the peritoneal cavity following IP administration of the PoLigel may be responsible for the improvement in efficacy that has been observed in our previous studies.
3.2 Introduction

IP chemotherapy involves the administration of therapeutic agents directly into the peritoneal cavity to achieve high local concentrations of drug for an extended period of time while minimizing systemic exposure. DTX, an anti-mitotic cytotoxic drug, has been investigated for IP therapy [60] due to its high molecular weight (807.9 g/mol), hydrophobic nature (water solubility: 5-6 µg/mL) and hepatic metabolism (over 90%) [88, 267, 268]. Taxotere® is the clinically used formulation of DTX, and when administered IP in patients with cancers localized to the peritoneal cavity, results in higher drug concentrations in the peritoneal cavity than in the systemic circulation. A Phase I study by Morgan et al. in patients with peritoneal malignancies including ovarian and gastric cancers showed the mean pharmacokinetic advantage, defined as the ratio of peritoneal AUC to plasma AUC over a 24 hour period, to be 181 following IP administration of Taxotere® [64]. Similarly, Fushida et al. observed a pharmacokinetic advantage of 515 (i.e. AUC_{peritoneal_{0-24h}}/AUC_{plasma_{0-24h}}) in gastric cancer patients following IP administration of Taxotere® [269]. Although IP administration of bulky, hydrophobic drugs such as DTX initially results in a high pharmacokinetic advantage, these drugs are eventually absorbed through peritoneal capillaries into the systemic circulation, followed by quick elimination via hepatic metabolism [270, 271].

Prolonged drug exposure has been shown to increase tumor responsiveness to chemotherapeutics [93-96, 98]. For this reason, lengthening the time that chemotherapeutics are retained within the peritoneal cavity may be beneficial for treatment of peritoneal localized cancers. One approach that has been used to increase the retention time of drugs in the peritoneal cavity is formulation of drugs in excipients such as surfactants or advanced delivery systems. For example, Taxotere® consists of DTX solubilized in the non-ionic surfactant Polysorbate 80. To date there are only reports on the levels of DTX in the peritoneal cavity and plasma up to 24 hours post IP administration of Taxotere® [272-274]. Longer studies on Taxotere® tissue and plasma distribution following IP administration are warranted. As well,
Polysorbate 80 has been shown to cause toxicity, alter the cellular uptake of DTX and interfere with its activity [7, 89, 90, 94]. To further increase the retention of drugs in the peritoneal cavity and circumvent issues with Polysorbate 80, drug delivery vehicles such as nanoparticles, microspheres and hydrogels have been explored. Nanoparticles can prolong the peritoneal residence time of free drug; however, they are typically cleared within two days through absorption into the lymphatic circulation [202-204]. Microspheres have been shown to lead to further improvements in peritoneal retention of drugs in comparison to nanoparticles [203, 204]. However, they have also been associated with drawbacks including foreign body reactions, residual polymer filaments in abdominal tissues several months after treatment [153], and development of peritoneal adhesions which can be potentially lethal [203, 275]. To date the majority of the microsphere systems evaluated for IP administration of drugs have been formed from polyester-based polymers and as such the issues with these systems may in part be attributed to the materials employed [218, 228-230].

The incorporation of free drugs or nanoparticulate-based delivery systems into implantable or injectable hydrogels can further prolong retention times. Yeo et al. accomplished this by incorporating nanoparticles within an in situ crosslinkable hydrogel, which increased the retention time of the nanoparticles within the peritoneal cavity from two days [275] to one week [276]. Similarly, Grant et al. incorporated drug-loaded nanoparticles into a polymer-lipid implantable film [217], which provided peritoneal drug release over several weeks following IP implantation in mice [218, 234]. With this goal in mind, we have recently developed and characterized an injectable biocompatible and biodegradable polymer-lipid hydrogel formulation (PoLigel) for localized IP delivery of DTX [98]. Sustained delivery of DTX using the PoLigel formulation has resulted in greater inhibition of disease progression, compared to Taxotere® administered IP at equivalent doses, in two distinct orthotopic models of ovarian cancer [94]. In order to understand the relationship between drug distribution and efficacy, the current study
compares the tissue distribution and pharmacokinetics of DTX administered IP in the PoLigel formulation and Taxotere®.

### 3.3 Materials and Methods

#### 3.3.1 Materials

DTX was purchased from Jari Pharmaceutical Co. (Jiangsu, China). Chitosan was purchased from Marinard Biotech Inc. (Quebec City, Canada). Taxotere® (40mg/mL) was purchased from Sanofi-Aventis. Hydrogen peroxide, ePC, GTMAC and LA were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada). Scintigest Tissue Solubilizer was purchased from Thermo Fisher Scientific (Waltham, USA). Ready Safe Scintillation Cocktail was purchased from Beckman Coulter (Mississauga, Canada). All other chemicals were reagent grade and used as received.

#### 3.3.2 Preparation of the Polymer-Lipid Formulation

The polymer-lipid hydrogel formulation (i.e. PoLigel) was prepared as outlined elsewhere [98]. In brief, a WSC [236] was dissolved in distilled deionized water to prepare a 4.2% (w/v) solution. DTX was dissolved in anhydrous ethanol and dried under nitrogen to form a thin layered film and then placed under vacuum for 24 h to remove any residual solvent. An ePC-LA solution (ePC to LA ratio of 1:4 w/w) was used to re-suspend the DTX film. Finally the WSC solution was added to the ePC-LA-DTX solution and vortexed for 1 min (drug to material ratio of 1:8 w/w). Samples were sterilized under UV-light (Sterilizer T209, Intercosmetics, Canada) for 3 h prior to use in animals.

#### 3.3.3 Analysis of Plasma and Tissue Distribution of Drug

All animal studies were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee and the Canadian Animal Care Council. Female CD-1 mice (6-8 weeks
old, 20 g) purchased from Charles River (St. Constant, Canada) were used. Mice were injected IP (DTX dose: 7mg/kg) with PoLigel or Taxotere® in the lower left quadrant of the peritoneal cavity, with an injection depth of 1 cm using a 25 gauge needle. In previous studies the PoLigel formulation was shown to provide sustained release of 7 mg/kg over a five day period [98]. Each treatment group was further randomized according to the length of therapy (1, 3, 6, 12, 18, 24, 48, 72, 96 and 120 h). At specific time points, mice (n = 4) were anesthetized and sacrificed by exsanguination via cardiac puncture. Plasma, heart, liver, spleen, stomach, intestine, kidney and peritoneal layer were collected for HPLC analysis.

HPLC work was conducted as outlined elsewhere [98]. In summary, an Agilent Series 1100 HPLC (Agilent Technologies, Canada) equipped with a Waters 4.6 mm x 250 mm column (XTerra® MS C18, 5 µm particle size) and Waters 3.9 x 20 mm guard column (XTerra® MS C18, 5 µm particle size), Waters Dual Absorbance Detector 2487 (Waters, USA) and ChemStation software (Agilent Technologies, Canada) was used for analysis. The wavelength of detection for DTX was 227 nm. A mobile phase of 60% 0.01 M PBS (pH=10) and 40% acetonitrile was used with a flow rate of 1 ml/min and an injection volume of 20 µL per sample. An internal standard of paclitaxel (10 µg/mL) was used for all HPLC analysis. The LOD was 5 ng/mL and 10 ng/mL for plasma and tissues, respectively. The LOQ was 10 ng/mL and 20 ng/mL for plasma and tissues, respectively.

3.3.4 Analysis of Intraperitoneal Distribution of Drug

Twelve mice received PoLigel-³H-DTX (DTX dose: 7 mg/kg, 0.002% ³H-DTX w/w) in the lower left quadrant of the peritoneal cavity, with an injection depth of 1 cm using a 25 gauge needle. At specific time points, mice (n = 4) were anesthetized and sacrificed by exsanguinations via cardiac puncture. Distal and proximal tissue sections (liver, intestine, kidney and peritoneal layer) with respect to the site of PoLigel injection (Figure 3.1) were collected for analysis of radioactivity. Scintillation counting was used to quantify the amount of radioactivity in each
tissue sample. Each tissue sample was homogenized at a 10% (w/v) concentration in deionized distilled water using a tissue homogenizer (Wheaton, USA). 100 µL of tissue homogenate or plasma was added into scintillation vials containing 1 mL Scintigest Tissue Solubilizer solution. Samples were kept at 55°C for 2 h, followed by the addition of 200 µL of 30% (v/v) hydrogen peroxide. The samples were kept at 55°C for an additional 30 min, after which each sample received 4 mL Ready Safe Scintillation Cocktail followed by scintillation counting (Beckman Coulter LS 5000TD, Beckman instruments Inc., USA). The amount of drug in each sample was quantified using a calibration curve.

3.3.5 Statistical Data Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). A two-sample t-test was used to measure statistical significance between pairs of results. For statistical analyses among three or more groups, ANOVA was used and subsequent multiple comparisons with Bonferroni correction were performed if statistical significance was detected by the ANOVA. A p-value < 0.05 was considered statistically significant.

3.4 Results

The plasma profile for Taxotere® showed a 3-fold higher DTX concentration compared to the PoLigel at 1 hour post-administration, and by 72 hours the levels in the plasma fell below the detection limit of the assay (Figure 3.2). Plasma levels of 0.140 µg/mL ± 0.048 at 1 hour post PoLigel administration were measured and, by 24 hours, the DTX plasma levels decreased by two fold. Following this, sustained DTX plasma levels of 0.060 µg/mL ± 0.010 per day were observed (Figure 3.2). The distribution of DTX in tissues is shown in Figure 3.3. The average concentrations of drug in tissues for the first 24 hours following administration of Taxotere® were as follows: peritoneal layer (2.5 ± 1.1 µg/g), stomach (11 ± 4 µg/g), spleen (8.9 ± 1.3 µg/g),
intestines (3.8 ± 2.1 µg/g), liver (1.4 ± 0.2 µg/g), kidneys (0.57 ± 0.31 µg/g) and heart (0.45 ± 0.16 µg/g). At the end of the five day period, only intestine, stomach and peritoneal layer showed detectable DTX concentrations (Figure 3.3A).

The average concentration of drug in tissues for the first 24 hours following PoLigel administration was as follows: peritoneal layer (3.8 ± 0.9 µg/g), stomach (1.4 ± 0.8 µg/g), spleen (0.66 ± 0.32 µg/g), intestines (1.4 ± 0.8 µg/g), liver (0.76 ± 0.48 µg/g), kidneys (0.21 ± 0.11 µg/g) and heart (0.077 ± 0.07 µg/g). DTX concentrations in the peritoneal layer were 200-fold higher than concentrations found in plasma. The distribution of DTX in the peritoneal cavity following IP administration of the PoLigel was also investigated. In order to assess this, tissues (i.e. peritoneal layer, kidney, liver and intestine) were separated into proximal and distal sections relative to the site of PoLigel injection (Figure 3.1). No statistically significant difference (p>0.05) in DTX concentration was seen between the proximal and distal sections of all tissues evaluated (Figure 3.4).
Figure 3.1 (A) Post-mortem view of the PoLigel in the peritoneal cavity of a female CD-1 mouse (distal and proximal is relative to site of PoLigel injection). (B) Chemical structures of drug and material components of the PoLigel.
Figure 3.2 DTX plasma levels following IP administration of PoLigel or Taxotere® in female CD-1 mice (total DTX dose: 7mg/kg). By 72 hours DTX levels in the plasma fell below the detection limit of the assay for the Taxotere® group. The results represent mean ± SD (n = 4).
Figure 3.3 DTX tissue distribution following IP administration of (A) Taxotere® or (B) PoLigel in female CD-1 mice (total DTX dose: 7mg/kg). The results represent mean ± SD (n = 4).
Figure 3.4 Concentrations of DTX in tissue sections that were proximal and distal to the site of injection of the PoLigel formulation (see Figure 3.1) in female CD-1 mice (total DTX dose: 7mg/kg). (A) Peritoneal layer, (B) liver, (C) intestine and (D) kidney. No significant differences ($p > 0.05$) in DTX concentration between proximal and distal sites were seen. The results represent mean $\pm$ SD ($n = 4$).
3.5 Discussion

Localized IP drug delivery in a sustained manner can provide significant therapeutic advantages for peritoneal localized diseases by ensuring high drug concentrations at the target site, extended drug exposure, and lower systemic toxicity. Strategies to extend drug retention time in the peritoneal cavity using drug delivery systems such as nanoparticles, microspheres and hydrogels have been investigated [98, 202-204, 217]. Of these, hydrogels have been shown to enable the most significant improvements in drug retention, within the peritoneal cavity following IP administration. Implantable [217] and injectable [98, 219] hydrogels have been developed by our laboratory for IP localized and sustained delivery of taxanes (i.e. PTX and DTX). Sustained plasma drug levels have been observed in murine models for up to one month post administration of these hydrogel systems [98, 234]. Furthermore, evaluation of efficacy in murine orthotopic models of ovarian cancer have demonstrated significant reduction in disease burden upon treatment with the taxane-containing hydrogel systems when compared to treatment with the FDA approved formulations of these drugs [94, 96, 98, 123]. Recently we have shown that sustained DTX exposure (i.e. via the PoLigel) results in greater antitumor efficacy than Taxotere® administration in ovarian cancer xenografts, which was due to greater tumor cell kill and reduced proliferation and angiogenesis [94]. We believe that the differences seen in efficacy between the two formulations may be justified by their peritoneal drug distribution profile.

Following IP administration of either PoLigel or Taxotere®, high concentrations of DTX in the peritoneal layer and stomach were observed compared to other peritoneal tissues such as kidney, liver, spleen and intestine. The peritoneal layer is composed of mesothelium and connective tissue layers. The main transport route for drugs into the systemic circulation from the peritoneal cavity is through the capillaries found within the peritoneal layer [277, 278]. Since this layer is largely composed of poorly vascularised connective tissue, the blood capillary density is low. High molecular weight lipophilic drugs such as DTX have a slow uptake rate from
the peritoneal cavity as compared to other agents [254]. This characteristically slow uptake of DTX into capillaries, compounded with the low blood capillary density of the peritoneal layer and sustained drug release from the PoLigel, results in greater drug accumulation within the connective tissue layers of the peritoneum compared to other tissues. Similar to our findings, Marchettini et al. [252] reported high concentrations of DTX (administered as Taxotere®) in the abdominal wall (i.e. peritoneal layer) and in the omentum up to 24 hours following IP administration. The high drug concentrations in the peritoneal cavity and homogenous drug tissue distribution (i.e. distal and proximal to the PoLigel) suggest the application of the PoLigel formulation in the treatment of cancers confined to the peritoneal cavity such as ovarian, colorectal and gastrointestinal which in advanced stages form metastatic lesions along the peritoneal surface [279]. In addition, damage to the peritoneal surface due to cytoreductive surgery of the lesions is known to encourage further peritoneal metastasis [256]. Overall, sustained drug levels observed in the peritoneal layer after treatment with the PoLigel could allow for greater efficacy in advanced stage metastatic peritoneal cancers.

DTX is highly hydrophobic and, for this reason, the non-ionic surfactant Polysorbate 80 is currently used as the formulation vehicle for this drug (Taxotere®, Sanofi-Aventis). Post administration Polysorbate 80 forms nano-sized micelle-like structures that solubilize DTX [7]. As shown in the tissue distribution profiles (Figure 3.3A) this formulation results in some retention of the drug within peritoneal tissues. However, this surfactant is associated with hypersensitivity reactions, and has been shown to alter cellular uptake, tissue distribution, and the activity of DTX [7, 89, 90, 94]. Therefore, to further prolong drug retention within the peritoneal cavity and to circumvent these issues drug delivery strategies such as PoLigel must be considered.
3.6 Conclusions

The influence of two different formulation strategies (i.e. Taxotere® and PoLigel) on the peritoneal and plasma levels of DTX was evaluated. For the first time the long term (i.e. > 24 hours) tissue and plasma distribution of DTX was assessed following IP administration of Taxotere®. Importantly, 72 hours following the IP injection of Taxotere® the drug levels in plasma were undetectable and only detectable levels in the intestine, stomach and peritoneal layer tissues were observed. In contrast, sustained DTX tissue and plasma levels were observed following IP administration of the PoLigel over the 120 hour period. In summary, the PoLigel formulation increases tissue and plasma drug retention and provides sustained DTX levels compared to the current clinically used Taxotere® formulation, which may explain the improvement in efficacy that has been observed in our previous studies.

3.7 Acknowledgments

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CHAPTER 4. An Injectable Depot System for Sustained Intraperitoneal Chemotherapy of Ovarian Cancer Results in Favorable Drug Distribution at the Whole Body, Peritoneal and Intratumoral Levels

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All experiments and data analyses were carried out by P. Zahedi. J. Stewart developed the MATLAB® algorithms. Animal experiments were performed with technical assistance from R. De Souza. Immunohistostaining was performed by the Pathology Research Program, University Health Network.

This chapter was written primarily by P. Zahedi.
4.1 Abstract

The current study characterizes the impact of DTX distribution on efficacy following sustained IP chemotherapy in murine models of ovarian cancer. A polymer-lipid biodegradable depot (PoLigel) was used to deliver DTX in a sustained manner over 21-days following IP administration. Distribution and efficacy studies were carried out in SCID mice bearing SKOV3 IP solid tumors or C57BL/6 mice with ID8 IP ascites fluid. In addition, a subcutaneous (SC) SKOV3 model was used to determine whether systemic drug levels that result from IP administration of the PoLigel influence antitumor efficacy. Immunostained IP and SC SKOV3 tumor sections were used to study cell death, intratumoral drug distribution and tumor penetration. Sustained concentrations of DTX were observed in plasma, tissue, tumor and ascites over the entire study period. Drug accumulation was several fold greater in tumors and ascites when compared to plasma levels. Sustained chemotherapy resulted in significant inhibition in tumor burden and ascites volume. IP tumors showed greater cell death compared to the SC tumors as seen by higher TUNEL and caspase-3 expression. At the intratumoral level, DTX distributed more towards the core of IP tumors compared to the SC tumors. Tumor penetration of drug from nearest blood vessel was 1.5 fold greater in the IP tumors than the SC tumors. Overall, favorable drug distribution at the whole-body, peritoneal and intratumoral levels in combination with local and systemic sustained drug exposure contribute to the high efficacy observed. These results support the clinical use of IP sustained chemotherapy for ovarian cancer.
4.2 Introduction

The peritoneal cavity is the principal site of ovarian cancer both at diagnosis and relapse [280]. The delivery of chemotherapeutics through IP administration into the peritoneal space has the potential to expose the cancer to higher concentrations of drug over longer periods of time while minimizing systemic toxicity [60]. Phase III trials have demonstrated a substantial survival advantage following IP chemotherapy in patients with optimally debulked ovarian cancer when compared to intravenous chemotherapy [57-59]. Nevertheless, the use of IP chemotherapy has remained limited due to local toxicities and complications associated with prolonged use of indwelling catheters required to deliver the chemotherapeutics [71].

Drug delivery systems including liposomes, nanoparticles and microspheres have been explored for IP chemotherapy in an effort to overcome catheter-related problems. However, liposomes and nanoparticles are rapidly cleared from the peritoneal cavity by absorption into the lymphatic circulation [202-204], thus requiring frequent administration. Microspheres are retained longer in the peritoneal cavity [203, 204, 281], although local toxicities caused by material components have been documented [153]. Another approach to increase drug retention and minimize frequent dosing has been the use of IP implantable drug delivery systems. Grant et al. have developed a PTX loaded implant for IP chemotherapy of ovarian cancer [217]. This implant was shown to be biocompatible, provide sustained drug levels in the peritoneal cavity and lead to greater efficacy compared to standard intermittent chemotherapy [96, 123, 218, 234].

Sustained IP chemotherapy with DTX, a semi-synthetic analogue of PTX, holds much promise for ovarian cancer therapy. Firstly, high peritoneal DTX levels are attainable upon IP administration, as clinical studies have shown 152-207 fold greater DTX concentrations in the peritoneal cavity when compared to systemic drug levels [254]. Furthermore, DTX is a cell cycle specific drug [99] and only a small percentage of cancer cells are found at vulnerable phases (i.e. G2/M) at any given time [100]. Achieving sustained DTX levels ensures the drug is
present when more cells cycle through these phases, potentially resulting in greater cell death. Recently an injectable depot (PoLigel) was developed in order to achieve sustained delivery of DTX in the peritoneal cavity [98]. The physicochemical properties of the PoLigel formulation as well as molecular interactions and organization of materials within PoLigel have been evaluated and published elsewhere [98]. Sustained release of DTX from the PoLigel delivery system was confirmed in mice with 4% of the total DTX loaded being released per day [98]. The PoLigel formulation does not induce local toxicity or result in a systemic inflammatory response and is progressively degraded \textit{in vivo}, with $7.4 \pm 5.0\%$ of the original injected mass remaining four weeks following IP administration [98, 241]. The PoLigel resulted in a significant decrease in tumor burden and ascites fluid accumulation when compared to intermittent IP administration of Taxotere®, the clinically used formulation of DTX [94]. Additionally, studies evaluating the impact of sustained versus intermittent chemotherapy at the cellular and molecular levels revealed that sustained DTX resulted in greater cell death, less angiogenesis, less cell proliferation and a reduction in the development of drug resistance [94, 122].

Another limitation that has hindered widespread clinical use of IP chemotherapy, administered using an indwelling catheter, has been the heterogeneous drug distribution that results within the peritoneal cavity [282-284]. In order to improve drug distribution a large volume of fluid is instilled into the peritoneal cavity at time of treatment, which has been shown to result in significant pain and patient discomfort [282]. Alternatively, the PoLigel has been shown to result in a homogeneous distribution of drug within the peritoneal cavity, of healthy CD-1 mice, without the need for fluid co-administration [98]. As well, compared to IP administration of Taxotere® in healthy CD-1 mice, the PoLigel results in sustained peritoneal drug levels [285]. The present study investigated DTX distribution at the whole-body, peritoneal and intratumoral levels in disease bearing mice in an effort to further understand the improved efficacy that results from sustained IP DTX delivery. Furthermore, as the PoLigel provides sustained plasma levels of DTX, the influence of sustained systemic drug exposure on
antitumor efficacy was explored. This was accomplished by evaluating efficacy following IP administration of PoLigel in a SC murine model.

4.3 Materials and Methods

4.3.1 Materials

DTX was purchased from Jari Pharmaceutical Co. (Jiangsu, China). Tritium labeled DTX (3H-DTX) was purchased from American Radiolabeled Chemicals (St. Louis, USA). Chitosan was purchased from Marinard Biotech Inc. (Quebec City, Canada). ePC and LA were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada). All other chemicals were reagent grade and used as received.

4.3.2 Cell Culture

The SKOV3 human ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, USA). The murine epithelia ovarian cancer cell line ID8 was a gift from Dr. Jim Petrik (University of Guelph, Canada) [286]. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 mg/mL streptomycin), grown as a monolayer at 37°C in 5% CO₂ and 90% relative humidity.

4.3.3 Preparation of Polymer-Lipid Formulation

The PoLigel was prepared as outlined elsewhere [98]. In brief, a WSC synthesized by conjugating glycidyltrimethylammonium chloride onto the chitosan backbone [236] was dissolved in distilled deionized water to prepare a 4.2% (w/v) solution. DTX was dissolved in anhydrous ethanol and dried under nitrogen and then placed under vacuum for 24 h to remove any residual solvent. To the DTX containing vial, ePC and LA (1:4 w/w) were added and vortexed until the drug film dissolved. Finally the WSC solution was mixed with the ePC-LA-DTX
solution and vortexed for 1 min (drug to material ratio of 1:8 w/w). Samples were sterilized under UV-light for 3 h prior to use in animals.

4.3.4 Biodistribution

All studies were conducted in 6-8 week old female SCID or C57BL/6 mice (Charles River, Canada) using sterile techniques and in accordance with the guidelines of the University of Toronto Animal Care Committee and the Canadian Animal Care Council. Two IP models and one SC model were used. For the IP models, SCID mice (n = 4) received $1 \times 10^6$ SKOV3 cells and C57BL/6 mice (n = 4) received $2.5 \times 10^6$ ID8 cells suspended in 200 µL PBS via IP injection into the peritoneal cavity. Seven and fourteen days post inoculation with SKOV3 and ID8 cells, respectively, mice were injected IP with the PoLigel (DTX dose: 32 mg/kg, 0.002% 3H-DTX) in the lower left quadrant. For the SC model, SCID mice (n = 4) were inoculated SC in the right flank with $2 \times 10^6$ SKOV3 cells suspended in 200 µL PBS. Seven days post inoculation (tumors ~ 2 mm), mice were injected IP with the PoLigel (DTX dose: 32 mg/kg) in the lower left quadrant. Control animals for each model (n = 4) were injected IP with 20 µL sterile saline solution post cancer cell inoculation. Weight loss was monitored by regular body weight measurements, and the presence of all other endpoints was examined visually by animal care technicians and veterinarians. Endpoints requiring humane euthanasia included excessive muscle wasting, abdominal distention, hypothermia, inactivity, and weight loss in excess of 20%. Plasma, tumors, ascites, liver, spleen, stomach, intestine, heart, kidney and peritoneal layer were collected from the IP models at various time points and analyzed using scintillation counting as described previously [285]. Plasma and tumors were collected from the SC model on day 21 post treatment and analyzed using HPLC as detailed elsewhere [98].
4.3.5 Immunohistochemistry

IP and SC SKOV3 tumors were processed and immunostained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), caspase-3 and CD-31 as previously described [96]. In brief, after initial processing tumor sections were incubated with active caspase-3 antibody (1/50 dilution overnight), CD-31 primary antibody (1/50 dilution overnight), or with biotin-nucleotide cocktail and DNA polymerase 1 for 1 h at 37°C for TUNEL. Sections were counterstained with Mayer’s haematoxylin. IP SKOV3 tumors were immunostained during a previous study [94], but re-analyzed using an improved image analysis technique as outlined below. Each tumor section was digitized using a bright-field scanner (ScanScope XT, Aperio Technologies Inc.) at 20x magnification with a resolution of 0.5 microns/pixel.

4.3.6 Intratumoral Distribution

Semiquantitative analysis of the intratumoral distribution of DTX in IP and SC SKOV3 tumor sections immunostained for caspase-3 was carried out using a customized image analysis program written in MATLAB® (version 7.9.0.529, R2009b). The border of each tumor section was first delineated by thresholding the image in order to remove any background noise. Next, the caspase-3 stained regions in the original immunostained tumor section were determined based on an empirically chosen threshold of the color intensity. Finally, the minimum Euclidean distance between each caspase-3 region to the periphery of the tumor section was calculated and pooled into three separate regions (i.e. edge, inner and core) with equidistant thicknesses.

4.3.7 Tumor Penetration

DTX tumor penetration from nearest blood vessel was analyzed in the IP and SC SKOV3 tumor sections immunostained for caspase-3 and CD-31 using a customized MATLAB® algorithm. Digitized images of caspase-3 and CD-31 immunostained tumor sections were first superimposed and rigidly registered by manual selection of points of similarity between the
images. Then, each caspase-3 and CD-31 region was thresholded based on empirically chosen thresholds of color intensity. Each caspase-3 region was sorted based on nearest distance relative to CD-31 vasculature. Finally distances were binned into 10 µm regions and expressed as a function of total signal intensity.

4.3.8 Quantification of Cell Death and Microvessel Density

For quantification of tumor cell death in the IP and SC SKOV3 tumor sections the percentage of TUNEL and caspase-3 immunostaining were measured. The microvessel density for each tumor section was calculated as the percent total area within tumor section that stained positively for CD-31. ImageScope software (Aperio Technologies Inc., Version 10) combined with the built-in Positive Pixel Count algorithm was used for analysis.

4.3.9 Efficacy Studies

Tumor weight and ascites volume were measured in the IP SKOV3 and ID8 models, respectively, post mortem in order to assess efficacy of the PoLigel. For the SC SKOV3 model, tumors were measured every 3-4 days post inoculation using a vernier caliper in 2 dimensions. Tumor volume was calculated as follows: \( \frac{(\text{length} \times (\text{width})^2)}{2} \), where length is the longest diameter and width is the shortest diameter perpendicular to length. Efficacy outcome for the IP SKOV3 model was calculated as: \( \frac{(\text{mean tumor weight}_{\text{control}} - \text{mean tumor weight}_{\text{PoLigel}}) \times \text{mean tumor weight}_{\text{control}}}{100}\% \). Efficacy outcome for the IP ID8 model was calculated as: \( \frac{(\text{mean ascites volume}_{\text{control}} - \text{mean ascites volume}_{\text{PoLigel}}) \times \text{mean ascites volume}_{\text{control}}}{100}\% \). Efficacy outcome for the SC SKOV3 model was calculated as: \( \frac{(\text{mean tumor volume}_{\text{control}} - \text{mean tumor volume}_{\text{PoLigel}}) \times \text{mean tumor volume}_{\text{control}}}{100}\% \). Endpoints requiring humane euthanasia included weight loss in excess of 20%, excessive muscle wasting, abdominal distention, hypothermia and inactivity. Weight loss was monitored by regular body weight measurements and the other endpoints were examined visually by animal care technicians and veterinarians.
All mice were sacrificed once any one of the endpoints was reached by at least 50% of the animals.

4.3.10 Statistical Data Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). A two-sample t-test was used to measure statistical significance between pairs of results. For statistical analyses among three or more groups, ANOVA was used and subsequent multiple comparisons with Bonferroni correction were performed if statistical significance was detected by the ANOVA. A p-value < 0.05 was considered statistically significant.

4.4 Results

4.4.1 Whole-body and Peritoneal Distribution of DTX

DTX distribution was assessed in two models of ovarian cancer, one producing high tumor burden with minimal to no ascites (IP SKOV3) and the other only ascites fluid (IP ID8). Constant concentrations of DTX were detected in plasma, tissues, tumors and ascites following IP administration of the PoLigel over the entire study period. The highest concentrations were seen in the peritoneal layer for both models. Tumors and ascites showed the second highest levels of DTX for the IP SKOV3 and IP ID8 models, respectively. Tissues located in the peritoneal cavity including spleen, liver, kidneys, stomach and intestine had higher drug levels than systemic concentrations. Plasma and heart had the lowest levels of DTX overall. The average drug accumulation in tumors, tissues and plasma for the IP SKOV3 model was as follows (Figure 4.1): tumor (6.7 ± 1.4 µg/g), peritoneal layer (12 ± 1 µg/g), intestine (1.1 ± 0.3 µg/g), stomach (0.98 ± 0.30 µg/g), liver (0.55 ± 0.21 µg/g), spleen (0.33 ± 0.15 µg/g), kidney (0.19 ± 0.08 µg/g), heart (0.09 ± 0.05 µg/g) and plasma (0.036 ± 0.006 µg/mL). Figure 4.2 summarizes the distribution of DTX in tumors harvested from the IP SKOV3 model with respect to distance from
the site of PoLigel injection (i.e. proximal or distal). The differences in DTX concentrations between proximal and distal tumor samples were not statistically significant.

The average drug accumulation in ascites fluid, tissues and plasma for the IP ID8 model was as follows (Figure 4.3): ascites (8.4 ± 2.0 µg/mL), peritoneal layer (17 ± 3 µg/g), intestine (1.8 ± 0.7 µg/g), stomach (1.9 ± 0.7 µg/g), liver (0.77 ± 0.23 µg/g), spleen (0.40 ± 0.18 µg/g), kidney (0.31 ± 0.13 µg/g), heart (0.09 ± 0.04 µg/g) and plasma (0.037 ± 0.009 µg/mL). The ID8 model, characterized by large ascites volume, showed higher concentrations of DTX in all tissues located in the peritoneal cavity compared to the IP SKOV3 model.

For the SC SKOV3 model plasma and tumor concentrations of DTX were 0.045 ± 0.007 µg/mL and 3.1 ± 1.0 µg/g on day 21 post treatment, respectively. DTX levels observed in tumors and ascites on day 21 post treatment were 200, 69 and 258 times greater than levels in plasma for the IP SKOV3, SC SKOV3 and IP ID8 models, respectively.
Figure 4.1 Time dependent distribution of DTX in tissue and tumor following IP administration of PoLigel in SCID mice bearing SKOV3 ovarian cancer (total DTX dose: 32 mg/kg). The inset shows DTX plasma levels over time. The results represent mean ± SD (n = 4).
Figure 4.2 (A) Schematic illustrating distal and proximal tumors relative to site of PoLigel injection. The PoLigel was injected IP into the lower left quadrant of mice. (B) Concentrations of DTX in tumors proximal and distal to the site of injection of PoLigel in SCID mice bearing SKOV3 ovarian cancer (total DTX dose: 32 mg/kg) over 21 days. There was no statistical difference ($p > 0.05$) in DTX concentration between proximal and distal tumor sites. The results represent mean ± SD ($n = 4$).
Figure 4.3 Time dependent distribution of DTX in tissue and ascites following IP administration of the PoLigel in C57BL/6 mice bearing ID8 ovarian cancer (total DTX dose: 32 mg/kg). The inset shows DTX plasma levels over time. The results represent mean ± SD (n = 4).
4.4.2 Intratumoral Distribution and Tumor Penetration of DTX

Caspase-3 activation was used as an indirect method to measure the intratumoral distribution of DTX in IP and SC tumors (Figure 4.4a). Assessing the activity of a cytotoxic drug with markers of cell death allows for semiquantitative analysis of intratumoral drug distribution [136]. The IP tumors showed greater ($p < 0.05$) distribution of drug towards the core of the tumors compared to SC tumors (Figure 4.4b).

Similar to intratumoral distribution, caspase-3 (representing DTX) and CD-31 (i.e. blood vessels) immunostaining were used to assess the tumor penetration of drug from nearest blood vessels (Figure. 4.5a). DTX diffused 1.5-fold further from blood vessels in the IP tumors compared to the SC tumors (Figure. 4.5b). The mean distances traveled from the nearest blood vessels were $96 \pm 32\, \mu m$ and $73 \pm 15\, \mu m$ for the IP and SC tumors, respectively. A significant difference was not observed between the microvessel densities of the two SKOV3 models, as measured by CD-31 immunostaining, with values of $4.6 \pm 1.4\%$ and $4.2 \pm 2.5\%$ area for the sections from the IP and SC tumors, respectively.
Figure 4.4 (A) Representative SKOV3 IP and SC tumor section used to measure intratumoral distribution of DTX following sustained drug delivery via the PoLigel (total DTX dose: 32 mg/kg). The distribution of caspase-3 activation was used to indirectly assess the intratumoral distribution of the drug. The image processing of tumor sections was performed using a customized MATLAB algorithm. (B) The intratumoral distribution of DTX in IP and SC SKOV3 tumors. Each tumor section was segmented into three regions with equidistant thicknesses. * represents difference between IP and SC tumors (p < 0.05). The results represent mean ± SD (n = 4).
Figure 4.5 (A) Representative SKOV3 IP and SC tumor sections used to measure the tumor penetration of DTX following sustained delivery via the PoLigel (total DTX dose: 32 mg/kg). The distance between caspase-3 activation (green) and CD-31 immunostaining (red) was used to measure tumor penetration following image processing using a customized MATLAB algorithm. (B) DTX tumor penetration in (i) IP and (ii) SC SKOV3 tumors. \( D_{\text{mean}} \) represents the average distance DTX traveled from the nearest blood vessel. The results represent mean ± SD (n = 4).
4.4.3 In vivo Cell Death

Immunohistochemistry of IP and SC tumor sections was performed for TUNEL and caspase-3 activation to assess cell death (Fig. 6a). The amount of TUNEL and caspase-3 immunostaining was greatest in the PoLigel treated tumors (Fig. 6b). For both the IP and SC tumors the PoLigel resulted in 1.7-fold higher TUNEL immunostaining compared to non-treated controls. Caspase-3 immunostaining was 2.8-fold and 1.3-fold higher following PoLigel treatment compared to non-treated controls for the IP and SC tumors, respectively. Only in the IP model was there a significant increase (p < 0.05) in both TUNEL and caspase-3 immunostaining after PoLigel treatment. Furthermore, there was significantly more (p < 0.05) caspase-3 immunostaining observed in the IP tumors post treatment compared to the SC tumors.

4.4.4 Efficacy

The ability of the PoLigel to inhibit tumor growth and reduce ascites volume was assessed in IP and SC SKOV3 and IP ID8 models of ovarian cancer, respectively (Appendix I). In the IP SKOV3 model a tumor burden inhibition of 88 ± 12% was seen compared to non-treated control animals (p < 0.05). Similarly, significant (p < 0.05) efficacy was observed in the ID8 model, evidenced by a decrease in ascites volume of 94 ± 4%. A significant difference (p < 0.05) in SC SKOV3 tumor volume was observed 10 days post IP administration of the PoLigel compared to non-treated control tumors, and a tumor burden inhibition of 72 ± 6% was seen on day 21 post treatment (p < 0.05).
Figure 4.6 (A) Representative SKOV3 IP and SC tumor sections illustrating the effect of sustained DTX delivery via the PoLigel on tumor cell death (total DTX dose: 32 mg/kg). Tumors were immunostained for TUNEL and caspase-3 activation for assessment of cell death. (B) Percentage of TUNEL and caspase-3 positive staining for PoLigel and non-treated control tumor sections. Trends observed for the IP tumors are in agreement with previously published data [94]. * represents differences among groups (p < 0.05). The results represent mean ± SD (n = 4).
4.5 Discussion

To date there have been a limited number of preclinical [272-274] and clinical [64, 287] studies that have examined the distribution of DTX in the peritoneal cavity following IP administration for ovarian cancer treatment. As a result there is inadequate understanding of the distribution of DTX in tissue, tumor and ascites following IP administration. Of the limited number of studies that have been performed, only DTX plasma and peritoneal fluid levels have been documented following bolus IP administration [64, 287]. Furthermore, no studies have examined the influence of ovarian cancer disease parameters such as tumor burden and ascites volume, as well as length of therapy on the distribution of DTX following IP administration. On average, 75% of patients are diagnosed with advanced ovarian cancer which typically presents with both tumors and ascites fluid [288]. The extent of tumor burden can vary and can depend on the cancer subtype and grade [280, 289]. Ascites, the pathologic accumulation of fluid in the peritoneal cavity, tends to accumulate in volumes up to 25 liters in individuals afflicted with ovarian cancer [290]. Close to 80% of patients with ovarian cancer present with ascites at the time of diagnosis [291].

To isolate the effects of solid tumors and ascites, the SKOV3 and ID8 models of ovarian cancer, which predominantly form solid tumors and abundant ascites, respectively, were used. As seen by us and other research groups IP inoculation of SKOV3 cells in mice results in the formation of solid tumors, appearing throughout the peritoneal cavity including on the peritoneal layer, diaphragm and intestine [94, 289, 292, 293]. On the other hand, high ascites fluid accumulation has been observed following IP administration of ID8 cells in mice [94, 286]. The cause of this ascites fluid build-up is not yet fully understood, although studies have suggested the involvement of anti-vascular endothelial growth factor (VEGF). A study that evaluated a series of ID8 cell lines demonstrated that VEGF production by these cells was directly correlated with the volume of ascites accumulation in vivo [294]. If ID8 cells are injected directly
under the ovarian bursa of mice, both ascites fluid and solid tumors form throughout the peritoneal cavity [286, 294].

Following IP administration of PoLigel the highest concentration of DTX was localized in the peritoneal layer in both SKOV3 and ID8 IP ovarian cancer models. A combination of factors including the slow uptake rate of DTX due to its high molecular weight and lipophilicity [254], the low blood capillary density within the peritoneal layer [270, 278] and sustained DTX release from the PoLigel explain the high accumulation of drug within the peritoneal layer. Similar findings have been reported for DTX post IP administration in other rodent models [98, 252, 285]. Cancers located in the peritoneal cavity such as ovarian cancer are known to form metastatic lesions along the peritoneal lining, particularly in advanced stages of the disease [279]. Therefore, sustained concentrations of drug in the lining can be beneficial in preventing the development and progression of metastatic lesions. High drug concentrations were also seen in tumor masses and ascites fluid. In comparison to plasma levels, the accumulation of DTX was several fold greater at diseased sites located in the peritoneal cavity. High local peritoneal drug concentrations are ideal for maximizing therapeutic efficacy, while low plasma drug levels keep systemic toxicity to a minimum.

Higher levels of DTX accumulated in the peritoneal tissues of the IP ID8 model, characterized by abundant ascites fluid, when compared to drug concentrations in the same tissues in the IP SKOV3 model. This indicates that the presence of ascites fluid increases DTX retention in the peritoneal cavity. Mechanistically ascites formation occurs as malignant cells secrete proteins, growth factors, and cytokines that cause neovascularization, angiogenesis, and lymphatic obstruction resulting in the buildup of excess fluid within the peritoneal cavity [250]. DTX easily binds to proteins such as albumin which are found in abundance in ascites fluid [234]. These compounding factors slow the clearance of DTX from the peritoneal cavity leading to higher drug concentrations in peritoneal tissues.
Comparable DTX distribution to all tumor sites in the peritoneal cavity was observed, with no significant difference in DTX concentrations in tumors proximal and distal to the site of PoLigel administration. In a previous report, similar trends were seen in proximal and distal sections of the peritoneal layer, kidney, liver and intestine of healthy female CD-1 mice following IP administration of the PoLigel [285]. This homogeneous distribution is favorable in contrast to the poor peritoneal distribution achieved in various clinical studies [282-284]. Specifically, in a study published by Dawson et al. it was found that up to 70% of patients had poor peritoneal drug distribution [282]. In the current study it is shown that sustained drug levels over long periods of time may enable uniform drug distribution to be achieved throughout the peritoneal cavity, without the need for co-instillation of large volumes of fluid.

Sustained DTX delivery via the PoLigel resulted in a significant inhibition in tumor burden and reduced ascites fluid volume compared to non-treated mice. As evidenced by the distribution data, this efficacy is a result of the high peritoneal drug concentrations achieved, sustained drug exposure, and homogenous distribution of drug within the peritoneal cavity. To further understand the high efficacy observed following sustained IP chemotherapy, markers of cell death (i.e. TUNEL and caspase-3) were measured. The amount of cell death was greater in the PoLigel treated tumors compared to non-treated controls for both the IP and SC SKOV3 models. Furthermore, cell death was greater for the IP tumors compared to SC tumors. The efficacy trend observed in this study for the IP SKOV3 model is in agreement with previously published work using bioluminescence imaging to assess efficacy following PoLigel [94]. The trend observed in the IP ID8 model is also in agreement with our previously published study [94]. Similarly, PTX delivered in a sustained manner via the IP route has been shown to result in excellent antitumor efficacy against IP SKOV3 tumors when compared to that achieved with intermittent regimens [96, 123].

The PoLigel provides sustained DTX levels not only locally, but also systemically over an extended time period. To understand whether the sustained plasma drug levels have an effect
on antitumor efficacy, SKOV3 tumors were induced SC and mice were treated IP with PoLigel. Although greater antitumor efficacy was observed in the IP model, the low sustained DTX levels in plasma yielded significant efficacy against SC tumors. This demonstrates that, while localized drug exposure has a greater influence on efficacy outcomes for IP chemotherapy, sustained systemic recirculation of drug into tumors also contributes to antitumor efficacy. Sustained systemic drug levels may also be beneficial for reaching distal metastatic disease sites not located in the peritoneal cavity which can be found in the advanced stages of the cancer.

Poor drug penetration in solid tumors is a potential mechanism of chemotherapy failure [144]. At present there is limited quantitative in vivo data on the penetration and distribution of taxanes in solid tumors. Quantitative assessment of the distribution of taxanes is challenging given that they are not fluorescent compounds. PTX tissue penetration has been studied via the detection of radiolabeled drug in tumor sections [295, 296] and histocultures [95, 297]. Although these studies illustrated low PTX tumor penetration, quantitative analyses were not performed. PTX penetration has also been studied in vitro using multicellular spheroids [298] and multilayered cell cultures [134, 295, 299], although subsequent analysis to quantify drug penetration was not carried out. The only quantitative in vivo report on PTX and DTX penetration has shown drug diffusion to be limited to about 100 µm from blood vessels following systemic administration of radiolabeled drugs in a SC tumor model [295]. Another approach to analyze the intratumoral distribution and penetration of drugs is to identify markers of their activity on cancer cells, such as inhibition of cell proliferation or induction of cell death [136]. In this study, tumor sections immunostained for apoptosis via caspase-3 activation were used to assess intratumoral distribution and penetration of DTX. IP and SC tumors from non-treated control mice were also analyzed in order to compare the differences in caspase-3 activation between controls and drug treated tumors (Appendix I). Comparable caspase-3 activation was observed for the control tumors irrespective of disease site, and levels were lower than drug treated tumors. Sustained DTX delivery resulted in good intratumoral distribution of DTX to all...
areas of the IP tumors, with increased distribution to the tumor core. In the IP tumors DTX diffused an average of over 20 µm farther from blood vessels compared to SC tumors. Comparable levels of CD-31 expression were observed for the IP and SC tumors, suggesting that the differences observed in penetration are due to sustained local and systemic drug exposure rather than differences in tumor vascularization. However, it is noted that tumor penetration is also known to depend on the biological properties of tumors (e.g. composition and structure of the extracellular matrix) which can vary greatly depending on tumor type and site.

Although the results of this study are promising, there are significant physiological differences between mice and humans that must be taken into account for translation of this treatment strategy to humans. In this regard, a logical next step towards translation of this technology is evaluation of the PoLigel formulation in a larger animal model including assessment of the distribution of drug in the peritoneal cavity. A significant barrier to successful implementation of treatment strategies that rely on depot delivery systems is the clinical expertise that is required for effective administration. The PoLigel formulation will likely be more effective if administered at the time of cytoreductive surgery. An imaging technique could be used to guide optimal administration of the formulation at specific sites within the peritoneal cavity. Medical imaging is currently being explored as a non-invasive approach to guide intratumoral and peritumoral implantation of delivery systems [300]. Intraoperative image-guided surgery was recently pursued in ovarian cancer patients [301]. The integration of imaging into disease staging, cytoreduction and drug delivery will provide personalized medicine and may lead to improved outcomes for ovarian cancer patients.
4.6 Conclusions

The present study shows that sustained IP delivery of DTX via an injectable biodegradable depot delivery system leads to optimal DTX distribution throughout the peritoneal cavity of mice bearing ovarian cancer without the need for fluid co-administration. Furthermore, prolonged sustained systemic drug levels provided by the formulation after IP administration were sufficient to induce antitumor efficacy in SC tumors. This suggests that high efficacy in peritoneal tumors, as observed here and in previous studies, is a result of a combination of both local and systemic sustained drug exposure. Overall, results presented herein show that sustained IP drug delivery results in favorable drug distribution at the whole-body, peritoneal and intratumoral levels contributing to the high degree of efficacy observed. This sustained IP chemotherapy approach may have applications in the treatment of other cancers localized in the peritoneal cavity and may also be efficacious for distal metastatic sites that are only reached by the systemic circulation.

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CHAPTER 5. Combination Drug Delivery Strategy for the Treatment of Multidrug Resistant Ovarian Cancer

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All experiments and data analyses were carried out by P. Zahedi. Quantification of mRNA expression levels were done by R. De Souza. Animal experiments were performed with technical assistance from R. De Souza. Partition coefficient and solubility parameters where calculated using computational software by L. Huynh.

This chapter was written primarily by P. Zahedi.
5.1 Abstract

The onset of MDR in ovarian cancer is one of the main causes of treatment failure and low survival rates. Inadequate drug exposure and treatment-free periods due to intermittent chemotherapy select for cancer cells overexpressing drug efflux transporters, resulting in resistant disease. The present study examines the sustained administration of the chemotherapeutic agent DTX alone and in combination with cepharanthine (CEP), a potent drug efflux transporter inhibitor. DTX and CEP were delivered via the intraperitoneal route in a sustained manner using an injectable polymer-lipid formulation. In vitro, the combination strategy resulted in significantly (p < 0.05) more apoptosis, greater intracellular accumulation of DTX, and lower DTX efflux in ovarian cancer cells showing the MDR phenotype. In vivo, sustained treatment with DTX and CEP showed significantly greater (p < 0.05) tumor inhibition (91 ± 4%) in a murine model of multidrug resistant ovarian cancer compared to sustained DTX treatment (76 ± 6%) and was more than twice as efficacious as intermittent DTX treatment. Overall findings from these studies highlight the impact of sustained delivery of mono and combination therapy in the management of refractory ovarian cancer displaying the MDR phenotype.
5.2 Introduction

Conventional treatment for ovarian cancer includes cytoreductive surgery followed by intermittent chemotherapy with platinum and taxane chemotherapeutic agents administered at their MTD [5]. This standard regimen results in a complete response rate of 40-60%; however, more than 90% of patients are believed to relapse after a median period of 18 months due to the emergence of MDR [125, 302]. The MDR phenotype is a phenomena by which cancer cells develop the ability to survive in the presence of structurally and functionally different chemotherapeutic agents [124]. Cells that either innately possess or acquire MDR are selected for during the course of intermittent chemotherapy. The most common mechanism responsible for the MDR phenotype is the overexpression of ATP-binding cassette (ABC) drug efflux transporters such as P-glycoprotein (Pgp) [126, 128]. This overexpression results in greater efflux of chemotherapeutic agents, leading to intracellular drug levels below cytotoxic concentrations. Tumors from patients with MDR have been shown to overexpress Pgp [303]. Different approaches to overcome MDR have been pursued including use of drug efflux transporter inhibitors, synthesis of more active drug analogues, development of drug conjugates or prodrugs and utilization of advanced drug delivery systems [138]. To improve the effectiveness of chemotherapy in resistant disease, some investigators have explored combination delivery of chemotherapeutic agents and drug efflux transporter inhibitors via advanced delivery systems such as nanoparticles [139-143].

Treatment-free periods are required during traditional intermittent chemotherapy at the MTD to allow healthy tissues to recover from exposure to high doses of cytotoxic agents. These drug-free periods allow for selection of cells with the MDR phenotype between each treatment cycle, leading to progressively resistant disease [128, 302]. Studies have demonstrated that low doses of chemotherapeutic agents administered on a more frequent, prolonged or sustained schedule can increase tumor responsiveness when compared to intermittent dosing at the MTD [94, 96, 123, 266, 304]. As well Ho et al. reported that sustained delivery of PTX did not induce
Pgp overexpression in ovarian cancer tumors, whereas significant upregulation was observed following intermittent chemotherapy [149].

A polymer-lipid formulation (PoLigel) that provides sustained peritoneal delivery of DTX, a semi-synthetic analogue of PTX has been developed [98]. Sustained IP delivery of DTX via PoLigel-(DTX) was found to be more efficacious than intermittent IP therapy in a SKOV3 xenograft model and a murine ID8 syngeneic model, resulting in inhibition of tumor burden and ascites fluid, respectively [94]. The present study investigated whether DTX delivered via the PoLigel in a sustained manner can overcome MDR in ovarian cancer, and whether the addition of cepharanthine (CEP), a drug efflux transporter inhibitor, to the PoLigel-(DTX) formulation can further enhance this effect. CEP, a biscoclaurine alkaloid, competitively binds to the efflux transporters Pgp [305] and MRP7 [246] which have been shown to confer resistance to DTX when overexpressed in cancer cells [247-250].

5.3 Materials and Methods

5.3.1 Materials

DTX was purchased from Jari Pharmaceutical Co. (Jiangsu, China). ³H-DTX was purchased from American Radiolabeled Chemicals (St. Louis, USA). CEP was purchased from LKT Laboratories Inc. (St. Paul, USA). Taxotere® (40mg/mL) was purchased from Sanofi Aventis (West Laval, Canada). Chitosan was purchased from Marinard Biotech Inc. (Quebec City, Canada). GTMAC, ePC, LA, 3-[4,5-dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT reagent), 1.0 N sodium hydroxide solution (NaOH) and Bradford Reagent were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada). Primer sequences were purchased from Sigma Genosys (Oakville, Canada). Ready Safe Scintillation Cocktail was purchased from Beckman Coulter (Mississauga, Canada). TRIzol®, DNasel, RPMI1640 cell culture medium, fetal bovine serum and penicillin-streptomycin were purchased from Invitrogen (Burlington, Canada). Carboxyfluorescein FLICA Apoptosis Detection Kit Caspase Assay was purchased from
Immunochemistry Technologies (Bloomington, USA). ABI High Capacity cDNA Reverse Transcription Kit was purchased from Applied Biosystems (Streetsville, Canada).

5.3.2 Cell Culture

The human ovarian cancer cell line HeyA8 and its taxane-resistant counterpart HeyA8-MDR were purchased from M.D. Anderson Cancer Center (Houston, USA). HeyA8 and HeyA8-MDR cells were maintained in RPMI 1640 cell culture medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 mg/mL streptomycin). Both cell lines were kept as a monolayer at 37°C in 5% CO₂ and 90% relative humidity.

5.3.3 Determination of mdr1 and mrp7 mRNA Expression

The mRNA levels of \textit{mdr1}, which encodes for Pgp, and \textit{mrp7}, which encodes for MRP7, in HeyA8 and HeyA8-MDR cells were measured by quantitative real-time PCR (RT-qPCR). Cells grown in a monolayer were washed with PBS pH 7.4 three times, lysed with 1 mL of TRIzol® and total RNA was isolated from cell lysates as per manufacturer’s instructions. Following treatment with DNaseI, single-stranded cDNA was synthesized from 2 µg RNA using the ABI High Capacity cDNA Reverse Transcription Kit as per the manufacturer’s protocol. Amplification using quantitative RT-qPCR was performed on an Applied Biosystems 7900HT machine equipped with a 384-well reaction block, using SYBR green chemistry with 20 ng of cDNA product and specific primers following a method previously described [306]. Primer sequences used were as follows: mdr1-forward: 5’-TGCTCAGACAGGATGTGAGTTG-3’; mdr1-reverse: 5’-AATTACAGCAAGCCTGGGAACC-3’; mrp7-forward: 5’-CATGCAAGCCACGCGGAACG-3’; mrp7-reverse: 5’-AAGCTGGGGCTGGTGAGGGT-3’. The mRNA levels for each gene were normalized to cyclophilin, and mRNA ratios are presented as a percentage of control values.
5.3.4 Formulation Preparation and Characterization

Polymer-lipid formulations (PoLigel) were prepared as described elsewhere with minor modifications [98]. In summary, a WSC was synthesized by conjugating GTMAC onto the chitosan backbone [236]. The WSC was dissolved in distilled deionized water to prepare a 4.2% (w/v) solution. DTX and various ratios of DTX plus CEP (DTX+CEP) were dissolved in anhydrous ethanol and dried under nitrogen to form a thin layered film and then placed under vacuum for 24 h to remove any residual solvent. An ePC-LA solution (1:4 w/w) was used to resuspend the DTX and DTX+CEP films. Finally the WSC solution was added to the ePC-LA-(DTX or DTX+CEP) solution and vortexed for 1 min. The final DTX and DTX+CEP to material ratios were 1:8 (w/w). All formulations were sterilized under UV-light (Sterilizer T209, Intercosmetics, Canada) for 3 h prior to use.

Computational software was employed to estimate the octanol to water partition coefficient (log $P_{o/w}$) and solubility parameters ($\delta$) for DTX, CEP and material components of the PoLigel (i.e. WSC, LA and ePC) as outlined previously [307]. In vitro formulation stability was assessed by placing PoLigel formulations in vials containing 0.01 M PBS (pH 7.4) with 2 mg/ml lysozyme stored at 37°C and taking turbidity measurements over time. Turbidity analysis was conducted using a UV spectrophotometer at $\lambda = 700$ nm (Cary 50 UV-VIS spectrophotometer, Varian Inc., USA). In vitro drug release was performed by placing PoLigel formulations in vials containing 0.01M PBS (pH 7.4) with 0.2% lysozyme and 4% albumin (vol %) incubated at 37°C. At set time points aliquots were removed for analysis by HPLC and replaced with fresh release media. A detailed outline of sample preparation for HPLC analysis can be found elsewhere [98]. The wavelengths of detection used for CEP and DTX were 284 nm and 227 nm, respectively. A mobile phase of 52% water and 48% acetonitrile was used for DTX and 20% water and 80% acetonitrile for CEP.
5.3.5 Cytotoxicity Studies

HeyA8 and HeyA8-MDR cells were seeded onto 96-well plates or 6-well plates at a cell density of $5 \times 10^3$ cells/well or $1 \times 10^6$ cells/well, respectively. After 24 h the cell culture media was aspirated and replaced with fresh cell culture media containing varying concentrations of DTX, CEP or a combination of DTX and CEP (10:1, 1:1 and 1:10 w/w) in solution form and DTX or DTX and CEP (10:1 w/w) in the PoLigel. Following a 72 h incubation period, cell viability was assessed using the MTT assay. For the MTT assay, each well was aspirated and replaced with 5 mg/mL MTT reagent (in 0.01 M PBS, pH 7.4), followed by a 3 h incubation period. Extraction buffer was added to each well to solubilize the MTT crystals. Cell viability was measured by optical absorbance at $\lambda = 570$ nm using a Spectra Max Plus microplate reader (Molecular Devices, USA). Cells incubated with cell culture medium alone (i.e. untreated) were considered to be 100% viable and used as controls. The IC$_{50}$ values or the concentrations at which the cell growth inhibition was 50% compared to untreated controls were estimated from the dose-response curves.

5.3.6 Determination of Combination Index (CI)

Using a method developed by Chou and Talalay [253, 254] the CI for DTX and CEP combination treatments in solution form and in the PoLigel were determined. Firstly, the growth inhibitory results from each treatment were plotted using the median-effect plot [i.e. log(fraction of nonviable cells/fraction of viable cells) versus log(treatment concentration)] to determine whether the treatment combinations were mutually nonexclusive (i.e. agents acting independently or having different actions) or mutually exclusive (i.e. agents share similar modes of action). The CI values were calculated as follows:

For mutually nonexclusive combination,

$$CI = \frac{D_1}{(D_m)_1} + \frac{D_2}{(D_m)_2} + \frac{D_1 D_2}{(D_m)_1(D_m)_2}$$
For mutually exclusive combination,

\[
CI = \frac{D_1}{(D_m)_1} + \frac{D_2}{(D_m)_2}
\]

\((Dm)_1\) and \((Dm)_2\) represent the IC\(_{50}\) of treatments 1 and 2 applied separately, while, \(D_1\) and \(D_2\) are IC\(_{50}\) of treatments 1 and 2 applied as a combination. CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively [308].

5.3.7 Apoptotic Activity

For quantitative analysis of apoptosis HeyA8 and HeyA8-MDR cells were seeded onto 6-well plates at a density of \(1 \times 10^6\) cells per well. Apoptosis was induced by treating cells with DTX or a combination of DTX and CEP (10:1 w/w) in solution form or in the PoLigel. The concentration of each treatment was set to equal the IC\(_{50}\) concentration of free DTX (i.e. 1.35nM for HeyA8 cells and 264nM for HeyA8-MDR cells). For CEP only treated cells the corresponding IC\(_{50}\) concentrations were used for each cell line. After 72 h the cells were washed twice with PBS, trypsinized and re-suspended at a cell density of \(5 \times 10^3\) cells/µL in PBS. 10 µL of FLICA 30X solution was added to 300 µL of cell solution followed by 1 h incubation at 37°C. After incubation 1 mL 1X wash buffer was added followed by centrifugation at 4000 rpm for 5 min at room temperature. The supernatant was aspirated and the wash was repeated twice. Following removal of the final 1X wash buffer the cells were re-suspended in 400 µL PBS. Fluorescence of each cell solution was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using a dual-scanning microplate spectrofluorometer (Spectra GeminiXS, Molecular Devices, USA). Caspase-3/7 activity was reported as fold change in activation relative to untreated control cells.
5.3.8 DTX Intracellular Uptake and Efflux

For uptake and efflux studies HeyA8 and HeyA8-MDR cells were seeded onto 6-well plates at a density of $1 \times 10^6$ cells per well. Cells were treated for 4 h at 37°C with 10 nM DTX or a combination of DTX and CEP (10:1 w/w) in solution form and in the PoLigel. All drug samples had been spiked with 0.001% of $^3$H-DTX for analysis purposes. For uptake studies following 4 h treatment, cells were washed twice with PBS, lysed with 500 µL of 1.0 N NaOH solution, and collected in scintillation vials. For efflux studies following 4 h treatment, cells were washed twice with PBS then fresh RPMI 1640 cell culture medium was added. At set time points (i.e. 30, 60, 90 and 120 mins) cells were washed, lysed and added to scintillation vials as above. A 4 mL aliquot of scintillation cocktail was added to each vial followed by scintillation counting (Beckman Coulter LS 5000TD, Beckman instruments Inc., USA). Final DTX concentrations in the cell lysates were determined using a standard curve and were normalized to cell protein content as determined by the Bradford protein assay.

5.3.9 Efficacy Studies and Levels of DTX in Tissues

All animal studies were approved by the University of Toronto Animal Care Committee and adhered to the guidelines of the Canadian Animal Care Council. Female SCID mice of 6-8 weeks old (Charles River) were inoculated IP with $1 \times 10^6$ HeyA8 or HeyA8-MDR cells suspended in 200 µL PBS (pH 7.4). Seven days later mice were randomly grouped (n = 6 per group) and treated with one of the following: (1) non-treated control; (2) drug free PoLigel formulation; (3) DTX PoLigel formulation (i.e. sustained DTX therapy); (4) DTX-CEP 10:1 w/w PoLigel formulation (i.e. sustained DTX-CEP therapy); and (5) Taxotere® (i.e. intermittent DTX therapy). Groups 1 and 2 received one 20 µL IP injection of sterile saline or drug-free PoLigel, respectively. Groups 3 and 4 received one IP injection of PoLigel-DTX or PoLigel-(DTX+CEP) (DTX dose: 32 mg/kg), respectively. Group 5 received an IP injection of Taxotere® once per week for three weeks (DTX dose: 32 mg/kg). Weight loss was monitored by regular body weight
measurements, and the presence of all other endpoints was examined visually by animal care technicians and veterinarians. Endpoints requiring humane euthanasia included excessive muscle wasting, abdominal distention, hypothermia, inactivity, and weight loss in excess of 20%. All mice were sacrificed concurrently once any of these endpoints was reached by at least 50% of animals. After a 21-day treatment period, endpoints for control animals were reached. Mice from all treatment groups were anesthetized and sacrificed by exsanguination via cardiac puncture. Tumors, liver, spleen, intestine, and kidneys were collected. Anti-tumor efficacy was calculated as: 

\[
\%\text{Efficacy} = \frac{(W_{\text{untreated}} - W_{\text{treated}})}{W_{\text{untreated}}} \times 100,
\]

where \(W_{\text{untreated}}\) is the mean tumor weight of non-treated control animals and \(W_{\text{treated}}\) is the mean tumor weight of treated animals. DTX levels in liver, spleen, intestine and kidneys were quantified using a previously developed HPLC assay [98].

5.3.10 Statistical Data Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). A two-sample t-test was used to measure statistical significance between pairs of results. For statistical analyses among three or more groups, ANOVA was used and subsequent multiple comparisons with Bonferroni correction were performed if statistical significance was detected by the ANOVA. A p-value < 0.05 was considered statistically significant.

5.4 Results

5.4.1 Characterization of Polymer-Lipid Formulations

Three different ratios of DTX to CEP (i.e. 1:1, 1:10 and 10:1) (w/w) were formulated into the PoLigel. Turbidity measurements taken over a one week period were used to assess the effect of DTX:CEP loading ratio on \textit{in vitro} PoLigel formulation stability. A greater ratio of CEP to DTX resulted in lower formulation stability as indicated by higher turbidity values (Appendix II). Both
the 1:1 and 1:10 (w/w) DTX:CEP formulations showed poor stability in solution, disintegrating within 24 h post incubation. On the other hand, the 10:1 (w/w) DTX:CEP formulation demonstrated a good stability profile, as it refrained from disintegrating over time and turbidity values remained near zero. This formulation showed a comparable stability profile to our previously developed PoLigel-(DTX) formulation [98]. Based upon these results, all subsequent studies involving the PoLigel-(DTX+CEP) formulation were done using the 10:1 (w/w) DTX:CEP ratio.

The drug release profile of DTX from the PoLigel-(DTX+CEP) formulation was assessed and compared to that from PoLigel-(DTX) (Figure 5.1). In both cases, a biphasic release profile was obtained, characterized by a more rapid release phase during the initial 24 h (i.e. in terms of % released per day) and a sustained, slower drug release phase for the remainder of the study period. During the initial 24 h, the PoLigel-(DTX+CEP) formulation released 7.9% of total DTX and 3.0% of total CEP. This is similar to the 8% DTX released from the PoLigel-(DTX) formulation during the initial 24 h. Following this initial phase, a sustained release rate of 1.9 ± 0.3% of DTX and 0.68 ± 0.28% of CEP per day occurred from PoLigel-(DTX+CEP). The PoLigel-(DTX) formulation released 2.4 ± 0.1% of DTX per day.
Figure 5.1 *In vitro* release of DTX from the PoLigel-(DTX) and DTX and CEP from the PoLigel-(DTX+CEP) (10:1 w/w) in 0.01M PBS (pH 7.4) with 2 mg/mL lysozyme and 40 mg/mL albumin at 37°C over 21 days. The results represent mean ± SD (n = 3).
5.4.2 Cytotoxicity Studies and Combination Effects

The 50% inhibitory concentrations (IC₅₀) of DTX and CEP as single agents and in combination in taxane-sensitive and taxane-resistant ovarian cancer cells are summarized in Table 5.1. Quantitative PCR was used to compare the mRNA expression of mdr1 (which encodes for Pgp) and mrp7 in HeyA8 and HeyA8-MDR cells (Appendix II). After normalization to cyclophilin expression, the mRNA concentration of mdr1 in HeyA8-MDR cells was shown to be 3.1 times greater (p < 0.05) than in HeyA8 cells (1.27 ± 0.19 versus 0.41 ± 0.12), and mrp7 expression is 3.3 times greater (p < 0.05) in HeyA8-MDR cells than in HeyA8 cells (16.68 ± 2.67 versus 5.02 ± 0.87). DTX IC₅₀ values were found to be 1.35 ± 0.13 nM and 264 ± 38 nM, in HeyA8 and HeyA8-MDR cells, respectively. This demonstrates the high level of resistance of HeyA8-MDR cells to DTX, which require 200-fold more DTX than HeyA8 cells for the same amount of cell death. In HeyA8-MDR cells, the IC50 value for PoLigel-(DTX+CEP) was 440-fold, 43-fold and 3.3-fold lower than that for DTX, DTX+CEP (10:1 w/w) and PoLigel-(DTX) (p < 0.05), respectively. In the taxane-sensitive HeyA8 cells, exposure to PoLigel-(DTX) resulted in the lowest IC₅₀ value, followed by PoLigel-(DTX+CEP). It must be noted that the drug-free PoLigel did not induce a significant cytotoxic effect over a range of concentrations tested in both cell lines (Appendix II). At the highest concentration of drug-free PoLigel, the cell viability was 91% and 95% in the sensitive and resistant cell lines, respectively. Overall the drug-free PoLigel showed a lack of in vitro cytotoxicity, which is in agreement with our previous work in L929 mouse fibroblasts and HeLa cervical cancer cells [241]. The PoLigel-(DTX+CEP) resulted in the highest synergistic effect as seen from the CI values measured by the Chou and Talalay method in both cell lines compared to DTX+CEP combination treatments in solution form (Table 5.1).
Table 5.1 The 50% inhibitory concentration (IC$_{50}$) values and combination index (CI) of DTX and CEP delivered either as single agents or in combination to taxane-sensitive (HeyA8) and taxane-resistant (HeyA8-MDR) ovarian cancer cells. Cells were exposed to the different treatments for 72 h. The results represent mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>HeyA8 $\text{IC}_{50}$ [nM]</th>
<th>HeyA8-MDR $\text{IC}_{50}$ [nM]</th>
<th>Cl$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX solution</td>
<td>1.35 ± 0.13</td>
<td>264 ± 38</td>
<td></td>
</tr>
<tr>
<td>CEP solution</td>
<td>$1.19 \times 10^4$ ± 0.20</td>
<td>$1.33 \times 10^4$ ± 0.41</td>
<td></td>
</tr>
<tr>
<td>PoLigel-(DTX)</td>
<td>0.610 ± 0.02</td>
<td>2.01 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>DTX+CEP (1:1 w/w) solution</td>
<td>2.17 ± 0.75</td>
<td>0.82</td>
<td>0.1</td>
</tr>
<tr>
<td>DTX+CEP (1:10 w/w) solution</td>
<td>30.5 ± 3.4</td>
<td>2.25</td>
<td>0.04</td>
</tr>
<tr>
<td>DTX+CEP (10:1 w/w) solution</td>
<td>1.98 ± 0.39</td>
<td>1.34</td>
<td>0.08</td>
</tr>
<tr>
<td>PoLigel-(DTX+CEP) (10:1 w/w)</td>
<td>1.09 ± 0.20</td>
<td>0.73</td>
<td>0.601 ± 0.041</td>
</tr>
</tbody>
</table>

$^a$ CI < 0.1, very strong synergism; Cl = 0.1-0.3, strong synergism; Cl = 0.3-0.7, synergism; Cl = 0.7-0.85, moderate synergism; Cl = 0.85-0.90, slight synergism; Cl = 0.90-1.10, nearly additive; Cl = 1.10-1.20, slight antagonism; Cl = 1.20-1.45, moderate antagonism; Cl = 1.45-3.3, antagonism; Cl = 3.3-10, strong antagonism; and, CI > 10, very strong antagonism [308, 309].
5.4.3 Cellular Apoptotic Activity

To further confirm the therapeutic potential of combination therapy, apoptosis was quantified by caspase-3/7 activity levels in both taxane-sensitive and taxane-resistant ovarian cancer cells (Figure 5.2). Greater caspase-3/7 activity was seen upon HEYA8 treatment with PoLigel-(DTX+CEP) or PoLigel-(DTX) in comparison to that obtained with DTX+CEP (10:1 w/w) or DTX (p < 0.05). When HEYA8-MDR cells were treated with PoLigel-(DTX+CEP), more caspase-3/7 activity was observed relative to that obtained following treatment with DTX, DTX+CEP (10:1 w/w) or PoLigel-(DTX) (p < 0.05). Treatment with PoLigel-(DTX+CEP) resulted in a 3.6-fold increase in caspase-3/7 activity when compared to DTX alone in the HEYA8-MDR cells. Both the PoLigel-(DTX+CEP) and PoLigel-(DTX) resulted in more than a 2-fold increase in caspase-3/7 activity when compared to free DTX in the HEYA8 cells.
Figure 5.2 Caspase-3/7 activation, as measured by relative increase to control (non-treated cells), in HeyA8 and HeyA8-MDR ovarian cancer cells. Cells were exposed to the different treatments for 72 h. Statistical significance was seen when caspase-3/7 activity associated with PoLigel-(DTX+CEP) (10:1 w/w) and PoLigel-(DTX) were compared to DTX and DTX+CEP (10:1 w/w) in both cell lines (p < 0.05). Within each cell line: * represents significant difference from DTX and DTX+CEP (10:1 w/w) (p < 0.05); ‡ represents significant difference from PoLigel-(DTX) (p < 0.05). The results represent mean ± SD (n = 3).
5.4.4 Cellular Uptake and Efflux of DTX

The cellular uptake and efflux of DTX were quantified in both cell lines to assess transporter activity upon combination therapy. Significantly higher cellular levels of DTX resulted when both cell lines were treated with either PoLigel-(DTX+CEP) or PoLigel-(DTX) compared to DTX and DTX+CEP (10:1 w/w) (p < 0.05) (Figure 5.3). In the taxane-resistant cells incubation with PoLigel-(DTX+CEP) resulted in significantly higher accumulation of DTX in comparison to PoLigel-(DTX). The PoLigel-(DTX+CEP) resulted in a 3.1-fold, 1.9-fold and 1.3-fold higher DTX accumulation in the HEYA8-MDR cells compared to DTX, DTX+CEP and PoLigel-(DTX), respectively.

The efflux of DTX from HeyA8 cells was not significantly different irrespective of the treatment approach (Figure 5.4A). Overall, two hours post-treatment, approximately 75% of the total DTX added was still retained in the HeyA8 cells. On the other hand, the amount of DTX effluxed from the HeyA8-MDR cell line that overexpresses drug efflux transporters changed depending on treatment. After two hours only 22% of DTX was effluxed from cells that had originally been treated with the PoLigel-(DTX+CEP), whereas 47% was effluxed from cells that had been exposed to free DTX (Figure 5.4B). Combination delivery of CEP and DTX via the PoLigel formulation significantly (p < 0.05) decreased DTX efflux in the HeyA8-MDR cell line compared to DTX, DTX+CEP (10:1 w/w) and PoLigel-(DTX) treatment for 2 hours (Figure 5.7).
**Figure 5.3** Cellular uptake of DTX in HeyA8 and HeyA8-MDR ovarian cancer cells. Cells were exposed to the different treatments for 4 h. Statistical significance was seen in drug uptake when PoLigel-(DTX+CEP) (10:1 w/w) and PoLigel-(DTX) were compared to DTX and DTX+CEP (10:1 w/w) in both cell lines (p < 0.05). Within each cell line: * represents significant difference from DTX and DTX+CEP (10:1 w/w) (p < 0.05); ‡ represents significant difference from PoLigel-(DTX) (p < 0.05). The results represent mean ± SD (n = 3).
Figure 5.4 DTX efflux from (A) HeyA8 and (B) HeyA8-MDR ovarian cancer cells. Cells were exposed to the different treatments for 4 h, then washed followed by addition of fresh media. After 120 minutes a statistically significant decrease in DTX efflux was seen when HeyA8-MDR cells were treated with the PoLigel-(DTX+CEP) (10:1 w/w) compared to DTX, DTX+CEP (10:1 w/w) and PoLigel-(DTX) (* = p < 0.05). The results represent mean ± SD (n = 3).
5.4.5 Anti-tumor Efficacy

The ability of PoLigel-(DTX+CEP) to inhibit tumor growth was assessed in taxane-sensitive and taxane-resistant ovarian cancer models (Figure 5.5). In the taxane-sensitive model, a tumor growth inhibition of $88 \pm 3\%$ was achieved with PoLigel-(DTX+CEP) treatment, which was not statistically different from the $94 \pm 4\%$ tumor inhibition obtained with PoLigel-(DTX) treatment. Both PoLigel-(DTX+CEP) and PoLigel-(DTX) treatment resulted in greater anti-tumor efficacy in the taxane-sensitive model than could be achieved with intermittent Taxotere® ($75 \pm 9\%, p < 0.05$). In the taxane-resistant model, PoLigel-(DTX+CEP) treatment resulted in a $91 \pm 4\%$ inhibition in tumor burden, which was significantly greater than treatment with either PoLigel-(DTX) or intermittent Taxotere® ($p < 0.05$). Treatment with PoLigel-(DTX) led to a $76 \pm 6\%$ anti-tumor efficacy, whereas significantly lower efficacy ($36 \pm 17\%$) was achieved with intermittent Taxotere® treatment ($p < 0.05$).

5.4.6 Tissue Distribution

In order to verify whether combination treatment with a drug efflux transporter inhibitor caused greater DTX accumulation in healthy tissues, drug levels in liver, spleen, intestine and kidney were compared between mice in the PoLigel-(DTX) and PoLigel-(DTX+CEP) treatment groups. The average drug accumulation in liver, spleen, intestine and kidneys following 21-days treatment with PoLigel-(DTX+CEP) were as follows: liver ($0.47 \pm 0.11 \mu g/g$), spleen ($0.41 \pm 0.09 \mu g/g$), intestine ($0.91 \pm 0.33 \mu g/g$) and kidney ($0.23 \pm 0.10 \mu g/g$). These values were not significantly different ($p > 0.05$) from values obtained following 21-days treatment with PoLigel-(DTX): liver ($0.51 \pm 0.08 \mu g/g$), spleen ($0.30 \pm 0.11 \mu g/g$), intestine ($1.2 \pm 0.6 \mu g/g$) and kidney ($0.13 \pm 0.08 \mu g/g$).
Figure 5.5 Anti-tumor efficacy of PoLigel-(DTX+CEP) (10:1 w/w) in HeyA8 and HeyA8-MDR ovarian cancer models (total DTX dose: 32 mg/kg). Significant difference was observed between the PoLigel-(DTX+CEP) (10:1 w/w) group compared to all other treatment groups in the HeyA8-MDR tumor model (p < 0.05). All mice were sacrificed 21 days post treatment initiation. * represents significant difference from Taxotere® (p < 0.05); ‡ represents significant difference from PoLigel-(DTX) (p < 0.05). The results represent mean ± SD (n = 6).
5.5 Discussion

Current intermittent chemotherapy used in the clinical setting for ovarian cancer therapy has been shown to eventually result in the development of MDR. Overexpression of drug efflux transporters plays a prominent role in the MDR phenotype. Previous work in pre-clinical models of ovarian cancer has shown that eliminating treatment-free periods using sustained drug delivery improves therapeutic outcomes in comparison to intermittent chemotherapy [90-92, 205] and, unlike intermittent therapy, does not lead to upregulation of Pgp [149]. This study examined the impact of sustained delivery of DTX or the combination of DTX and CEP in taxane-resistant and taxane-sensitive ovarian cancer models. CEP is a substrate of Pgp and directly interacts with and competitively binds to Pgp, thereby inhibiting its transport activity [305]. Furthermore, CEP is a competitive inhibitor of MRP7 [246], a member of the MRP family of ABC transporters [310]. MRP7 is the most structurally unrelated member of the MRP family and has a unique resistance profile, as it is the only MRP protein capable of transporting taxanes [311]. In a survey involving various natural product chemotherapeutic agents, MRP7 exhibited the greatest activity towards DTX; in fact, it has been shown to confer resistance to DTX [248, 249]. MRP7 has a commonality with Pgp, in that both are the only ABC transporters with established activity toward taxanes [311]. Based on the inhibitory effect of CEP on these two efflux transporters that confer strong resistance to DTX, CEP was selected for this combination delivery strategy.

Sustained delivery of DTX alone and in combination with CEP was achieved by loading the compounds into a previously developed polymer-lipid based formulation (i.e. PoLigel) [98]. The PoLigel forms a gel implant in situ following IP injection, allowing for sustained and localized peritoneal delivery of active agent(s). Due to greater hydrophobicity of CEP, its release rate from the PoLigel was slower than that of DTX. Both DTX and CEP showed an initial burst release phase which can be attributed to the drugs being associated with the surface of the
PoLigel. This was followed by a sustained release phase, which can be attributed to DTX and CEP partitioning into the hydrophobic regions (i.e. LA and ePC) within the PoLigel.

The PoLigel consists of both hydrophilic and hydrophobic regions. The hydrophilic regions are composed of water-soluble chitosan and the hydrophobic regions are made up of LA and ePC. Both DTX and CEP are hydrophobic in nature, with log P_{o/w} values of 2.45 and 7.02, respectively. Therefore, DTX and CEP will likely partition into the hydrophobic regions of the PoLigel. CEP has an almost 3-fold higher log P_{o/w} than DTX. The solubility parameter of CEP (21.03 (J/cm^3)^(1/2)) is closer to that of both LA (19.82 (J/cm^3)^(1/2)) and ePC (17.2 (J/cm^3)^(1/2)) as compared to DTX (24.26 (J/cm^3)^(1/2)), which indicates that CEP is likely to be more miscible with these formulation components. In this way it was expected that CEP would be more slowly released from the PoLigel formulation (Figure 5.2).

In both taxane-resistant and taxane-sensitive cells, delivery of DTX in a sustained manner via the PoLigel (i.e. PoLigel-(DTX)) provided a clear benefit over bolus DTX given as a single agent or in combination with CEP. In vitro, exposure to PoLigel-(DTX) resulted in a lower IC_{50}, higher caspase 3/7 activity indicative of more apoptosis, and greater DTX uptake than bolus DTX or bolus DTX+CEP combination in both cell lines. In the taxane-resistant cell line, greater intracellular DTX accumulation also resulted from PoLigel-(DTX) treatment. Previous studies have shown that prolonged drug delivery can overcome MDR by decreasing drug efflux when compared to bolus [312] and intermittent [149] treatment, although the underlying mechanisms are not yet well understood. The lower DTX efflux observed with PoLigel-(DTX) in taxane-resistant cells compared to bolus DTX treatment may be attributed to the materials in the PoLigel formulation. Pharmaceutical excipients such as surfactants, polymers (both natural and synthetic), and phospholipids can inhibit drug efflux transporter activity, leading to enhanced drug uptake and lower drug efflux [194]. Two components of the PoLigel formulation, specifically chitosan derivatives [258-260] and phosphatidylcholine [261-263] have been shown to inhibit drug efflux transporters.
*In vivo*, sustained DTX therapy delivered by the PoLigel resulted in greater anti-tumor efficacy than intermittent DTX therapy in both the sensitive and resistant tumor models. Studies have demonstrated that chemotherapy on a more frequent, prolonged or sustained manner in drug sensitive disease provides superior anti-tumor effects over the traditional intermittent treatment at the MTD [90-92, 204, 205]. Our results show that sustained chemotherapy can also overcome MDR disease, which confirms previous observations that show prolonged [312] or more frequent [244] drug exposure leads to a greater response in MDR disease.

Although treatment with sustained DTX via the PoLigel provided a benefit in sensitive cells both *in vitro* and *in vivo*, the addition of CEP to the formulation did not lead to further improvement in taxane-sensitive (i.e. HEYA8) cells and tumors. In these cells, treatment with PoLigel-(DTX+CEP) resulted in a similar IC$_{50}$ value, degree of apoptosis, intracellular DTX accumulation and efflux as treatment with PoLigel-(DTX). The level of anti-tumor efficacy that resulted from treatment with PoLigel-(DTX) or PoLigel-(DTX+CEP) also did not significantly differ. These results are not surprising as HEYA8 cells are almost 200 times more sensitive to DTX than their resistant counterparts, HEYA8-MDR cells. The expression of efflux pumps responsible for DTX transport is also much lower in the HEYA8 cells than in the HEYA8-MDR cells; thus, inhibition of these transporters by CEP does not provide an advantage in HEYA8 cells or the HEYA8 tumor model.

When drug efflux pumps are overexpressed, as is the case in HEYA8-MDR cells, the combination of DTX with an inhibitor of transporters that are responsible for efflux of this drug from cells should significantly improve therapeutic outcomes [264]. Indeed, we have shown that the DTX+CEP combination resulted in very strong synergism in HEYA8-MDR cells. This holds true whether the combination is administered in bolus form or sustained via the PoLigel, although treatment with the latter shows greater synergism. The IC$_{50}$ value upon PoLigel-(DTX+CEP) exposure to HEYA8-MDR cells decreased by more than 3-fold when compared to PoLigel-(DTX), and treatment with bolus DTX+CEP resulted in an 11-fold lower IC$_{50}$ than bolus
DTX. This demonstrates that CEP significantly enhances cell kill by DTX in MDR cells. PoLigel-(DTX+CEP) resulted in more apoptosis, greater intracellular DTX uptake, and lower DTX efflux than PoLigel-(DTX) in MDR cells. A benefit was also seen in vivo, as greater tumor inhibition resulted from PoLigel-(DTX+CEP) therapy in the MDR ovarian cancer model. As seen in this study, sustained delivery of DTX+CEP via the PoLigel is important, as there were no differences between bolus DTX and bolus DTX+CEP in terms of apoptotic index, intracellular DTX uptake and efflux. These results demonstrate at the cellular and tumor levels that the combination of chemotherapy with an inhibitor of drug efflux transporters, when delivered in a sustained manner, is highly beneficial in the treatment of MDR disease.

The addition of drug efflux transporter inhibitors to the treatment of MDR cancers should theoretically provide a solution for recurrent disease; however, toxicities due to greater drug accumulation in healthy tissues and unfavorable pharmacokinetics have limited their use clinically [264]. Organs such as liver, kidney and intestine that express drug efflux transporters at high levels could potentially be affected by an inhibitor such as CEP when administered intraperitoneally [313]. We hypothesized that delivering the inhibitor (i.e. CEP) in a sustained manner would diminish undesirable effects in healthy tissues, which would be exposed to low doses of CEP at any given time. Indeed, there were no differences in DTX levels in liver, kidney, intestine and spleen following PoLigel-(DTX+CEP) or PoLigel-(DTX) treatment. DTX concentrations in these organs were in fact comparable to levels seen in a study conducted in healthy female CD-1 mice treated with the PoLigel-(DTX), whereby no signs of tissue toxicities were observed [98].
5.6 Conclusions

The evolution of MDR in ovarian cancer following traditional intermittent chemotherapy at the MTD is the leading cause of treatment failure, resulting in very poor clinical outcomes. Strategies that allow for enhanced drug uptake and retention in cancer cells displaying the MDR phenotype can have a profound impact on the management of refractory disease. In this study, we have examined DTX and combination of DTX and CEP delivered in a sustained manner via a polymer-lipid based formulation for the treatment of drug-resistant ovarian cancer. Combination therapy resulted in more apoptosis, higher DTX uptake, lower efflux, and greater anti-tumor efficacy. Sustained delivery of DTX and CEP did not cause greater accumulation of DTX in healthy tissues when compared to sustained delivery of DTX alone, suggesting that this treatment strategy would not increase toxic side effects. Overall, these results demonstrate the clear benefit associated with DTX and CEP combination therapy when delivered in a sustained manner as a treatment for refractory ovarian cancer.

5.7 Acknowledgements

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CHAPTER 6. Summary and Future Directions

6.1 Thesis Conclusions and Summary of Findings

The overall goal of this thesis was to develop and characterize a biocompatible and biodegradable injectable depot sustained DTX delivery system (PoLigel) for IP chemotherapy of ovarian cancer. Chapter 2 outlined the preparation, physicochemical characterization and in vivo performance of the PoLigel. The stability of the formulation was confirmed by turbidity measurements and attributed to specific molecular interactions and organization between the material components. The PoLigel afforded sustained DTX release with a tunable release rate based on initial drug loading. No signs of toxicity or inflammation were observed following IP administration of the PoLigel in healthy CD-1 mice. At DTX doses of 28.8 mg/kg and 19.2 mg/kg, the PoLigel showed significant tumor inhibition of 87% and 74%, respectively, in an orthotopic SKOV3 ovarian cancer murine model. Furthermore, in previous studies the therapeutic effect of DTX administered in PoLigel was compared to DTX administered in Taxotere® [94, 97]. In all tumor models the PoLigel formulation of DTX resulted in superior efficacy. These results prompted further studies to gain insight into the mechanisms by which administration of DTX in PoLigel results in this superior therapeutic effect.

Firstly, the distribution of DTX was investigated in healthy CD-1 mice (Chapter 3) following IP administration as PoLigel or Taxotere®. Three days after IP administration of Taxotere®, no detectable levels of DTX were measured in plasma, while sustained DTX plasma levels were observed in mice administered PoLigel. At five days post IP administration of Taxotere®, only low concentrations of DTX were detected in the intestine, stomach and the peritoneal layer whereas all tissues and plasma showed sustained DTX levels in mice that received PoLigel. These results demonstrated that sustained DTX levels seen in the peritoneal cavity following IP administration of the PoLigel may be responsible for the improvement in
efficacy that has been observed in our previous studies compared to bolus intermittent Taxotere®.

Studies in Chapter 4 investigated DTX distribution at the whole-body, peritoneal and intratumoral levels in disease bearing mice in an effort to further understand the improved efficacy that results from sustained IP DTX delivery via the PoLigel. Given that administration of DTX in Taxotere® did not result in sustained levels of drug in plasma and tissues (Chapter 3), we did not pursue assessment of the distribution of DTX following administration in Taxotere® in these studies. Furthermore, as the PoLigel provides sustained plasma levels of DTX, the influence of sustained systemic drug exposure on antitumor efficacy was explored. This was accomplished by evaluating efficacy following IP administration of PoLigel in a SC murine model. Sustained concentrations of DTX were observed in plasma, tissue, tumor and ascites over the entire study period. Drug accumulation was several fold greater in tumors and ascites when compared to plasma levels. Sustained chemotherapy resulted in significant reduction in tumor burden and ascites volume. IP tumors showed greater cell death compared to the SC tumors as seen by higher TUNEL and caspase-3 expression. At the intratumoral level, DTX distributed more towards the core of IP tumors compared to the SC tumors. Tumor penetration of drug from nearest blood vessel was 1.5 fold greater in the IP tumors than the SC tumors. Overall, favorable drug distribution at the whole-body, peritoneal and intratumoral levels in combination with local and systemic sustained drug exposure are key factors which contribute to the high efficacy observed.

The onset of multidrug resistance (MDR) in ovarian cancer is one of the main causes of treatment failure and low survival rates. Inadequate drug exposure and treatment-free periods due to intermittent chemotherapy select for cancer cells overexpressing drug efflux transporters, resulting in resistant disease. Previous work has shown downregulation of a key drug efflux transporter in ovarian cancer tumors following sustained PTX chemotherapy, whereas upregulation was observed in tumors treated with an intermittent PTX dosing schedule. Effect of
sustained DTX delivery alone and in combination with the drug efflux transporter inhibitor CEP via the PoLigel was studied (Chapter 5). The combination strategy resulted in more apoptosis, greater intracellular accumulation of DTX, and lower DTX efflux in ovarian cancer cells showing the MDR phenotype. As well, sustained treatment with DTX and CEP showed significantly greater tumor inhibition in a murine model of MDR ovarian cancer compared to sustained DTX treatment and was more than twice as efficacious as intermittent Taxotere® treatment. These findings highlight the impact of sustained delivery of mono and combination therapy in the management of refractory ovarian cancer displaying the MDR phenotype.

Overall, this is the first report of an injectable delivery system that provides sustained DTX delivery following IP administration. It has been shown that this treatment strategy results in superior efficacy in different murine models of ovarian cancer owing to favorable peritoneal drug distribution, high drug accumulation in tumors and ascites, prolonged local and systemic drug exposure, more cellular apoptosis, greater drug uptake and lower drug efflux. In conclusion, results presented here encourage the clinical investigation of IP sustained chemotherapy for ovarian cancer treatment.

### 6.2 Future Directions

#### 6.2.1 Sustained Docetaxel in Other Models of Ovarian Cancer

The validation of our findings in other models of ovarian cancer is warranted. Studies presented in this thesis show the advantages of sustained DTX therapy in models of ovarian cancer that have been established from immortalized cell lines. These models included the following subtypes: clear cell (SKOV3), serous (ID8) and undifferentiated (HeyA8) epithelial ovarian cancer. The most common subtype of epithelial ovarian cancer is high grade serous [13]. Since the ID8 model of serous ovarian cancer employed here resulted only in the formation of ascites fluid, it would be interesting to establish tumors by injecting ID8 cells into the ovarian bursa, thereby allowing the assessment of this treatment strategy on a high grade serous tumor model.
in an immunocompetent mouse model [286]. Other cell lines can be used to establish high grade serous tumors when injected IP into immunocompromised mice, such as OVCAR-3, CAOV-3 and OAW-42 [314].

Nevertheless, the use of established, immortalized cell lines has its limitations. Continuous culture is known to cause changes to genotype, and selection of subpopulations can occur over time [315]. This may not only hinder reproducibility of experimental results, but may also deviate from the phenotype of the original tumor. The latter is especially concerning when the goal is to model, for example, a specific subtype. An alternative is to xenograft cells from primary patient tumors into immunocompromised mice [316]. These models are arguably more clinically relevant, as they allow for accurate reproduction of the heterogeneity of human tumors, and may recapitulate the histology of the primary tumor [317]. Future studies should consider the use of primary ovarian epithelial tumors of different subtypes to understand how sustained chemotherapy can impact each subtype.

It would also be interesting to evaluate sustained chemotherapy in low-grade serous ovarian cancer. This subtype is comprised of tumors characterized by low proliferation [318]. Perhaps for this reason, they frequently display extreme drug resistance to PTX and CRBP chemotherapy, which targets rapidly proliferating cells. Although this subtype is non-invasive and often is curable with surgery alone, about 30% of these cancers do recur, at which point successful treatment becomes a great challenge [318]. It is plausible that the constant presence of these drugs in a sustained chemotherapy setting may improve response of low-grade tumors. Modeling low-grade tumors is a great challenge due to the slow-growing nature of these cells. Recently however, the first ever model of low-grade serous ovarian cancer has been successfully established [319]. Such a model could be used in future studies to investigate whether sustained chemotherapy provides a therapeutic solution for this highly resistant subtype.
6.2.2 Docetaxel and Carboplatin Sustained Combination Therapy

Studies in this thesis focused primarily on sustained IP DTX delivery. Furthermore, the sustained combination of a drug efflux transporter inhibitor with DTX showed increased efficacy in a MDR model of ovarian cancer. Currently, in the clinical setting, combination chemotherapy is used, specifically co-treatment with taxane and platinum agents. Improvement in efficacy in a murine model of ovarian cancer following IP sustained monotherapy with CRBP compared to intermittent CRBP IP therapy has been shown (Nick Zhidkov, Piquette-Miller Laboratory, MSc Thesis). In addition, clinical trials have shown CRBP administered daily in combination with radiotherapy to improve efficacy with no major toxicities observed [320-322]. The combination of CRBP and DTX seems to be a promising combination, as a high response rate of 67% was reported upon weekly CRBP and DTX in patients with recurrent ovarian cancer [84]. The combination of DTX and CRBP as first-line therapy in ovarian cancer has shown similar efficacy to PTX and CRBP, with significantly less neurotoxicity [44]. Future studies should investigate whether the combination of sustained CRBP therapy with sustained DTX therapy can further increase antitumor efficacy and extend survival in models of ovarian cancer. As well, both DTX and CRBP intratumoral tumor penetration and distribution should be assessed following combination sustained delivery.

6.2.3 Docetaxel and Anti-angiogenic Agent Sustained Therapy

Several clinical trials have shown improved response rates when chemotherapy is combined with an anti-angiogenic agent [323]. One such anti-angiogenic agent is bevacizumab (Avastin®), the first VEGF monoclonal antibody to have undergone evaluation for ovarian cancer therapy [324]. The combination of chemotherapy and bevacizumab in Phase I and II trials in ovarian cancer patients has proven to be efficacious [325]. Currently, a Phase III trial is underway comparing weekly IV PTX plus every 3-week IV CRBP (Arm 1) against the same weekly IV PTX plus every 3-week IP CRBP (Arm 2) and against IV PTX plus IP CDDP and IP
PTX (Arm 3) [326]. Notably, bevacizumab, given both during cytotoxic therapy and as maintenance therapy, is included in all arms of the study. Studies have also shown that the addition of bevacizumab to weekly PTX, chemotherapy significantly increases efficacy in ovarian cancer when compared to weekly chemotherapy alone [327, 328]. This has been attributed to not only bevacizumab’s anti-angiogenic action, but also its effect on tumor vessel normalization, which enhances chemotherapeutic delivery and perfusion [328]. Taxanes have also been shown to have anti-angiogenic properties of their own [329-331]. As well, metronomic chemotherapy, which uses shorter treatment-free periods between each cycle, has been shown to have an anti-angiogenic effect [91]. Addition of anti-angiogenic agents to metronomic chemotherapy has shown an even more enhanced anti-angiogenic effect [332]. Since the addition of an anti-angiogenic agent to chemotherapy has proven to be advantageous, future studies should assess whether the addition of the anti-VEGF antibody bevacizumab to a sustained chemotherapy regimen is beneficial. It is possible that sustained chemotherapy provides enough anti-angiogenic activity that the inclusion of this agent may prove to be redundant. Previous work has already demonstrated that sustained chemotherapy with DTX decreases angiogenesis in tumors compared to intermittent DTX chemotherapy [94]. The extent of DTX intratumoral distribution and penetration should also be investigated upon combination with bevacizumab.
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Appendix I: Chapter 4 Supplemental Figures

Figure A1.1 Anti-tumor efficacy post IP treatment with the PoLigel (total DTX dose: 32 mg/kg) in (A) SCID mice bearing IP SKOV3 tumors and C57Bl6 mice bearing IP ID8 ascites fluid and (B) SCID mice bearing SC SKOV3 tumors. Treatment started 7, 14 and 7 days post inoculation for the IP SKOV3, IP ID8 and SC SKOV3 models, respectively. Control mice were injected IP with 20 µL sterile saline solution. All mice were sacrificed 21 days post treatment initiation. * represents significant difference between PoLigel and control (p < 0.05). The results represent mean ± SD (n = 4).
Figure Al.2 (A) Intratumoral distribution of caspase-3 activation in IP and SC SKOV3 tumors extracted from non-treated mice 21 days post inoculation. (B) Distribution of distance of caspase-3 activation from nearest blood vessel in (i) IP and (ii) SC SKOV3 tumors extracted from non-treated mice 21 days post inoculation. The results represent mean ± SD (n = 4).
Appendix II: Chapter 5 Supplemental Figures

Figure AII.1 *In vitro* characterization of stability of various PoLigel formulations. Turbidity measured as absorbance of 0.01 M PBS (pH 7.4) solution containing PoLigel blend, as a function of time. The results represent mean ± SD (n = 3).
Figure AII.2 mRNA concentrations of \textit{mdr1} and \textit{mrp7} genes normalized to that of cyclophilin house-keeping gene for the HEYA8 and HEYA8-MDR ovarian cancer cells. For each gene: * represents a significant difference between HEYA8-MDR and HEYA8 cells (p < 0.05). The results represent mean ± SD (n = 6).
Figure A11.3  In vitro cytotoxicity evaluation of drug-free PoLigel tested at different volumes in HEYA8 and HEYA8-MDR ovarian cancer cells following a 72 hour incubation period. The results represent mean ± SD (n = 3).