Impact of Chemotherapy Dosing Schedule on Ovarian Cancer Tumor Responsiveness

by

Raquel S. M. G. De Souza

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

In Canada, ovarian cancer kills about 67% of diagnosed patients, largely due to difficulties in early diagnosis. Current treatment consists of debulking surgery and intermittent chemotherapy every three weeks. This approach leads to insufficient drug concentrations at disease sites, and long treatment-free intervals cause accelerated tumor proliferation and drug resistance, resulting in a 5-year survival rate of only 25-35%. Drug resistance development is the ultimate cause of the majority of patient deaths. Improvements yielding more effective treatment are fundamental for successful management of this disease. This thesis investigated a continuous chemotherapy strategy devoid of treatment-free intervals for ovarian cancer treatment.

A biocompatible, biodegradable polymer-lipid injectable formulation PoLi\textsubscript{gel}, was used for continuous DTX delivery. The formulation was well tolerated; no alterations in body weight, behaviour, histology of peritoneal tissues, or interleukin-6 levels were seen in CD-1 mice treated with the PoLi\textsubscript{gel}. Continuous DTX therapy via the PoLi\textsubscript{gel} was considerably more efficacious than intermittent therapy, resulting in significantly less tumor burden and ascites fluid in models of human and murine ovarian cancer. Continuous therapy resulted in less tumor cell proliferation and angiogenesis, and more tumor cell death than intermittent DTX.
The presence and length of treatment-free intervals was shown to contribute to the development of drug resistance. Eliminating these intervals by continuous dosing resulted in superior antitumor efficacy in both chemosensitive and chemoresistant xenograft models of human ovarian cancer, and prevented drug resistance increase after a 21-day treatment period. Survival studies revealed that intermittent dosing led to a mild survival prolongation of 36% and 10% in chemosensitive and chemoresistant models, respectively, whereas continuous DTX prolonged survival by a striking 114% and 95%. Although long-term continuous chemotherapy substantially improved survival, increased drug resistance mechanisms were found at the endpoint. Overall, results presented here encourage the clinical implementation of continuous chemotherapy due to greater achievable therapeutic advantages.
Acknowledgements

I would like to express my deepest gratitude to Dr. Micheline Piquette-Miller for providing me with incredible opportunities and welcoming me into her laboratory as a summer student multiple times, a project student, and finally, a graduate student. Dr. Piquette-Miller has been a wonderful mentor who always encourages creative, independent work, providing outstanding guidance while still allowing for the development of independent problem solving in her students. I am grateful for her continuous support, reassurance, trust, kindness, encouragement, and for always believing in me. With absolute certainty, my research career would not have happened without her.

I am fortunate to have had wonderful advisory committee members – Dr. Christine Allen, Dr. Linda Penn and Dr. Ted Brown. At each of our encounters, they encouraged, challenged, supported and guided me in such a kind and helpful manner that always motivated me to do my best work. I thank them for their valuable suggestions throughout the past four years, and especially for their constant encouragement. They inspired and motivated me greatly. I also thank my external appraiser Dr. Barbara Vanderhyden for thoroughly reviewing my thesis and providing very helpful comments, and Dr. Rob Macgregor for participating in my defense and contributing with valuable insights.

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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>1x/week</td>
<td>once per week</td>
</tr>
<tr>
<td>3x/week</td>
<td>three times per week</td>
</tr>
<tr>
<td>ABC</td>
<td>adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Akt2/akt2</td>
<td>v-akt murine thymoma viral oncogene homolog 2</td>
</tr>
<tr>
<td>Bcl2/bcl2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Casp3</td>
<td>caspase 3</td>
</tr>
<tr>
<td>CHART</td>
<td>continuous hyperfractionated accelerated radiotherapy</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTX</td>
<td>docetaxel</td>
</tr>
<tr>
<td>DTX-PoLigel</td>
<td>docetaxel-loaded polymer-lipid injectable implant</td>
</tr>
<tr>
<td>ePC</td>
<td>egg phosphatidylcholine</td>
</tr>
<tr>
<td>EOC</td>
<td>epithelial ovarian cancer</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>GTMAC</td>
<td>glycidyltrimethylammonium chloride</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HeyA8-MDR</td>
<td>taxane resistant HeyA8 cells</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ID8-luc</td>
<td>ID8 cells transfected with a luciferase gene</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal / intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous / intravenously</td>
</tr>
<tr>
<td>LA</td>
<td>lauric aldehyde</td>
</tr>
<tr>
<td>LCI</td>
<td>lauric chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>mdr1</td>
<td>multidrug resistance gene 1</td>
</tr>
<tr>
<td>MRP7/mrp7</td>
<td>multidrug resistance associated protein 7</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum tolerable dose / doses</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>OSE</td>
<td>ovarian surface epithelium / epithelial</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>plating efficiency</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PoLigel-LA</td>
<td>polymer-lipid injectable implant formulated with lauric aldehyde</td>
</tr>
<tr>
<td>PoLigel-LCl</td>
<td>polymer-lipid injectable implant formulated with lauric chloride</td>
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<tr>
<td>PS80</td>
<td>polysorbate 80</td>
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<tr>
<td>PTX</td>
<td>paclitaxel</td>
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<tr>
<td>Rpn2/rpn2</td>
<td>ribophorin II</td>
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<tr>
<td>RT-qPCR</td>
<td>real time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency / immunodeficient</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SKOV3-luc</td>
<td>SKOV3 cells transfected with a luciferase gene</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume</td>
</tr>
<tr>
<td>WSC</td>
<td>water-soluble chitosan</td>
</tr>
<tr>
<td>w/w</td>
<td>weight to weight</td>
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</table>
Chapter 1

General Introduction


1.1 Ovarian cancer

Over 200,000 women are diagnosed yearly with ovarian cancer worldwide, a disease that remains one of the most lethal cancers to affect women (1, 2). One of the greatest challenges in the management of this disease is the absence of specific symptoms and a lack of adequate screening strategies. If diagnosed at its earliest stage, the 5-year survival rate of this disease can be as high as 94% (3); however, the absence of symptoms until late stages makes early diagnosis difficult. Patients with ovarian cancer experience ordinary, unspecific symptoms that include urinary urgency, abdominal pain and bloating, loss of appetite and early satiety (3). The best method to diagnose ovarian cancer upon suspicion of the disease remains transvaginal ultrasound, although this is not considered to have enough specificity or sensitivity to be used as a reliable screening test (3). For these reasons, about 80% of patients are diagnosed at late stages, when the disease has become metastatic and difficult to treat (2, 4, 5).

Types of ovarian cancers include epithelial, sex cord-stromal and germ cell (Fig. 1.1) (6). Sex cord-stromal tumors account for 7% of ovarian cancers, and arise from stromal fibroblasts and ovarian cells that produce hormones. Germ cell tumors account for 4% of cases, occur
Figure 1.1: Ovarian cancer classification according to origin, histological characteristics and grade.
predominantly in young women and are highly curable (7). Epithelial ovarian cancer (EOC) is the most common form of the disease, accounting for 80-90% of cases (7, 8). EOC is sub-classified into four main histological subtypes (Fig. 1.1). The papillary serous histology, which has a similar architecture to the fallopian tube epithelium, comprises 75% of EOCs, followed by mucinous and endometrioid, each accounting for 10% of cases (9, 10). Endometrioid carcinoma is associated with endometriosis and is histologically similar to uterine and endometrioid cancers, while mucinous carcinoma histology resembles endocervical glands or gastrointestinal epithelium (11). The remaining 5% of cases are clear cell, Brenner tumors, undifferentiated carcinomas, and others (9). Clear cell tumors are also associated with endometriosis and are morphologically similar to serous and endometrioid tumors (11).

Serous EOCs are further subdivided into high- and low-grade tumors (Fig. 1.1). Tumor grade is highly predictive of survival. While high-grade serous ovarian cancer is associated with a low 5-year survival of 10-20% (12), low-grade tumors lead to a 5-year survival rate of 40-56% (6, 12-14). High-grade tumors presents at 55 to 65 years of age, whereas the age of onset of low-grade serous ovarian cancer is earlier, at 45 to 57 years (15). Low-grade tumors are slow growing and clinically indolent (12). These tumors have greater molecular similarity to normal ovarian epithelial cells than to invasive cancers (16). They can reach a large size while still confined to the ovary and are, therefore, detected before metastasis in the majority of cases (17). Yet, since low-grade cancers are slow-growing, they do not respond as well to chemotherapy as high-grade tumors (15, 16). High-grade tumors comprise 90% of serous ovarian cancers (12). They are aggressive tumors that quickly metastasize throughout the peritoneal cavity, a state with which 85% of these tumors present (12). Since these tumors proliferate quickly, they are more responsive to chemotherapy than low-grade tumors. Some definite differences in gene expression patterns exist between high and low grade tumors, the most significant example being that mutations in the p53 gene are present in up to 80% of high
grade cancers, whereas low-grade tumors have a functional p53 (18), (12). This suggests that p53 status is a key branching point between the development of high-grade or low-grade tumors. High-grade tumors are further characterized by epigenetic silencing of BRCA genes, which occurs in 80% of cases (12). Low-grade tumors are characterized by KRAS, BRAF or ERBB2 gene mutations (15).

About 90% of ovarian cancers are sporadic, with no known genetic component (16). The 10% of cases that arise from genetic susceptibility involve mutations in mismatch repair or BRCA genes, which are inherited in an autosomal dominant pattern (6, 16). The BRCA genes encode for tumor suppressors that are essential for genomic stability (19). Mutations that render these genes non-functional lead to genomic aberrations and account for 90% of genetically linked ovarian cancers (20). The remaining 10% of cases with a genetic component have been associated with Lynch syndrome or hereditary nonpolyposis colon cancer, both of which involve defects in mismatch repair (16). The lifetime likelihood of developing ovarian cancer is 39%-46% in women with BRCA1 mutations, 10%-20% in women with BRCA2 mutations, and 9%-12% in women with Lynch syndrome (21-23). Women with BRCA mutations may opt to undergo prophylactic oophorectomy to reduce the risk of ovarian cancer, or bilateral salpingo-oophorectomy to decrease the risk further, although a risk of peritoneal cancer will still remain (24).

EOC is thought to arise from the surface epithelium that covers the ovary, or from inclusion cysts that form as a result of ovulation, as these are constantly exposed to hormones or chemokines that could lead to transformation (10). Cells in the ovarian surface epithelium (OSE) are sensitive to environmental stimuli (25). A few hypotheses exist that attempt to explain the mechanism of EOC development. The incessant ovulation hypothesis is based on the observation that women with fewer ovulatory cycles due to multiple pregnancies, oral contraceptive use, early menopause or late menarche show a 50% decreased risk of
developing ovarian cancer (17, 26-28). It is proposed that the OSE is damaged at each ovulation event and must be repeatedly repaired, increasing the potential for transformation of OSE cells. In addition to the mechanical damage, inflammation that occurs at each ovulation may play a role in ovarian carcinogenesis, as inflammation causes cellular stress that may lead to genetic damage and malignancy (29). The gonadotropin hypothesis proposes that repeated exposure to follicle-stimulating and luteinizing hormones, which occurs at each ovulation, contributes to transformation of OSE cells (16). This is based on the fact that these hormones promote cell proliferation and have been shown in preclinical studies to induce oncogene expression and promote tumor growth and angiogenesis (16, 30, 31). A third hypothesis suggests that stimulation by hormones, including estrogens, progesterones and androgens contributes to OSE cell transformation. This is especially the case with androgens, as conditions associated with elevated androgen levels such as polycystic ovarian syndrome and hirsutism increase ovarian cancer risk (32). Androgens are the most abundant hormones in developing follicles, which in turn are exposed to OSE cells that are rich in androgen receptors (33, 34). These hormones promote proliferation, although no evidence currently exists to support their causative role in transformation.

Evidence suggests that a combination of various factors leads to this disease, as no single hypothesis has yet been able to fully explain ovarian cancer development (16). Recent evidence suggests that a significant number of high grade serous ovarian cancers arise from the fallopian tube and implant on the ovarian epithelium (35, 36). Supporting this theory is the fact that serous carcinoma histologically resembles the fallopian tube epithelial architecture (11). The fallopian tubes make contact with the ovaries during ovulation through the fimbriae, and it has been suggested that this can transfer malignant cells to the OSE (37). In fact, examination of fallopian tube fimbriae of genetically predisposed women who underwent prophylactic surgery has revealed pre-malignant lesions (38).
1.2 Diagnosis

Early diagnosis of ovarian cancer remains the greatest challenge in the successful management of this disease. Symptoms are vague and nonspecific, including abdominal pain, bloating, difficulty eating, early satiety, and urinary urgency or frequency (3, 39). These symptoms are not predominant in early stage disease, and are often completely absent when the cancer is confined to one ovary, thus screening based on these symptoms is not sensitive or specific (6, 40). Upon presentation and recognition of symptoms, pelvic examination is conducted to detect a mass, followed by ultrasound and measurement of CA 125 levels (6). Pelvic examination may only be able to detect large masses, as the ovaries are deep within the peritoneal cavity (41). Elevation of blood CA 125 levels occurs upon disruption of the peritoneum, and levels significantly increase in 90% of advanced cases and in 50% of early disease cases (6). However, it is also found elevated in pregnancy and other benign conditions, and some ovarian cancer patients do not show elevated levels of CA 125 (3, 42). Vaginal ultrasound has high sensitivity in detecting ovarian cancer and is the best method for ruling out this disease, although its specificity is low (43). If ultrasound findings are not clear, which occurs in about 20% of cases, MRI can be used (43). CT scanning is then used in advanced cases to evaluate metastasis and to assess the possibility for surgery and need for neoadjuvant chemotherapy (44). The disease is staged during cytoreductive surgery, as staging using current imaging modalities is not sufficiently accurate (41, 45).

The International Federation of Gynecology and Obstetrics (FIGO) has assigned four stages of ovarian cancer disease progression (Figure 1.2): during Stage I, disease is confined to the ovaries; Stage II involves one or both ovaries and the disease has spread to the pelvic area; Stage III involves both ovaries with peritoneal metastasis beyond the pelvis and/or regional lymph nodes; and Stage IV involves distant metastasis beyond the peritoneal cavity (6, 46). An important predictive factor for survival in ovarian cancer patients is the stage at which the
disease is diagnosed (45). The five-year survival rates for Stage I and II are over 90% and 70%-80%, respectively (41). Diagnosis rarely happens at these stages, and mostly occurs during Stages III and IV when survival drops to 20%-30% and less than 5%, respectively (41). Only 19% of cases are diagnosed before the disease has metastasized beyond the ovaries (42). It is important to note that, although disease stage greatly influences survival, another important factor in predicting survival is the grade of ovarian cancer, which has been discussed in section 1.1.

Routine screening in the general population is not currently recommended due to the low overall incidence of ovarian cancer, which is 17 per 100,000 (3). Women with a genetic predisposition to the disease can undergo annual monitoring using CA 125 levels, pelvic exams and ultrasound, usually after the age of 35 (3, 6, 47). Current screening strategies to detect early stage disease are not effective and have not improved survival rates, thus optimizing treatment strategies is the solution for successfully managing ovarian cancer (48).
Figure 1.2: Pictorial representation of the four FIGO stages used to classify ovarian cancer progression. Adapted from images copyright of Terese Winslow produced for the US National Cancer Institute. Permission to reproduce was granted by the copyright holder on June 6th 2011.
1.3 Treatment

The first step in ovarian cancer treatment is usually cytoreductive surgery (49). Optimal debulking is currently defined as complete removal of all macroscopic disease, as the amount of residual disease after surgery is a determining factor of survival (48, 50). This is due to various factors, including the fact that smaller tumor masses proliferate faster and are more sensitive to chemotherapy, and the elimination of tumors that are large, poorly vascularised and possibly chemoresistant (48). In addition to removal of tumor masses, debulking surgery also involves complete removal of the uterus, both ovaries and fallopian tubes (44). Surgery is typically followed by systemic chemotherapy. In the case of early stage disease, surgery alone leads to over 90% survival (51).

Initially, chemotherapy consisted of cyclophosphamide or melphalan administered as single agents, which remained first-line treatment until the late 1970’s (48). In 1973, cisplatin was the first platinum compound to be introduced for the treatment of epithelial ovarian cancer after clinical trials showed a 5% improvement in 2- and 5-year survival rates over current treatment (52). In the early 1980’s, carboplatin was introduced as a less toxic platinum compound, and in 1998 it became a substitute for cisplatin, as the efficacy of both drugs were similar (52). Both cisplatin and carboplatin exert their cytotoxic action through alkylating DNA. Taxanes, introduced in the 1990’s, are microtubule-stabilizing compounds that bind to and promote polymerization of tubulin subunits and inhibit depolymerization by reducing the critical concentration of subunits required for microtubule formation (53). This stability leads to Bcl2 phosphorylation, which deactivates its anti-apoptotic action (54).

Paclitaxel (PTX) was the first taxane to show anti-tumor activity (53). Studies by the Gynecologic Oncology Group (GOG) showed that the combination of intravenous (i.v.) cisplatin and PTX resulted in enhanced activity when compared to i.v. cisplatin and cyclophosphamide, the latter being the standard treatment at the time (55). Although very promising, the cisplatin-
PTX combination resulted in serious neurotoxicity. The combination of carboplatin and PTX was then evaluated. Studies demonstrated equivalent efficacy to cisplatin and PTX, but with less neurotoxicity (56, 57). Consequently, the current first-line treatment of ovarian cancer consists of maximal cytoreductive surgery followed by systemic combination chemotherapy with a platinum (cisplatin or carboplatin) and a taxane (PTX or docetaxel) agent administered every 3 weeks for 6 cycles (58), with the most widely used regimen consisting of PTX and carboplatin in combination (59). Since cisplatin and carboplatin have shown equivalent efficacy, carboplatin has been preferentially used due to its more favourable toxicity profile (60). Upon completion of chemotherapy, patients with no evidence of disease are monitored for disease recurrence, whereas those in which disease remains present undergo second-line therapy (44).

Docetaxel (DTX) is a semi-synthetic analog of PTX that has shown many advantages, including higher cytotoxicity to cancer cells and stronger binding to the taxane drug target β-tubulin than PTX (61, 62). It has a slower cellular efflux rate and higher solubility, allowing for higher achievable intracellular concentrations (63). Various in vitro studies have shown DTX to be up to 12 times more potent than PTX (53, 64). In addition, Bcl-2 phosphorylation enabled by DTX occurs at 1/100th the concentration needed for PTX to have this effect (63). Further, DTX has been clinically shown to be active in ovarian cancer patients who relapsed after therapy with PTX plus platinum (65). A compilation of phase II trials shows similar response rates resulting from the two taxanes (53). In a phase III study, DTX-carboplatin combination resulted in equivalent efficacy in ovarian cancer patients as PTX-carboplatin, with a more favourable toxicity profile (61, 66, 67). While PTX-carboplatin induced irreversible sensory neuropathy, DTX-carboplatin caused neutropenia, an easily manageable event (66, 67). Since this study, various groups have consistently shown low incidence of neurotoxicity with DTX treatment and high occurrence with PTX treatment (62, 68-70). While PTX-carboplatin remains the standard of care, it is likely that DTX will eventually replace PTX much in the same manner that carboplatin has replaced cisplatin.
Although progress over the past decade has increased overall survival of patients from 17 months to over 65 months, current strategies still result in a low 5-year survival rate of 30% (2, 71). Unfortunately, it is an accepted reality that current treatment is rarely curative and is simply used to prolong survival (6). Most patients achieve complete clinical response upon first line treatment; however, over 85% eventually relapse with drug resistant disease (2). Improving first line treatment is essential to completely eradicate the disease and prevent relapse from occurring, as the first-line setting is the only time cure can still be achieved (9, 72).

1.4 Intraperitoneal chemotherapy

One of the characteristic features of ovarian cancer is its location in the peritoneal cavity (73). Ovarian cancers can successfully thrive in the peritoneal cavity and, in contrast to most other solid tumors, metastasis via the vasculature rarely occurs (11). Metastasis of ovarian cancer most often occurs by direct extension to neighbouring tissues or when cells exfoliate from the tumor and are disseminated throughout the peritoneal cavity via ascites fluid (11). The accumulation of ascites fluid occurs due to obstruction of lymphatic drainage by cancer cells and due to secretion of vascular endothelial growth factor (VEGF) by these cells, which contributes to increased vascular permeability and fluid build-up (74). The mesothelial layer that covers the peritoneal cavity and its tissues is easily invaded by ovarian cancer cells. Cells can also spread throughout the peritoneal cavity via the lymphatic system (75). Metastasis occurs primarily at the fallopian tube(s) and the other ovary, followed by the omentum and peritoneum (76). Since ovarian cancer remains in the peritoneal cavity for most of its natural life, the idea of administering chemotherapy directly into this site is logical (77). Localized chemotherapy for this disease can be achieved by intraperitoneal (IP) chemotherapy.

Upon conventional i.v. chemotherapy, compounds must travel through the systemic circulation to eventually reach disease sites (78). Drug concentrations that can reach the
peritoneal cavity via i.v. chemotherapy are limited, and i.v. doses cannot be increased beyond the maximum tolerated doses (MTDs) due to systemic toxicities (79). A rationale of IP chemotherapy is to achieve higher drug concentrations at disease sites than would be possible by i.v. delivery, while decreasing systemic toxicities that arise from systemic exposure (71, 80). Peak IP drug concentrations in the micromolar range can be achieved through IP administration, whereas nanomolar concentrations are observed after i.v. delivery (81). Three pivotal phase III clinical trials have shown the benefits of IP chemotherapy in optimally debulked, late stage ovarian cancer (Table 1.1) (82-85). The most recent of these trials, GOG 172, showed a median overall survival of 65.6 months after IP chemotherapy, compared to only 49.7 months resulting from i.v. administration (83). The outcome of this study led to an announcement from the National Cancer Institute stating that this treatment modality should become an integral part of ovarian cancer care (86).
Table 1.1: Pivotal Phase III trials assessing the feasibility of IP chemotherapy in advanced ovarian cancer. Adapted from Vergote et al. 2008 (87).

<table>
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<tr>
<th></th>
<th>GOG 104 (82)</th>
<th>GOG 114 (84)</th>
<th>GOG 172 (83)</th>
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<tr>
<td></td>
<td>i.v. regimen</td>
<td></td>
<td></td>
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<tr>
<td>i.v. cisplatin</td>
<td>100mg/m²</td>
<td>i.v. cisplatin</td>
<td>i.v. cisplatin</td>
</tr>
<tr>
<td>i.v. cyclophosphamide</td>
<td>600mg/m²</td>
<td>75mg/m²</td>
<td>75mg/m²</td>
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<tr>
<td>i.v. PTX</td>
<td>135mg/m²</td>
<td>i.v. PTX 135mg/m²</td>
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<th>GOG 104 (82)</th>
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<td></td>
<td>IP regimen</td>
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<tr>
<td>IP cisplatin</td>
<td>100mg/m²</td>
<td>i.v. carboplatin AUC9</td>
<td>IP cisplatin 100mg/m²</td>
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<tr>
<td>i.v. cyclophosphamide</td>
<td>600mg/m²</td>
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<td>i.v. PTX</td>
<td>135mg/m²</td>
<td>i.v. PTX 135mg/m²</td>
<td>i.v. PTX 135mg/m²</td>
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<tr>
<td>IP PTX</td>
<td>60mg/m²</td>
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<th>GOG 114 (84)</th>
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<tr>
<td></td>
<td>Overall survival</td>
<td></td>
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<tr>
<td>i.v. arm:</td>
<td>41 months</td>
<td>i.v. arm:</td>
<td>i.v. arm: 49 months</td>
</tr>
<tr>
<td>i.v. arm:</td>
<td>52 months</td>
<td>62 months</td>
<td></td>
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<tr>
<td>IP arm:</td>
<td>49 months</td>
<td>IP arm: 63 months</td>
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<td>IP arm:</td>
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The ideal agents for IP chemotherapy are those that have a slow clearance rate from the peritoneal cavity and that are quickly cleared from the systemic circulation in order to achieve the greatest pharmacokinetic advantage, defined as the ratio of drug concentration in the peritoneal cavity to levels in the systemic circulation (73). Upon IP delivery in patients, a pharmacokinetic advantage of 10-20-fold is achievable with cisplatin and carboplatin, and 200- and 1000-fold for DTX and PTX, respectively (Table 1.2) (73, 80, 88-91). The taxanes are particularly attractive candidates for IP chemotherapy due to their extensive retention in the peritoneal cavity and slow clearance rate to the systemic circulation (92). Both PTX and DTX have high molecular weight and are very lipophilic, leading to their retention at high concentrations in the peritoneal cavity over several days (93). A study in rats demonstrated that DTX has a rapid clearance rate from plasma; following i.v. administration, a rapid decrease occurs over 15 minutes, followed by a steady decrease over the next 75 minutes (79). This low plasma retention makes DTX attractive for IP delivery. The same study compared IP and i.v. DTX administration. The peak DTX concentration in plasma after i.v. administration was almost 40-fold greater than after IP administration, while concentrations in the peritoneal cavity were over 2500-fold greater with IP chemotherapy (79). This highlights that significantly higher local concentrations can be achieved with IP delivery, whereas only a limited concentration is able to reach the peritoneal cavity following i.v. administration.

PTX has been well established clinically as efficacious when administered IP and is currently the taxane of choice in most accepted IP regimens (71). It is likely that DTX may replace PTX in the IP setting as the taxane of choice due to its many advantages outlined earlier. Clinical studies assessing IP DTX reported that this was a well-tolerated approach, with no peritoneal toxicities arising (90).
Table 1.2: Pharmacokinetic (PK) advantage, defined as the peritoneal-to-plasma drug concentration ratio, following IP administration of taxanes in ovarian cancer patients

<table>
<thead>
<tr>
<th>Taxane</th>
<th>Reference</th>
<th>Dose</th>
<th>PK advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX</td>
<td>Markman 1992 (88)</td>
<td>25-200 mg/m²</td>
<td>996</td>
</tr>
<tr>
<td></td>
<td>Hofstra 2002 (89)</td>
<td>75 mg/m²</td>
<td>1350</td>
</tr>
<tr>
<td></td>
<td>Mohamed 2003 (94)</td>
<td>20 mg/m²</td>
<td>800</td>
</tr>
<tr>
<td>DTX</td>
<td>de Bree 2003 (95)</td>
<td>75 mg/m²</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td>Morgan 2003 (90)</td>
<td>100 mg/m²</td>
<td>181</td>
</tr>
</tbody>
</table>
1.5 Challenges and solutions for localized chemotherapy

Despite the survival advantages shown clinically, IP chemotherapy has not been widely adopted. This is partly due to the fact that IP chemotherapy is currently administered via an indwelling catheter (96, 97). Aside from drug toxicities, the second most common cause of IP chemotherapy discontinuation is catheter-related complications, such as infection, bowel perforations, rupture, occlusion, and fistula formation (80, 98). In fact, only 42% of patients in the IP arm of the pivotal GOG 172 trial were able to complete the full six cycles, mainly due to catheter issues (9). IP chemotherapy is also highly inconvenient for patients, as it is time consuming and often administered on an inpatient basis (99). Quality of life is significantly worse for patients receiving IP treatment when compared to i.v. (2, 5). Patient monitoring by staff must also be done more often than for i.v. chemotherapy, as acute abdominal pain and other side effects may arise without warning. As heterogeneous distribution is a prominent issue with IP chemotherapy that leads to some areas receiving no treatment, large volumes of saline solution are usually rapidly infused with the chemotherapeutic drug to ensure even distribution (91). This further contributes to abdominal pain and respiratory distress due to a sudden rise in abdominal pressure (96, 99). For these reasons, a dedicated team of staff must be assembled for proper IP administration and care. However, most physicians only see a few ovarian cancer cases per year, and may not be willing to invest time in learning a technique that will seldom be used (100). The survival benefits of IP chemotherapy are clear; it is therefore imperative that better delivery strategies be used to supply chemotherapeutics directly to the peritoneal cavity without the need for inpatient care and an indwelling catheter.

A possible solution to these problems is the use of biomaterials as long-term drug delivery systems that can retain the drug at the desired implant sites throughout the duration of treatment, obliterating the use of catheters. Polymers have been widely explored in the area of drug delivery due to their customizable nature which allows for the development of various
systems such as implants, pastes, hydrogels, and/or micro/nanoparticles (101, 102). The options for fabricating polymeric drug delivery systems are vast; however, one of the challenges in their use is biocompatibility (103). The immune response elicited by a polymeric material can result in adverse effects as well as influence drug release properties (104). An acute inflammatory response occurs upon implantation or injection of biomaterials, and activation of macrophages, foreign body giant cells and fibroblasts results in fibrous encapsulation of the system (103, 104). This encapsulation is a normal tissue response to a foreign material and does not necessarily denote a lack of biocompatibility, such as calcification and necrosis would (104). However, the extent of encapsulation and its effects on drug release will dictate the feasibility of the material. It is also important to consider the degradation products of a polymer, as these may elicit immune or toxic responses. Biodegradation is also important, since surgical removal of the delivery system would decrease patient compliance. Material properties must be carefully considered when designing drug delivery systems so that proper biodegradation can be ensured.

Although this approach seems promising, only a few polymer-based formulations have reached clinical testing, which are composed of synthetic polymers. These include the poly(lactic-co-glycolic acid) (PLGA) based localized injectable delivery system OncoGel™ (105) and the polyanhydride copolymer-based implantable system Gliadel® Wafer (106, 107). Many polymeric delivery systems for localized chemotherapy have failed to reach the market as results obtained preclinically often did not translate successfully into clinical development. An example is Paclimer®, a formulation of polyphosphoester microspheres for IP delivery of PTX. This formulation showed promise in preclinical studies but failed during Phase I testing due to biocompatibility issues associated with the polymer (108, 109).

Natural polymers are extracted from abundant natural resources (110). They are stable, non-toxic and are good candidates for developing biocompatible drug delivery materials (111).
Polysaccharides are biopolymers that consist of simple sugar units. Variations in the nature, molecular weight and structure of these units give rise to a large variety of polysaccharide-based polymers (112). These are generally inexpensive as they are abundantly found in nature and can be derived from algals (e.g. alginate), plants (e.g. pectin, guar gum), microbes (e.g. dextran, xanthan gum) and animals (e.g. chitin, chondroitin) (113). Chitosan is a linear natural polysaccharide composed of $\beta$-(1→4)-2-amido-2-deoxy-D-glucan (glucosamine) and $\beta$-(1→4)-2-acetamido-2-deoxy-D-glucan (acetyl glucosamine) units, and is obtained by deacetylation of chitin, which is found in crustaceans, insects and certain fungi (114). Chitosan has excellent biocompatibility and immunostimulatory activities, making it a good candidate for use in chemotherapy delivery systems (115, 116). Furthermore, the degradation products of chitosan (i.e. aminosugars) are nontoxic, non-immunogenic and noncarcinogenic (117, 118). Unfortunately, chitosan-based localized delivery systems have not yet reached clinical use. Achieving a high degree of purity is a great challenge, and contaminants or unreacted cross-linking agents, used to control drug release, can significantly impact chitosan’s safety profile (119). Furthermore, some chitosan derivatives are not degraded by natural enzymes (119). Once these and other issues have been resolved, chitosan may have clinical application, as it has shown much promise in preclinical studies.

One such example is an implantable chitosan-lipid film, termed PoLi$\text{film}$, developed for continuous, prolonged localized delivery of PTX (120). This formulation was shown to be biocompatible and provide continuous release both in vitro and in vivo when surgically implanted into the peritoneal cavity of mice over a period of 4 weeks (121-123). Although the PoLi$\text{film}$ was formulated with biodegradable components, the implant itself was found non-degraded and covered in a fibrous capsid 6 months after implantation into mice. This is a drawback that would require additional surgery to remove the implant. The requirement for surgical implantation of the PoLi$\text{film}$ also presented a disadvantage. For these reasons, an
injectable formulation with similar attributes and components, deemed PoLi\textsubscript{gel}, was subsequently developed (124-126).

The injectable PoLi\textsubscript{gel} formulation consists of the natural products chitosan, egg phosphatidylcholine (ePC), and lauryl aldehyde (LA) (124). These materials are biocompatible and biodegradable, thus eliminating the need to surgically remove them after the drug has been delivered. The PoLi\textsubscript{gel} is formulated with a water-soluble chitosan (WSC). This avoids the use of acids to dissolve this polymer, which inevitably leads to residual acid remaining when the formulation is prepared, which affects biocompatibility (127). The second component, egg phosphatidylcholine (ePC), is a major component of cell membranes (128). It is also a natural component of the peritoneum; thus, it is likely to be biocompatible (129). LA contributes hydrophobicity to the formulation through N-acylation of chitosan, which forms a stable matrix for drug delivery (130). The PoLi\textsubscript{gel} was formulated as a potential strategy for continuous chemotherapeutic treatment of ovarian cancer following cytoreductive surgery. The formulation was shown to be stable and capable of continuous release of DTX both \textit{in vitro} and \textit{in vivo} in healthy CD-1 mice, at a rate of 2.4 ± 0.7% and 4.4 ± 0.7% per day, respectively (131).

1.6 Chemotherapy dosing schedule

Standard chemotherapeutic agents are conventionally administered at their MTDs (132). In ovarian cancer therapy, these regimens include 3-4 week treatment-free intervals between chemotherapy doses, which is the time it takes for proliferating hematological precursor cells to repopulate and recover from the cytotoxicity (133, 134). However, these intervals also allow surviving, resistant tumor cells to repopulate at progressively faster rates after each treatment, eventually leading to drug resistant disease (135, 136). To address these limitations, various studies have explored more frequent dosing schedules with the goal of increasing efficacy while decreasing systemic toxicity (137).
PTX and DTX induce their cytotoxicity by stabilizing microtubules, generally during the G2/M phase of the cell cycle, thereby interfering with the normal functioning of the mitotic spindle (62). DTX has the ability to also act during the S phase by interfering with centrosome organization (62, 138). Nevertheless, both drugs are considered cell-cycle-phase specific. With short drug exposure times, most cells in a tumor are not at the critical target phase (139). More frequent exposure of tumor cells to such agents increases the probability of exposing a greater number of cells to the drug when they are at the vulnerable phase(s) of the cell cycle (140, 141). A clinical study of 96-hour “continuous” i.v. infusions of PTX resulted in disease stabilization in patients with metastatic melanoma, showing the potential of longer exposure of this drug (142).

Clinical trials have shown that shortening treatment-free intervals by weekly chemotherapy provides therapeutic efficacy in refractory ovarian cancer (Table 1.3) (69, 140, 141, 143, 144). Patients who no longer respond to PTX or DTX therapy every 3 weeks respond well when these drugs are administered on a weekly basis (145-147). A pivotal phase III clinical trial has recently shown that weekly PTX results in significant improvement in progression-free and overall survival when compared to the current standard of PTX every three weeks (148). With weekly dosing, side effects are either absent or much less severe when compared to standard chemotherapy (147). Phase II clinical trials in breast, prostate, and non-small cell lung cancers have shown high response rates with weekly DTX regimen in the first and second line settings (149). Clinical evidence consistently shows at least equivalent efficacy and a better safety profile as compared to conventional chemotherapy (149). A more frequent schedule of biweekly DTX has shown good tolerability and response rates in recurrent ovarian cancer (150-152).
Table 1.3: Phase II clinical trials assessing weekly administration of taxanes. Partially adapted from Baird 2010 (153).

<table>
<thead>
<tr>
<th>Taxane</th>
<th>Reference</th>
<th>Treatment</th>
<th>Overall Response Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX</td>
<td>Breier 1997 (154)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>Ota 2001 (155)</td>
<td>i.v. PTX weekly (70mg/m2)</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>Thirapakawong 2001 (156)</td>
<td>i.v. PTX weekly (60 and 80mg/m2)</td>
<td>43% and 62%</td>
</tr>
<tr>
<td></td>
<td>Kaern 2002 (157)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>56%</td>
</tr>
<tr>
<td></td>
<td>Markman 2002 (158)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Ghamande 2003 (159)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>Kita 2004 (160)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Dunder 2005 (161)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>Markman 2006 (162)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>Gladieff 2009 (163)</td>
<td>i.v. PTX weekly (80mg/m2)</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Berkenblit 2004 (164)</td>
<td>i.v. DTX weekly (30mg/m2)</td>
<td>6.9%</td>
</tr>
<tr>
<td></td>
<td>Kushner 2007 (69)</td>
<td>i.v. DTX weekly (35mg/m2)</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.v. carboplatin weekly (AUC=2)</td>
<td></td>
</tr>
<tr>
<td>DTX</td>
<td>Tinker 2007 (165)</td>
<td>i.v. DTX weekly (35mg/m2)</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>Gupta 2009 (166)</td>
<td>i.v. DTX weekly (30mg/m2)</td>
<td>25%</td>
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<td></td>
<td></td>
<td>i.v. topotecan weekly (3.5mg/m2)</td>
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Initial studies found that administration of cytotoxics on a more frequent basis inhibited tumor angiogenesis, which originated the idea of metronomic chemotherapy (167, 168). Drug resistant disease could theoretically be managed by shifting the drug target from tumor cells to endothelial cells. The roles of tumor angiogenesis to disease progression, drug resistance and poor therapeutic outcome, and the benefits of anti-angiogenic therapy are discussed in detail in section 1.7.2. Endothelial cells in developing tumor vessels are highly proliferative and can therefore be targeted by cytotoxic agents (169). However, this potential anti-angiogenic action of chemotherapeutics is obliterated by long treatment-free intervals which allow for recovery of the tumor vasculature (147). Metronomic chemotherapy consists of dosing multiple times per week, thereby shortening treatment-free breaks. Preclinical studies have shown that this strategy acts primarily via an anti-angiogenic action (147). As is the case with weekly dosing, preclinical studies have shown a decrease in side effects associated with the chemotherapeutic agents used (170). Phase I and II studies have shown satisfactory response rates with metronomic chemotherapy in various tumor types, including breast, prostate, and one study in ovarian cancer (170). A caveat of this dosing schedule is that increased efficacy has been mostly seen in the clinic upon combination with an anti-angiogenic targeted agent (171-175).

Although more frequent dosing seems promising, weekly and metronomic chemotherapy approaches still include treatment-free intervals. Alternatively, eliminating these treatment-free intervals through continuous chemotherapy is a promising strategy.

### 1.7 Chemotherapy drug resistance

The greatest challenge in cancer management is the development of resistance in tumors. Drug resistance leads to 90% of patient deaths with metastatic late-stage ovarian cancer (176-178). It may ensue due to: (a) changes in the tumor microenvironment that result in poor tumor drug
delivery and penetration (179, 180), (b) abnormal tumor vessel formation through irregular angiogenesis (181), (c) increased tumor cell proliferation (182), or (d) due to molecular alterations in drug targets, survival pathways (180, 183) or cell membrane drug transporter expression (184).

1.7.1 Drug distribution and penetration into tumors

The tumor microenvironment is highly heterogeneous, with a chaotic distribution of proliferative and quiescent cells, hypoxia and pH fluctuations, causing the concentration gradient of a drug within tumors and the distribution of drug sensitive and resistant cells to vary greatly (185). Hypoxia has been long known as a mechanism of chemotherapy resistance and significantly hinders drug delivery (186, 187). Cells that do not receive proper oxygenation due to poor blood flow within a tumor are quiescent and, as most anticancer drugs act on cycling cells, these cells are resistant to chemotherapy (188). Hypoxia also leads to an enrichment of cells with enhanced survival capabilities that may, in turn, be inherently resistant to chemotherapeutics (185). It also induces upregulation of P-glycoprotein (P-gp), a membrane efflux pump that induces multidrug resistance, as it is capable of removing multiple structurally and mechanistically distinct chemotherapeutics from cells (185).

Poor tumor drug penetration is an important contributor to drug resistance, and can occur due to a number of factors. Tumor cell density influences the extent of tumor drug penetration (187). Tumors don’t have properly functioning lymphatic drainage, which results in high interstitial fluid pressure that limits diffusion and proper movement of molecules throughout the tumor (188). Due to these and other factors, chemotherapeutics can only penetrate a maximum of 1-2mm into tumors upon single treatment (73). Taxanes have particularly poor penetration into tumors (189). However, prolonged presence of a drug has been shown to enhance its tumor penetration (185, 187). Continuous drug infusion can achieve this, as diffusion of the drug can be maintained for prolonged periods, resulting in a more uniform tumor
distribution than a single administration (185). This is especially the case with IP chemotherapy, in which tumors are bathed in chemotherapy for prolonged periods (92).

1.7.2 Angiogenesis

A high degree of tumor angiogenesis has been correlated with poor survival in ovarian cancer (190). Upon activation, VEGF induces the proliferation and recruitment of endothelial cells, which can form new blood vessels (190). It also increases the permeability of existing blood vessels (190). The permeability of vasculature contributes to the formation of ascites fluid, which facilitates dissemination of malignant cells throughout the peritoneal cavity. In fact, VEGF is essential for ascites formation in ovarian cancer (191). Anti-angiogenic agents cause pruning of immature blood vessels while normalizing existing tumor blood vessels, which are usually chaotically formed and leaky (188, 192). Reducing vessel permeability leads to reduced interstitial pressure in tumors, thereby improving drug delivery into tumors through vessels as well as direct tumor penetration upon locoregional treatment in the case of IP chemotherapy, which is greatly hindered by high interstitial fluid pressure within tumors (192, 193). Since blood flow within tumors is improved, hypoxic regions are also reduced (186). Furthermore, VEGF inhibition directly enhances the activity of taxanes, as VEGF induces the expression of the anti-apoptotic protein survivin, which maintains microtubule integrity (194).

Most cytotoxic agents have an anti-angiogenic nature. Since they are effective against rapidly proliferating cancerous cells, which are highly heterogeneous, they can easily attack dividing endothelial cells (195). Microtubule-disrupting agents such as the taxanes have anti-angiogenic activity (141). In fact, endothelial cells have a 10- to 100-fold greater sensitivity to taxanes compared to tumor cells (141). DTX is ten times more aggressive at targeting endothelial cells and preventing capillary sprouting than PTX (196, 197). In a more direct mechanism against angiogenesis, DTX has been shown to reposition the microtubule-organizing centre, which is directly involved in chemotaxis of endothelial cells towards hypoxic
regions (198). As discussed earlier, more frequent dosing of chemotherapeutics has an anti-angiogenic effect, which could partly explain why weekly chemotherapy is efficacious in disease resistant to the 3-weekly dosing schedule (141).

This beneficial activity, however, is hindered by the long treatment-free periods that currently exist in chemotherapy. During this period, damaged endothelial cells become repaired and form new vasculature, enhancing tumor growth (195). Rationally, decreasing this treatment-free period would prevent these cells from recovering, thus inhibiting angiogenesis (195). Impressive anti-angiogenic effects were shown when cyclophosphamide was administered at lower but more frequent doses in various tumor types (199). It has been shown that when PTX is administered continuously at a low concentration, vascular endothelial cells quickly undergo apoptosis (200). Interestingly, in another study that shows the susceptibility of endothelial cells to taxanes, DTX was shown to be ten times more aggressive at targeting these cells and preventing capillary sprouting than PTX (196, 197). Thus, it is important to study the effects of sustained and localized DTX delivery on angiogenesis at the tumor site. In 2004, it was shown that the commercial formulation vehicles of both PTX and DTX (Cremophor-EL and polysorbate 80, respectively) nullify the anti-angiogenic activity of these taxanes (201). A novel delivery vehicle that eliminates the need for these formulation vehicles would allow taxanes to perform their anti-angiogenic function.

1.7.3 Tumor cell repopulation

Tumor repopulation, a significant contributor to treatment failure, is the accelerated proliferation of surviving clonogenic tumor cells during or after therapy that occurs without changes to the intrinsic sensitivity of cells to the drugs used (133, 202, 203). This is often observed in solid tumors that initially respond well to therapy, followed by tumor regrowth upon subsequent treatment cycles (133). When cytotoxic treatment eradicates a population of cells within a tumor, a response occurs during the treatment-free periods between courses of chemotherapy that
causes clonogenic tumor cells to regenerate at a faster rate than prior to treatment, hence the termed accelerated repopulation (204-206). These cells that are capable of regenerating the tumor have often been considered to be cancer stem cells (133, 204).

In radiotherapy, increased repopulation with each treatment cycle has been shown to occur by various studies. Radiotherapy is administered in one daily fraction, five times per week, over 6-7 weeks (207). In 1997, a randomized trial showed that multiple small fractions (three daily fractions over 12 days) of radiation at smaller doses improves local control and survival when compared to conventional radiotherapy (207). This treatment strategy is known as CHART, or continuous, hyperfractionated, accelerated radiotherapy (207). Immediately after a radiation fraction has been delivered, cytotoxic doses are achieved. A few hours later, however, surviving cells proliferate and repopulate the tumor during the treatment-free period (136, 208). While the underlying mechanisms have still not been completely elucidated, it is believed that cells on the surface of the tumor are vulnerable to radiotherapy due to their proliferative state and proximity to blood vessels. Once these have been killed and the surface layer of the tumor becomes stripped, the microenvironment of inner quiescent cells is altered, allowing for improved nutrition. During treatment-free periods, these cells then re-enter the cell cycle and proliferate (209). The rate of proliferation has been shown to accelerate with treatment time in radiotherapy (136, 207, 208).

In chemotherapy, repopulation should play an even larger role, since treatment-free periods and treatment times are much longer than in radiotherapy (210). In a study in rats that had been implanted with gliosarcoma cells, the doubling time of tumor cells decreased from 40 hours to 26 hours after a single chemotherapy dose (211). Another study showed a two-fold increased production of clonogenic cells in a murine sarcoma model two weeks after chemotherapy (212). As with radiotherapy, a potential strategy to limit repopulation is to decrease the treatment-free period while maintaining low toxicity to normal tissues (210).
recent study shows that intermittent PTX administration causes an increased proliferation rate in ovarian xenograft tumors, whereas continuous PTX did not impose changes in proliferation (209). This strategy eliminates the treatment-free periods, which is when repopulation would occur.

### 1.7.4 Drug resistance at the cellular level

At the cellular level, drug resistance commonly arises upon changes to the expression levels of certain genes and proteins that interfere with the drug’s mechanism of action, inhibit intracellular drug accumulation, or enhance cellular survival capabilities. This section will discuss genes and their proteins that are known to lead to resistance to DTX when overexpressed.

Alterations in drug uptake or efflux that interfere with intracellular drug accumulation are of key importance in drug resistance (213, 214). The efflux transporter P-glycoprotein (P-gp), encoded by the *mdr1* gene, has been associated with multidrug resistance in many cancers (215), as it can efflux multiple structurally and mechanistically unrelated chemotherapeutic agents ranging in size from 300 to 2,000 Da, including DTX (184). The only other ABC transporter known to be capable of DTX transport is the product of the *mrp7* gene, MRP7 (216). This transporter is the most structurally distinct of all MRPs and, when overexpressed, conferred strongest resistance to DTX in a screen of various other anticancer agents (216). The product of the *rpn2* gene contributes to P-gp-mediated DTX resistance by stabilizing P-gp in the cell membrane (217). A previous study has shown that knockdown of RPN2 by siRNA leads to greater DTX retention within cancer cells, due to reduced P-gp glycosylation, thus resulting in greater cytotoxicity (217).

DTX promotes microtubule polymerization and stabilization by binding to β-tubulin subunits, which leads to cell cycle arrest and apoptosis (183). Overexpression of the β-tubulinIII isoform, observed clinically in resistant ovarian cancer, leads to DTX resistance, as the drug cannot effectively bind to this isoform (183). The action of DTX on microtubules can also be
hindered by stathmin1, a molecule found overexpressed in ovarian tumors (218). This molecule stimulates microtubule depolymerisation and interferes with taxane binding to β-tubulin subunits (219). Thioredoxin also obstructs this process by interfering with the redox regulation of tubulin cysteine residues, which microtubule assembly depends on (220). Thioredoxin expression has been shown to increase in tumors after DTX treatment (220).

Resistance to DTX can also occur due to increased cellular survival capabilities. The serine/threonine kinase Akt2 is the member of the protein kinase AKT/PKB family that most influences cell survival and proliferation in ovarian cancer, and is found overexpressed in 40% of cases (221). Knockdown of Akt2 by siRNA was shown to substantially increase DTX sensitivity in cancer cells (222). This kinase promotes DTX resistance by increasing transcription of pro-survival genes upon DTX exposure while inactivating or inhibiting the transcription of pro-apoptotic factors (223). Akt2 phosphorylation and activation is influenced by actinin4, a molecule that thereby indirectly contributes to DTX resistance (224). Survival is also promoted by the anti-apoptotic protein Bcl-2, which becomes deactivated upon phosphorylation by DTX (225). Overexpression of Bcl-2 can lead to the prevention of apoptosis and drug resistance (225).

Various approaches have been investigated to overcome drug resistant disease. The simultaneous use of structurally and mechanistically distinct chemotherapeutic agents is one such strategy, although it has not proven completely successful due to the emergence of multidrug resistance (180). The use of drug efflux transporter inhibitors has been extensively investigated, although their clinical implementation has been limited by extensive toxicities associated with their use (226). A promising approach to overcome resistance is modifying the chemotherapy dosing schedule, as previous studies have shown efficacy in refractory disease upon more frequent dosing (227, 228).
1.8 Rationale, hypothesis and objectives

Ovarian cancer is the deadliest gynaecological malignancy, mainly due to significant difficulties in diagnosis which cause 80% of patients to discover the disease only at late stages (2, 4, 5). Effective treatment strategies are, therefore, crucial in the successful management of ovarian cancer. Current first-line treatment of cytoreductive surgery followed by i.v. chemotherapy every 3 weeks has not been successful, leading to a 5-year survival rate of only 30% (71). Although patients initially respond to this approach, 85% relapse with drug resistant disease (2). IP chemotherapy has shown to significantly improve survival by achieving higher drug concentrations at disease sites than would be possible by i.v. delivery, while decreasing systemic toxicities (82-85). Unfortunately, catheter-related complications have limited its clinical use. It is therefore imperative that better IP chemotherapy delivery strategies be used to exacerbate the need for inpatient care and an indwelling catheter.

Chemotherapy is currently administered at MTD, followed by treatment-free intervals to allow recovery of healthy tissues (133). These intervals cause surviving resistant tumor cells to repopulate at progressively faster rates after each treatment (135, 136). This leads to drug resistant disease, which accounts for 90% of patient deaths with metastatic late-stage ovarian cancer (176-178). Resistance occurs due to changes in the tumor microenvironment that result in poor tumor drug delivery and penetration (179, 180), increased tumor cell proliferation (182), molecular alterations in drug targets, survival pathways (180, 183) or drug transporters (184). Clinical trials have shown that shortening treatment-free intervals by weekly chemotherapy administration provides superior therapeutic efficacy in ovarian cancer treatment (69, 140, 141, 143, 144). Although more frequent dosing seems promising and exposes long treatment-free intervals as a limiting factor in favorable clinical outcomes, weekly chemotherapy still incorporates these intervals. Complete elimination of drug-free breaks is a promising alternative.
This thesis project aimed at investigating the effects of continuous and localized DTX delivery on the efficacy and tumor responsiveness in ovarian cancer. Although PTX is currently used as first line therapy, its semi-synthetic analog DTX has shown numerous advantages and has been used as the model drug in these studies. Mechanisms underlying differences in efficacy between continuous and intermittent DTX delivery were explored. We employed the PoLi\textsubscript{gel} injectable depot-forming drug delivery system for continuous and localized DTX delivery.

1.8.1 Overall hypothesis

The complete elimination of treatment-free intervals by a continuous chemotherapy approach will result in superior antitumor efficacy than intermittent chemotherapy, both administered locally, in models of EOC due to better tumor responsiveness, specifically greater tumor cell death and less angiogenesis, repopulation, and molecular drug resistance.

1.8.2 Objectives

**Objective 1.** To determine the *in vitro* and *in vivo* biocompatibility and *in vivo* biodegradability of the PoLi\textsubscript{gel} formulation, the platform for continuous DTX delivery.

**Objective 2.** To assess the antitumor efficacy of continuous and intermittent DTX treatment in murine xenograft models of human and murine ovarian cancer.

**Objective 3.** To understand the impact of dosing frequency on tumor cell repopulation rates, apoptosis, and angiogenesis progression.

**Objective 4.** To determine the effects of continuous and intermittent DTX on the development and/or progression of molecular drug resistance.
1.9 Ovarian cancer models used in this thesis

Since 1969, xenograft tumor models of ovarian cancer have been indispensable tools for studying cancer cell biology and response to therapeutics, among other uses (229, 230). These models were at first mostly established subcutaneously in immunocompromised mice. Subcutaneous tumor models are still widely used due to ease of measuring disease progression over time, which is not possible with IP models without the aid of imaging modalities (231). Subcutaneous tumor models, however, do not reflect the disease representation of ovarian cancer in humans, in which tumors mostly metastasize throughout the peritoneal cavity (229). Peritoneally-localized tumor models can be achieved via inoculation of human ovarian cancer cells into the peritoneal cavity of immunocompromised mice. These are considered to be the best models of late stage ovarian cancer, and different cell lines can be used to establish certain histological subtypes of ovarian cancer (230). This thesis project employed xenograft models using the ovarian cancer cell lines SKOV3, HeyA8 and its resistant counterpart HeyA8-MDR.

The SKOV3 cell line was established from cells isolated from the ascites of an ovarian cancer patient. This cell line has a quick doubling time of 28.8 hours in culture (232). When inoculated IP in nude mice, the take rate of SKOV3 cells varies widely and has been reported to be 100% (232), 60% (233) and even as low as 29% (231), although studies by our group have shown a high take rate approaching 100% when these cells are inoculated in severe combined immunodeficient (SCID) mice. IP inoculation of SKOV3 cells results in the formation of multiple small solid tumors on the peritoneal layer, diaphragm, and mesentery. Large tumor masses can usually be seen in the pelvic area, near the intestinal area and attached to the omentum (231, 233). Variable amounts of bloody ascites fluid can also be present (231); in our experience, the volume is limited to a maximum of 200-300 μL and is often not present at all. The anatomical features of advanced ovarian cancer are well represented in IP SKOV3 xenografts (229). Tumors arising from the SKOV3 cell line were originally classified as serous epithelial
adenocarcinoma (229, 232). However, recent studies have classified SKOV3 tumors as having clear cell histology (230, 231). Ovarian clear cell carcinoma is a rare subtype of epithelial ovarian cancer, comprising only 5-10% of all cases (234). However, this subtype is highly resistant to standard chemotherapy regimens, with response rates being as low as 11% to first-line treatment, resulting in very poor disease outcome when diagnosed at late stages (234-236).

The HeyA8 cell line was established by passaging human epithelial ovarian tumor tissue in immunocompromised mice (232, 237). The primary tumor from which HeyA8 cells were derived was described as moderately differentiated papillary adenocarcinoma (232, 237). Interestingly, xenografts that form from these cells in mice are undifferentiated carcinomas (230, 231). Undifferentiated ovarian carcinomas are very rare but clinically aggressive, and are associated with a prognosis worse than clear cell carcinoma (238). When HeyA8 cells are injected IP into immunocompromised mice, large tumors can be observed, usually attached to the peritoneal layer, without ascites fluid accumulation (233). The doubling time of the HeyA8 cell line is 30 hours in culture (232). The take rate of HeyA8 cells varies from 100% (232) to 67% (231). As with SKOV3 cells, studies by our group have shown a take rate of 100% when HeyA8 cells are inoculated IP in SCID mice. In this thesis project, a taxane resistant HeyA8-MDR cell line was used in addition to HeyA8. This line was previously derived from the HeyA8 line by culturing these cells in paclitaxel-containing medium for 18 months until cells became resistant to 500 ng/ml paclitaxel (239).

The use of immunocompetent mice overcomes the caveat of xenograft models in immunocompromised mice, as many studies have demonstrated a role of the immune system in cancer progression and metastasis (240). An alternative is to develop ovarian cancer cell lines from the ovarian surface epithelium of immunocompetent mice. These cells can then be injected directly into the bursa that covers the murine ovary. This is considered a good model of early stage disease (230). The ID8 orthotopic model was developed in an effort to better understand
the early events of ovarian carcinogenesis in immunocompetent mice (241). Using this orthotopic model allows for studies of the immune system and its role in cancer progression and response to treatment (241). Its development was based on the observation that rodent ovarian surface epithelial cells can be immortalized when cultured in vitro (240). Epithelial ovarian cells from the ovaries of C57Bl/6 mice were isolated and cultured in vitro for more than 20 passages to establish the ID8 cell line (241). When injected into the ovarian bursa, ID8 cells give rise to extensive ascites fluid accumulation 90 days post-inoculation and solid tumors throughout the peritoneal cavity, which have a serous carcinoma histology, the most common histologic type of this disease (240, 242).

A caveat of this approach is that the ovarian bursa is not present in the human ovary, and may interfere with metastasis beyond the ovary (230). If the goal is to model advanced stage disease, as was the case in this thesis project, IP inoculation of cells is a superior alternative. When these cells are injected IP into C57Bl/6 mice, large volumes (about 10mL) of ascites fluid accumulate within 33 days (241). This extensive ascites fluid accumulation has been attributed in part to changes in VEGF expression, which contributes to vascular leakiness and fluid build-up (240). Solid tumor formation is not seen until at least 2 months post-inoculation (243).

The use of IP SKOV3, HeyA8 and HeyA8-MDR xenograft models in this thesis project has allowed for the assessment of a continuous chemotherapy strategy and its effect on drug resistance in several types of clinically aggressive and chemoresistant forms of highly proliferative, advanced epithelial ovarian cancers. The IP syngeneic ID8 model in immunocompetent mice allowed for the assessment of this dosing schedule on ascites volume accumulation in a model of advanced epithelial ovarian cancer.
1.10 Overview of thesis chapters

Studies addressing the aforementioned objectives are described in Chapters 2-5 of this thesis. Since the PoLi\textsubscript{gel} was the platform used throughout this project for localized and sustained drug delivery, establishing the biocompatibility of this system was an important first step. Chapter 2 describes the biocompatibility assessment of two potential PoLi\textsubscript{gel} formulations. At the time those studies were conducted, two formulations showed promise in terms of favorable continuous \textit{in vitro} drug release and other parameters. The biocompatibility of both formulations was assessed in order to choose the ideal formulation for use in subsequent studies. \textit{In vitro} cytotoxicity was evaluated in L929 and HeLa cell lines, and \textit{in vivo} biocompatibility was investigated subcutaneously and IP in healthy CD-1 mice. Furthermore, histological examination of peritoneal tissues was done to assess signs of local toxicity, and plasma interleukin-6 levels were measured as an indication of systemic inflammation. Biodegradation was also measured \textit{in vivo} over a period of four weeks, the period during which the PoLi\textsubscript{gel} releases its drug load.

The chosen PoLi\textsubscript{gel} formulation was used in studies described in Chapter 3. The antitumor efficacy of continuous DTX was assessed \textit{in vitro} using SKOV3 and ID8 human and murine ovarian cancer cell lines, respectively, followed by \textit{in vivo} assessment in SCID mice bearing SKOV3 tumors. Once this treatment strategy was deemed efficacious, continuous and intermittent DTX therapy were compared in SCID mice bearing SKOV3 tumors using bioluminescence imaging to track tumor progression over time. Possible mechanisms leading to differences in efficacy were then examined. Immunohistochemistry was used to assess the extent of tumor cell death, repopulation, and angiogenesis in these tumors. Repopulation of continuous and intermittent DTX was more closely examined \textit{in vitro}. Efficacy of these two dosing schedules was also assessed in C57/Bl6 mice bearing ID8 ascites.

Studies described in Chapter 4 addressed whether changes in chemotherapy dosing schedule affect the development, progression, or circumvention of drug resistance in
chemosensitive and chemoresistant ovarian cancer. This was done using the human ovarian cancer cell line HeyA8 and its DTX-resistant counterpart, HeyA8-MDR. SCID mice bearing HeyA8 or HeyA8-MDR tumors were treated with DTX intermittently (1x/week or 3x/week) or continuously for 21 days. RT-qPCR was used to measure the relative expression levels of genes coding for proteins that have been implicated in DTX resistance, including drug efflux transporters, molecules that interfere with this drug’s mechanism of action, and those that promote cell survival in the presence of DTX. Drug efflux transporter expression was further examined by Western blotting. In order to perform a direct comparison of gene expression between the various treatments, all groups were terminated on day 21, when control mice reached predetermined ethical endpoints.

Chapter 5 describes studies which aimed to understand whether long-term continuous chemotherapy lengthens survival and delays drug resistance in xenograft models of sensitive and resistant ovarian cancer. For this purpose, SCID mice bearing HeyA8 and HeyA8-MDR xenografts received DTX intermittently or continuously until any of the predetermined ethical endpoints were reached. Tumors from each group were analyzed for gene expression of the same DTX resistance-related genes examined in Chapter 4.

This thesis concludes with Chapter 6, which outlines overall conclusions and proposes future research directions.
Chapter 2

Biocompatibility of Injectable Chitosan-Phospholipid Implant Systems


Author Contributions: De Souza R performed all experiments and data analysis, and wrote the manuscript. Zahedi P formulated the PoLiigel, synthesized water-soluble chitosan, assisted in animal studies, and assisted in the writing of the manuscript. Histological staining was done by the Pathology Research Program (University Health Network).
2.1 Abstract

Injectable biomaterials are desirable therapeutic platforms due to minimal invasiveness and improved patient compliance, and are applicable in such areas as compound delivery and tissue engineering. The present work examined the biocompatibility of injectable blends composed of chitosan, phospholipid and LA, PoLi\textsubscript{gel}-LA, or lauric chloride (LCl), PoLi\textsubscript{gel}-LCl. \textit{In vitro} cytotoxicity was evaluated in L929 and HeLa cell lines. Both blends resulted in acceptable biocompatibility, although greater cell viability was seen with PoLi\textsubscript{gel}-LA. \textit{In vivo} biocompatibility was investigated subcutaneously and IP in healthy CD-1 mice. The PoLi\textsubscript{gel}-LA blend caused no local or systemic toxicities over a four-week period while the PoLi\textsubscript{gel}-LCl caused immediate local toxicity. Mice injected with PoLi\textsubscript{gel}-LA did not show physical or behavioural alterations, and body weight changes did not differ from control animals. Furthermore, histological examination of spleen and liver showed unaltered morphology. Interleukin-6 levels in mice injected with PoLi\textsubscript{gel}-LA did not differ from levels of control animals (6.91 ± 3.61 pg/mL versus 6.92 ± 5.02 pg/mL, respectively). Biodegradation occurred progressively, with 7.4 ± 5.02 % of the original injected mass remaining after four weeks. Results obtained herein establish the biocompatibility of PoLi\textsubscript{gel}-LA and indicate its potential for use in various localized therapeutic applications.
2.2 Introduction

Recently, injectable systems have garnered much attention for a variety of applications including tissue engineering, localized drug delivery and gene therapy (244-246). Injectability provides several desirable advantages such as ease of application, localization at target site, bypass of various physiological barriers and improved patient compliance and comfort. Polymers have emerged as the material of choice for use in a wide range of medical and pharmaceutical applications including fabrication/coating of biomedical devices, therapeutic delivery systems and tissue engineering (247-249). Injectable polymer-based microspheres, pastes and gels have been developed for drug delivery (250). However, drawbacks in the preparation of these injectables such as the frequent need of organic solvents for in situ polymer precipitation are limiting (246). While thermosensitive systems which convert from a liquid to semi-solid upon injection provide ease of application; the high temperatures required and potential for dose-dumping during conversion pose problems, especially in intraocular delivery (246, 251).

A number of polyester-based injectable localized anti-cancer drug delivery systems have been developed (252). Although considered to have good biocompatibility, polyesters lead to a foreign body response which encapsulates the delivery system, hindering drug release (253-256). As well, polyesters result in acidic byproducts that could degrade the compound being delivered (257, 258). Natural polysaccharides have the potential to circumvent issues attributed to synthetic polymers and have been investigated for injectable localized drug delivery systems due to their biocompatibility, biodegradability and lack of foreign body response (259, 260). Chitosan is a linear natural polysaccharide composed of β-(1→4)-2-amido-2-deoxy-D-glucan (glucosamine) and β-(1→4)-2-acetamido-2-deoxy-D-glucan (acetyl glucosamine) units. It is obtained by deacetylation of chitin, a polysaccharide widely found in nature (e.g. crustaceans, insects and certain fungi). Chitosan has found application in the drug delivery field due to its
excellent biocompatibility, low toxicity, immunostimulatory activities, antibacterial and antifungal action, and anticoagulant properties (261, 262). Furthermore, the degradation products of chitosan have been shown to be nontoxic, nonimmunogenic and noncarcinogenic (263). However, the application of chitosan has been limited due to its poor solubility and the acidic compounds normally required to dissolve chitosan compromise the biocompatibility of the system (264). Blends in this study employed use of a WSC, eliminating the need for acidic compounds during preparation (265).

Phospholipids have been utilized in many types of drug delivery applications, including nanoparticle, microparticle and liposome formulations (266-268). They naturally occur in nature as the major component of membrane phospholipid in eukaryotic cells. It has been reported that favorable interactions (i.e. hydrogen and ionic bonding) exist in blends of chitosan and phospholipids (269).

Our group has explored the development of chitosan-phospholipid drug delivery systems for the localized delivery of antineoplastic agents, PTX and DTX (121, 126, 131, 270, 271). Currently available formulations of these drugs employ non-ionic surfactants, which cause acute hypersensitivity reactions and various systemic toxicity issues (272, 273). Cremophor EL and polysorbate 80 (PS80) are currently used as formulation vehicles for PTX (Taxol®, Bristol-Myers Squibb) and DTX (Taxotere®, Sanofi-Aventis), respectively. In previous reports, a stable film composed of chitosan and phospholipid was developed for localized PTX delivery (121, 269-271, 274, 275). The implant was shown to be nontoxic, non-immunogenic, and successfully circumvented toxicities caused by Cremophor EL (121, 126, 271, 275). However, drawbacks of the film implant include the need for surgical implantation and slow biodegradation. For these reasons, injectable chitosan-phospholipid blends containing a fatty acid (LC1 or LA), were recently developed (126, 131).
Based on physicochemical characterization (stability, pH, morphology, rheology, drug loading and release), two blends were chosen for further examination (126, 131). The present study focused on the *in vitro* and *in vivo* cytotoxicity and biocompatibility assessment of two chitosan phospholipid blends containing either LCI (PoLi<sub>gel</sub>-LCI) or LA (PoLi<sub>gel</sub>-LA). *In vitro* cytotoxicity was assessed in L929 mouse fibroblast cells and HeLa cervical cancer cells. *In vivo* biocompatibility was evaluated in healthy CD-1 mice following both subcutaneous and IP injection.
2.3 Materials and methods

2.3.1 Materials

Chitosan (92.5% purity, 94.5% degree of deacetylation and $2.2 \times 10^5$ molecular weight) was purchased from Marinard Biotech Inc. (Rivière-au-Renard, QC, Canada). ePC, LCl, LA, glycyltrimethylammonium chloride (GTMAC) and PS80 were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada). All other chemicals were used without further purification.

2.3.2 Chitosan-phospholipid blend (PoLi$_{gel}$) preparation

A WSC derivative was prepared through conjugation of GTMAC on the chitosan backbone, using a method described elsewhere (265). 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 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) (265). The degree of substitution of GTMAC on the chitosan backbone was calculated to be 45% using an established titration method (276).

Chitosan-phospholipid blends were prepared as outlined elsewhere (131, 265). In brief, WSC was dissolved in distilled deionized water to form a 4.2% (w/v) solution and allowed to rest for 24 h. LCl or LA was used to dissolve ePC, and this mixture was added to the WSC solution at a material ratio of 1:4:1 w/w/w (WSC:(LCl or LA):ePC). The blend was sterilized under UV-light (Sterilizer T209, Intercosmetics, Canada) for 3 h prior to use.
2.3.3 Cell culture experiments

The cytotoxicity of the PoLi$_{gel}$-LCl and PoLi$_{gel}$-LA was evaluated in L929 mouse fibroblast cells and HeLa cervical cancer cells. Fibroblast cells are widely used in cytotoxicity studies of biomaterials, as they are the main cellular component of connective tissues (277). As sensitivity to cytotoxicity differs among different cell lines, HeLa cells, which display a dissimilar sensitivity to biomaterials, were used to verify the results (278). L929 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 μg/mL streptomycin). HeLa cells were maintained in DMEM medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Both cell lines were allowed to grow in a monolayer in a tissue culture flask incubated at 37 °C, gassed with 5% CO$_2$ and held at 90% relative humidity. Cells were counted by hemocytometry and seeded onto 96-well plates at a cell density of 5,000 cells/well. Using the extract dilution method of cytotoxicity (279); growth media was aspirated after 24 h and replaced with 100 μL of media that had been incubated for 48 hours with varying amounts (0.0013 to 9.37 μg/mL) of sterilized PoLi$_{gel}$-LA or PoLi$_{gel}$-LCl. Following a 48 h incubation period, cell viability was evaluated using the MTT assay method. Specifically, 100 μL of 5 mg/mL of thiazolyl blue tetrazolium bromide solution (in 0.01 M sterilized PBS, pH = 7.4) were added to each well, and plates were incubated for 3 h. Following incubation, 100 μL of extraction buffer (20% w/v SDS in a solution of 47.5% v/v dimethylformamide, 47.5% v/v H$_2$O, 2.5% v/v of 80% acetic acid, and 2.5% v/v of 1N HCl) were added to each well to solubilize the MTT crystals, and was left at room temperature for 12 hours. Finally, cell viability was measured by optical absorbance at $\lambda = 570$ nm using a Spectra Max Plus microplate reader (Molecular Devices, CA, USA). Cells incubated with media alone were employed as control and this was considered as 100% cell viability. 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. All cell culture experiments were repeated three separate times (n=3).
2.3.4 Animal studies

Healthy, 6-8 week old (20-25 g) female CD-1 mice (Charles River Laboratories) were used. For subcutaneous administration studies, 20 $\mu$L of sterilized PoLi$_{gel}$-LA or PoLi$_{gel}$-LCl were injected subcutaneously at a dorsal site of each mouse, using a 30 gauge needle under sterile conditions. For IP administration studies, 50 $\mu$L of sterilized PoLi$_{gel}$-LA were injected IP in the lower left quadrant of each mouse, with an injection depth of 1 cm using a 25 gauge needle under sterile conditions. As one application of this blend is to formulate the anticancer agent DTX, one group of mice was injected with PS80, the commercial vehicle of DTX. Mice were injected IP with 82.5 $\mu$L of PS80:ethanol:saline (1:1:2, v/v/v). This volume of PS80 would be required to administer the same amount of DTX that would be loaded into 50 $\mu$L of PoLi$_{gel}$. In this way, effects of the two vehicles can be equivalently compared. Control animals received 100 $\mu$L of saline solution. Mice were weighed and monitored throughout the treatment period. Sacrifices were performed 1, 2, 3 and 4 weeks post-injections. Liver, spleen, and tissues surrounding the blend were collected, rinsed with PBS and fixed in 4% paraformaldehyde solution for histological assessment. Fixed specimens were paraffin embedded; 5 $\mu$m sections were cut and stained with hematoxylin and eosin (H&E). Slides were examined using Zeiss Axiovert 135 TV light microscope (Carl Zeiss MicroImaging Inc., Germany) at 100-400 times magnification. Liver and spleen sections were examined for presence of inflammatory cells, fibrosis, and abnormal cell morphology. Thickness of the fibrous capsid surrounding the blend was considered to be the distance from the border of the fibrous tissue flanking the blend to the end of the tissue. This was measured using Zeiss Axiovert 135 TV light microscope (Carl Zeiss MicroImaging Inc., Germany) equipped with an ocular micrometer. Blood was collected by cardiac puncture from CD-1 mice for measurements of interleukin-6 (IL-6) and alanine aminotransferase levels in the plasma. The injection site was inspected for PoLi$_{gel}$-LA integrity and capsid formation. Any remaining blend was collected and weighed to assess degradation. The presence of splenomegaly was assessed by visual observations of spleen appearance. All
studies were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee and the Canadian Council on Animal Care.

2.3.5 Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). All results are expressed as means ± standard error of the mean (SEM). For comparisons among different treatment groups, Student’s t-test was used, with significance assigned at p < 0.05.
2.4 Results and discussion

2.4.1 In vitro biocompatibility

The impact of the PoLigel blends on cell viability was assessed in two cell lines, L929 mouse fibroblast cells and HeLa cervical cancer cells using the MTT assay. The extract dilution method was used, which involves a 48 hr incubation of blends in media at 37°C, followed by exposure of cells to the same media (279). This method was chosen over the direct exposure method to avoid confounding factors resulting from physical trauma to the cells due to sample weight (279). The PoLigel-LA showed excellent in vitro biocompatibility as seen in both L929 and HeLa cell lines (Fig. 2.1 A and B). Approximately 80% cell viability was seen at concentrations up to 1.56 µg/ml in both cell lines. This is consistent with injectable chitosan hydrogel formulations that employ chitosan with a high degree of deacetylation (280). Cell viability upon PoLigel-LCL exposure was significantly lower than for PoLigel-LA in both cell lines studied (Fig. 2.1 A and B).
Figure 2.1: Cytotoxicity evaluation of PoLiigel blends. (A) Cytotoxicity profile of PoLiigel-LA and PoLiigel-LCl in L929 cell line. (B) Cytotoxicity profile of PoLiigel-LA and PoLiigel-LCl in HeLa cell lines. (*) indicates greater viability by PoLiigel-LA treatment than by PoLiigel-LCl. Data are expressed as mean ± SEM (n = 3).
While PoLi\textsubscript{gel}-LA IC\textsubscript{50} values of 4.0 μg/mL and 4.1 μg/mL were seen in L929 and HeLa cells, we observed PoLi\textsubscript{gel}-LCl IC\textsubscript{50} values of 1.2 μg/mL and 0.2 μg/mL in these cell lines, respectively. The presence of chloride in PoLi\textsubscript{gel}-LCl could explain the cytotoxicity observed, as it has been shown that pharmaceutical products, such as surfactants, containing chloride result in very low IC\textsubscript{50} values \textit{in vitro} (281, 282). Consistent with our observations, another chitosan formulation that employs chloride caused high cytotoxicity to HEK293 cells even at low concentrations (283). In the presence of water, LCl undergoes hydrolysis, producing acidic byproducts which would decrease the environmental pH (126, 284). In fact, recent work has shown that PoLi\textsubscript{gel}-LCl creates a highly acidic environment of pH approximately 2.75 within hours when incubated in PBS (0.01M pH 7.4) (126, 285). Rapid degradation may also be a contributing factor, as the PoLi\textsubscript{gel}-LCl was found to significantly disintegrate within three hours of incubation \textit{in vitro} in lysozyme solution, whereas only minimal disintegration of the PoLi\textsubscript{gel}-LA occurred over a two-week incubation (131).

2.4.2 Subcutaneous administration

It is known that the host response to foreign materials in the peritoneum is more intensive than in subcutaneous sites (286). Therefore, examining the response of our blends subcutaneously was an important first step. No signs of inflammation were observed upon PoLi\textsubscript{gel}-LA subcutaneous injection and post injections, whereby the blend retained integrity under the skin for six weeks (Fig. 2.2 A and B). Furthermore, no fibrous encapsulation surrounding the PoLi\textsubscript{gel}-LA was observed post-mortem. Other existing experimental chitosan hydrogel systems have been shown to induce a thin fibrous capsid when injected subcutaneously, likely due to differences in composition and formulation (287, 288). It is plausible that the inclusion of ePC in the PoLi\textsubscript{gel}-LA formulation contributes to the absence of encapsulation, as this lipid is used in coatings of biomaterials to prevent protein adsorption (269). Direct comparison to other experimental chitosan systems would assist in determining the underlying factors involved in encapsulation.
Figure 2.2: PoLi_{gel}-LA subcutaneous injection was well tolerated, with no adverse signs over a 6-week period (A) and no in-situ inflammation was seen upon sacrifice (B). Subcutaneous injection of PoLi_{gel}-LCI caused severe ulcerative dermatitis within 24 hours (C) and extensive subcutaneous tissue damage was seen post-mortem (D).
Although we observed a relatively high degree of cytotoxicity of PoLi\textsubscript{gel}-LCl \textit{in vitro} which could be related to acidic pH changes, reports suggest that pH may not impact \textit{in vivo} biocompatibility as acids can be rapidly neutralized within the body (289, 290). For this reason the \textit{in vivo} effects of PoLi\textsubscript{gel}-LCl were examined. However, CD-1 mice injected subcutaneously with PoLi\textsubscript{gel}-LCl experienced ulcerative dermatitis (Fig. 2.2 C and D) within 24 hours of injection and were promptly euthanized. As such only PoLi\textsubscript{gel}-LA was selected for further \textit{in vivo} studies.

2.4.3 IP administration

Once the PoLi\textsubscript{gel}-LA biocompatibility was established subcutaneously, IP response to the blend was examined in CD-1 mice. Behavioural or physical changes such as abdominal swelling were not seen in treated mice following injection or on subsequent days. Throughout the study period, animals showed no signs of peritonitis, lethargy, muscle loss, dehydration or anorexia, symptoms associated with animal toxicity (271, 291). Body weight was monitored as weight losses, particularly those in excess of 10-20%, are indicative of toxicity in mice (292). In this study all mice experienced normal weight gain over four weeks (Fig. 2.3). Percent body weight change of mice injected with PoLi\textsubscript{gel}-LA did not differ from that of saline-injected control mice (n=3, p>0.05).
Figure 2.3: Body weight changes following PoLi$_{gel}$-LA or saline injections. Data are expressed as mean ± SEM (n=3). Weight loss of more than 20% body weight would indicate toxicity.
Levels of the systemic inflammatory mediator, interleukin-6, were measured to assess systemic inflammatory response to the blend. This cytokine is involved in foreign body reactions, thus serving as a good tool for biocompatibility assessment (293). Levels in animals injected with PoLi\textsubscript{gel}-LA remained low at an average of 6.91 ± 3.61 pg/mL (n=3), which was not different from the average value in non-treated animals, 6.92 ± 5.02 pg/mL (n=3, p>0.05). It has been shown that mice suffering from peritonitis, which can be caused by toxic compounds present in the peritoneum, have interleukin-6 levels over one thousand times greater than the levels measured in this study (294). Accordingly, no systemic inflammation occurred in the presence of the PoLi\textsubscript{gel}-LA. Post-mortem examinations revealed no visible signs of internal inflammation at the injection site at all time points. Fibrous encapsulation of PoLi\textsubscript{gel}-LA was not visible to the naked eye after four weeks (Fig. 2.4).
Figure 2.4: Post-mortem view of PoLi_{gel}-LA (indicated by arrow) 4 weeks post-injection. Fibrous encapsulation is not visible to the naked eye, and the gel has not dispersed over this period.
The PoLi$_{gel}$-LA has recently been shown to be a promising formulation strategy for localized delivery of DTX due to continuous drug release, \textit{in vitro} pH and stability profiles (131). As mentioned previously, commercially available DTX is formulated in PS80, a non-ionic surfactant which has been shown to cause hypersensitivity reactions likely due to histamine release resulting in inflammation and fluid retention (295, 296). Effects of PS80 administration were therefore compared to those of PoLi$_{gel}$-LA administration.

Spleen and liver are filtering organs likely to be affected by compounds administered IP, which would traffic through these tissues; thus, they were assessed histologically (297). As seen in Fig. 2.5A, IP PS80 administration led to abnormally high numbers of megakaryocytes throughout the spleen, although no effects were observed in the liver. Megakaryocytes are responsible for the production of platelets, which have been described as having a modulatory role in inflammation (298, 299). This inflammatory reaction may be related to the hypersensitivity reaction caused by PS80, since the spleen has a key role in immune responses (300, 301). Alternatively, this could be a toxic effect of PS80 itself as it passed through the spleen upon clearance from the peritoneal cavity. To date, alterations to the spleen by PS80 have not been reported likely because PS80 is normally administered as a drug vehicle rather than as a single agent, and i.v. over long infusions rather than IP in a bolus manner (273).
Figure 2.5: Representative histological sections of liver and spleen (A) and tissue surrounding PoLi_{gel}-LA (B) stained with H&E, four weeks after IP injection. Magnification: (A) x400 (B) x100.
In contrast to these findings, spleen and liver samples from mice injected with PoLi_{gel}-LA displayed normal architecture and did not differ from those of control mice upon histological observations, indicating that the presence of PoLi_{gel}-LA in the peritoneal cavity did not cause damage to these filtering tissues (Fig. 2.5A). There was a lack of fibrosis, necrotic tissue, and inflammatory cells. Moreover, plasma levels of the liver enzyme alanine aminotransferase, which is indicative of liver toxicity, was not significantly elevated in any treatment group. An additional way the immunogenicity of a material can be evaluated is by the occurrence of splenomegaly, or spleen enlargement and discoloration caused by the proliferation and storage of lymphocytes in the spleen (302). This was not observed over the entire study period, as visual inspection revealed that the size and colour of spleens from animals injected IP with PoLi_{gel}-LA did not differ from those of control animals. This shows the potential use of the PoLi_{gel}-LA blend in replacing drug vehicles such as PS80.

Histological analysis detected a very thin (3-5 μm) capsid surrounding the implant (Fig. 2.5 B). This is consistent with that seen with chitosan-phospholipid films, which induced minimal capsid formation (121). The observed capsid is part of the normal tissue response to implantation. When a biomaterial is implanted, injury to the site occurs inevitably, resulting in an inflammatory response within 2 to 3 weeks that is triggered by injured vascularized connective tissue (303). This typical wound healing cascade involves the formation of a fibrotic capsule that surrounds the material, which occurs upon implantation with even inert materials such as stainless steel implants (303-306). The capsid observed in this study is more than ten-fold thinner than that reported with a previously developed chitosan-based hydrogel (306). The fact that we observed only very mild tissue reaction could possibly be due to our use of highly deacetylated chitosan as a greater degree of chitosan deacetylation has been associated with milder tissue reaction (307). However, other significant differences in materials, methods and experimental conditions exist (306). As was noted in subcutaneous observations, the presence
of phosphatidylcholine lipids prevented a more severe tissue reaction (269). Capsid formation and tissue responses after extended exposure periods (2-3 months) should be further examined in future studies.

Biodegradability is an important aspect if the PoLi_{gel}-LA is to be used as a drug delivery platform, as biodegradability obliterates the need for surgical removal of the system after it has served its purpose, leading to better patient compliance and comfort (307). Studies of injectable chitosan hydrogels have shown that a high degree of deacetylation, as possessed by the chitosan used in this study, contributes significantly to degradability of the system (280). Degradation must occur at an acceptable rate; slow degradation can lead to a granuloma which causes oedema and pain (308). The PoLi_{gel}-LA blend degraded in the peritoneal cavity progressively over time, with only 7.4 ± 5.02 % of the original mass remaining after four weeks (Fig. 2.6). This is a significant improvement over previously developed chitosan films, which showed slow biodegradation, possibly necessitating surgical removal (269). Recent work demonstrated a gradual release of DTX from PoLi_{gel}-LA throughout a two-week study period, at a rate of 2.4% to 3.7% per day for various levels of drug loading (131).
Figure 2.6: Degradation profile of PoLi_{gel}-LA injected IP into CD-1 mice over a 4 week period. Data are expressed as mean ± SEM (n = 3).
2.5 Conclusions

Injectable systems are in high demand for various therapeutic applications due to ease of application, localization at target site, and improved patient comfort. We have demonstrated the *in vitro* and *in vivo* biocompatibility and biodegradability of an injectable chitosan-phospholipid blend containing LA over a period of four weeks. The blend did not cause local or systemic toxicities, as illustrated by normal architecture of liver and spleen, interleukin-6 levels, weight changes, and physical and behavioural patterns of animals. This injectable system overcomes issues with our previously developed films, such as the need for invasive surgical implantation and slow biodegradation. These results, coupled with favourable physicochemical properties addressed in previous studies, reveal promising potential of the chitosan-phospholipid blend containing LA as an injectable localized therapeutic delivery platform.

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Chapter 3

Continuous Docetaxel Chemotherapy Improves Therapeutic Efficacy in Murine Models of Ovarian Cancer


Author Contributions: De Souza R performed all experiments and data analysis, and wrote the manuscript. Zahedi P formulated the PoLi$_{gel}$, synthesized water-soluble chitosan, and assisted in animal studies. Moriyama EH assisted with bioluminescence imaging method development and experiments.
3.1 Abstract

Ovarian cancer is known as the silent killer for being asymptomatic until late stages. Current first-line treatment consists of debulking surgery followed by i.v. chemotherapeutics administered intermittently, which leads to insufficient drug concentrations at tumor sites, accelerated tumor proliferation rates and drug resistance, resulting in an overall median survival of only 2-4 years. For these reasons, more effective treatment strategies must be developed. We have investigated a localized, continuous chemotherapy approach in tumor models of human and murine ovarian cancers using the antineoplastic agent DTX. We show herein that continuous DTX therapy is considerably more efficacious than intermittent therapy, resulting in a greater decrease in tumor burden and ascites fluid accumulation. Immunohistochemical analyses show that continuous chemotherapy abrogates tumor cell proliferation and angiogenesis to the tumor microenvironment, leading to greater tumor cell death than intermittent DTX therapy. Overall, our results show greater therapeutic advantages of continuous over intermittent chemotherapy in the treatment of ovarian cancer.
3.2 Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies, with the majority of patients not surviving beyond 5 years post diagnosis (309). Symptoms of ovarian cancer are non-specific, such as bloating and constipation, and are usually not evident until late stages of the disease, when 75% of cases are diagnosed (310, 311). As early diagnosis remains a challenge, effective treatment strategies are needed to manage the disease. Current therapeutic approaches result in a median overall survival of only 2-4 years (311). First-line therapy of advanced ovarian cancer consists of surgical debulking of large tumors followed by carboplatin and PTX administered i.v. at the MTD (52). This leads to low drug concentrations at tumor sites and high levels in healthy tissues, resulting in dose-limiting toxicities (78, 79). An alternative strategy is to maintain low drug levels in the systemic circulation through localized delivery, consequently decreasing toxic side effects, and increasing local drug concentrations in the peritoneum, where ovarian cancer tumors and ascites reside. This can be achieved through IP administration. Based on clinical trials that have evaluated this treatment strategy, the National Cancer Institute has recommended that IP chemotherapy be considered for the treatment of advanced ovarian cancer (83). A major obstacle hindering the implementation of IP chemotherapy is the need for a catheter for drug delivery into the peritoneal cavity. In large clinical studies, catheter complications including obstructions, infections and bowel perforations accounted for 70% of treatment termination cases in IP chemotherapy treatment groups (81, 312).

Another major problem with current therapy is the administration of short bursts of chemotherapy on a 3-week cycle. Treatment-free periods are required after each dose to allow healthy tissues to recover from the cytotoxic damage (181), during which surviving tumor cells re-enter the cell cycle and resume proliferation at a progressively accelerating rate (209). Decreasing the treatment-free period by administering lower doses more frequently diminishes
the accelerated proliferation observed during intermittent therapy (313). This strategy, termed metronomic chemotherapy, targets not only tumor cells but also rapidly proliferating endothelial cells that give rise to neoangiogenesis essential for tumor viability (181). In contrast, during periods between intermittent chemotherapy, neoangiogenesis resumes and new blood vessels are established.

In this study, we have eliminated treatment-free periods to study the effects of continuous chemotherapy delivered locally to the peritoneal cavity. We previously developed an implantable polymer-lipid film capable of continuous release of PTX, which showed efficacy in an ovarian cancer xenograft model (209, 271). The need for surgical implantation and slow biodegradation led to the development of an injectable paste with similar properties composed of chitosan, phospholipids and LA, termed PoLi\textsubscript{gel}, which showed good biocompatibility and biodegradation (131, 314). Although PTX is used in first-line therapy, its analog DTX has demonstrated advantages, including slower cellular efflux and higher solubility, allowing for higher intracellular concentrations (63). In clinical trials, DTX resulted in equivalent response rates and has demonstrated activity against PTX-refractory carcinomas (52, 53, 315). In\textit{ vitro} studies have shown that DTX has higher affinity for microtubules and greater apoptosis induction and potency at inhibiting microtubule depolymerization than PTX (53, 63, 64). Thus, it is plausible that DTX may become part of first-line therapy. Continuous delivery of DTX holds much promise since this drug acts during specific phases of the cell cycle. Only a small percentage of the tumor cell population is vulnerable to DTX at a given time. Continuous DTX exposure allows cycling cells to reach the vulnerable phases, potentially resulting in greater tumor cell death.

The present study is the first to propose a continuous, low-dose DTX regimen for the treatment of cancer. We have compared the antitumor efficacy of this strategy to intermittent DTX administration in two distinct models of ovarian cancer — a SKOV3 xenograft model that is
highly aggressive and is characterized by large solid tumor formation, and a murine ID8 syngeneic model that progresses slowly, is significantly more chemosensitive and predominantly forms large amounts of ascites fluid. We chose to assess efficacy in both tumor models as late stage ovarian cancer is both characterized by tumor formation and large volumes of ascites fluid. The PoLi\_gel injectable formulation loaded with DTX (DTX-PoLi\_gel) was employed as a means for continuous DTX delivery to the peritoneal cavity. Effects of the two treatment strategies on SKOV3 tumor cell death, proliferation rates and angiogenesis were investigated throughout the treatment period.
3.3 Materials and methods

3.3.1 Cell lines

The SKOV3 human ovarian adenocarcinoma cell line was obtained from the American Type Culture Collection and maintained as previously (316). For bioluminescence imaging, SKOV3 cells were transfected with the luciferase gene (SKOV3-luc) and bioluminescence-viability correlation was done as before (317). The murine ovarian cancer cell line ID8 was a kind gift from Dr. Jim Petrik (University of Guelph, Ontario, Canada) (240). ID8 cells were maintained as described elsewhere (240). All cell lines were grown in monolayers at 37°C/5% CO₂ and 90% relative humidity.

3.3.2 Cytotoxicity of DTX

DTX IC₅₀ values for SKOV3-luc and ID8 cells were determined for use in in vitro experiments. Cells were seeded onto 96-well plates (5,000 cells/well) and exposed to 1.6x10⁻⁴ – 1.2x10² µM DTX in DMSO for 24, 48 and 72 h. Cell viability was determined by MTT. These experiments were repeated three separate times (n=3).

3.3.3 In vitro activity of DTX-PoLigel

The DTX-PoLigel formulation is a blend of WSC, LA, ePC and DTX at final material ratios of 1:4:1:0.71 (w/w/w/w), which was prepared as previously described (131, 314). SKOV3-luc and ID8 cells were seeded onto 6-well plates (4x10⁵ cells/well) and incubated with 5, 10, 20 and 30 µL of drug-free PoLiigel or DTX-PoLiigel corresponding to total DTX concentrations of 93, 186, 372, and 558 µM for 24, 48 and 72h. Cell viability was determined by MTT. These experiments were repeated three separate times (n=3).

3.3.4 Preliminary evaluation of DTX-PoLiigel efficacy

All animal studies were approved by the University of Toronto Animal Care Committee or the University Health Network Animal Care Committee and adhered to these guidelines and the
Canadian Council on Animal Care. Female 6-8 weeks old SCID mice (Charles River) received 1x10⁶ SKOV3-luc cells in 200µL medium IP. Seven days later, mice were injected IP with saline (controls) or DTX-PoLi₉gel (total DTX doses of 16, 24 and 32 mg/kg, released at a rate of 3.6% per day (n=6/group)). Mice were monitored daily for signs of lethargy, weight loss, and abdominal distention. Endpoints requiring humane euthanasia included excessive muscle wasting according to the “body conditioning scoring system” (318), abdominal distention, hypothermia, inactivity, and weight loss in excess of 20%. After a 21-day treatment period, tumor weight was assessed post-mortem. Antitumor efficacy was calculated as: \[\frac{\text{(mean tumor weight untreated)} - \text{(mean tumor weight treated)}}{\text{(mean tumor weight untreated)}} \times 100\%\]. Tumor and plasma DTX content was determined by HPLC using an established method (131).

3.3.5 Antitumor efficacy of continuous and intermittent DTX in SKOV3-luc xenografts.

Female 6-8 week old SCID mice (Ontario Cancer Institute) received 1x10⁶ SKOV3-luc cells in 200 µL serum-free medium IP. Seven days later, animals were grouped (n=12/group): (1) continuous therapy via DTX-PoLi₉gel; (2) intermittent therapy with Taxotere® (Sanofi Aventis); (3) drug-free PoLi₉gel controls and (4) non-treated controls. Group 1 received a single IP injection of DTX-PoLi₉gel (total DTX dose of 32 mg/kg, released at a rate of 3.6% per day). Since the DTX-PoLi₉gel releases 25% of its load weekly (131), group 2 received one IP injection of 8 mg/kg Taxotere® weekly for a total of 32 mg/kg. Group 3 received a single IP injection of drug-free PoLi₉gel in an equal volume as administered to group 2. Group 4 received one weekly IP injection of sterile saline. On days 7, 10, 14, 17, 21 and 24 post-inoculation, tumor burden was assessed by bioluminescence as before (317). Animals were monitored as described above. On days 14, 21 and 24, mice were sacrificed (n=4/group) for tumor immunohistochemistry. The study was terminated on day 24 when control animals reached endpoints. Changes in tumor volume for each mouse at every timepoint were calculated and averaged to illustrate percent changes within each group.
3.3.6 Antitumor efficacy of continuous and intermittent DTX in ID8 xenografts

Female 6-8 week old C57Bl6 mice (Charles River) received 2.5 x 10^6 ID8 cells in 200 µL PBS IP. After 14 days, animals were divided (n=10/group) and treated as described for SKOV3-luc xenografts. This model predominantly forms large volumes of ascites, thus abdominal girth was measured on days 14, 21, 25, 28, 32 and 35 post-inoculation using a measuring tape. Assessment by bioluminescence using ID8-luc cells was not successful due to weak signal and/or interference by the dark skin colour of the mice, as shaving of abdominal hair caused no improvement. Animals were monitored as described above. The study was terminated on day 35, as control and intermittently-treated mice reached endpoints. Ascites fluid volume was measured post-mortem.

3.3.7 Immunohistochemistry

SKOV3-luc tumors collected from mice on days 14, 21 and 24 were processed and immunostained for Ki67, caspase 3 (Casp3) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as described elsewhere (317), while CD-31 immunostaining consisted of incubation with a CD-31 primary antibody (1/50 dilution overnight), followed by steps described for Ki67 and Casp3. A Nikon Coolpix 990 camera mounted onto a Nikon Eclipse e400 microscope (Japan) was used to photograph a minimum of 10 fields per slide at 100x magnification, entirely covering each section. Structures were quantified and scored as before (317).

3.3.8 In vitro tumor cell proliferation

IC_{50} DTX concentrations (24 h) determined for ID8 and SKOV3-luc cells were used as the total DTX concentration per cycle. Cells were plated onto 6-well plates (4x10^5 cells/well) and incubated overnight. Plates of SKOV3-luc cells received one of the following treatments: (a) 5.3 µM DTX as Taxotere® (intermittent treatment); (b) Taxotere® vehicle (PS80 and 13% ethanol in
water) in an equivalent volume used in ‘a’ (intermittent control); (c) 1.7 µM anhydrous DTX in DMSO (continuous treatment); (d) DMSO volume equivalent to ‘c’ (continuous control) (n=3). ID8 cells were treated equally; however, the intermittent concentration was 0.62 µM and the continuous concentration was 0.21 µM. Three 3-day treatment cycles were performed. Intermittent treatments and controls were incubated with cells for 12 h then replaced with fresh medium. Sustained treatments and controls were replaced every 24 h to ensure equal drug exposure as the intermittent treatment. Proliferation was assessed by the clonogenic assay. Cells were plated at known numbers, and colony formation was allowed for 7 days (SKOV3-luc) or 4 days (ID8). Colonies were fixed with methanol and stained with 1% crystal violet (Sigma). Plates were scanned and colonies (>50 cells) were counted using Image J analysis software (NIH). Plating efficiency (PE) was calculated as [(viable colonies)+(cells plated)] and number of clonogenic cells was calculated as [(cells counted at each time point)xPE]. These experiments were repeated three separate times (n=3).

3.3.9  In vitro tumor cell death

Treatments were performed as described for “In vitro tumor cell proliferation” for one treatment cycle. At each day, MTT was used to assess cell viability. These experiments were repeated three separate times (n=3).

3.3.10  Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were done using Statistical Package for the Social Sciences (SPSS, version 16.0). For comparisons between groups, one-way analysis of variance (ANOVA) was used, and multiple comparisons with Bonferroni correction were performed upon statistical significance as determined by ANOVA. Significance was assigned at p < 0.05.
3.4 Results

3.4.1 Activity of continuous IP DTX exposure on ovarian cancer cells and tumors

To test whether continuous DTX provided by the injectable DTX-PoLi\textsubscript{gel} formulation shows activity against ovarian cancer cells, human ovarian adenocarcinoma cells (SKOV3-luc) and mouse ovarian cancer cells (ID8) were exposed to four volumes of drug-free PoLi\textsubscript{gel} or DTX-PoLi\textsubscript{gel} at total DTX concentrations of 93, 186, 372 and 558 µM, over 24, 48 and 72 h, at which times cell viability was assessed by the MTT assay. Drug-free PoLi\textsubscript{gel} did not impact cell viability regardless of concentration or exposure time. Upon exposure, SKOV3-luc cell viability ranged from 97.4±2.6% to 103.4±10.8% (n=3), and ID8 cell viability ranged from 96.5±8.9% to 99.7±8.5% (n=3). On the other hand, DTX-PoLi\textsubscript{gel} caused a decrease in cell viability in a concentration and time-dependent manner in both cell lines (Fig. 3.1A). This shows that continuous DTX exposure has the potential to suppress growth of tumors arising from these cells. Although metronomic DTX has been shown to be efficacious (319), this is the first study to use a continuous DTX regimen. To assess in vivo efficacy of continuous DTX therapy, SCID mice bearing SKOV3-luc tumor xenografts were injected IP with three doses of DTX-PoLi\textsubscript{gel}. At the end of the 21-day treatment period, tumor burden of mice treated with DTX-PoLi\textsubscript{gel} (continuous DTX) was assessed by weight and compared to that of non-treated controls. Consistent with in vitro results, continuous DTX therapy resulted in a dose-dependent tumor inhibition (Fig. 3.1B), which was 38±3% at the highest total dose of 32 mg/kg (n=6). This dose was chosen for subsequent comparative studies between continuous and intermittent DTX. Blood plasma and tumor DTX content was measured by HPLC. At the highest dose of 32 mg/kg, drug levels in tumors were 178±44 times greater than in plasma, which is consistent with the pharmacological advantage reported for IP administered DTX (320). These in vitro and in vivo observations establish that continuous DTX therapy, as provided by the DTX-PoLi\textsubscript{gel} formulation, is efficacious against ovarian cancer tumor growth.
Figure 3.1: In vitro (A) and in vivo (B) ovarian cancer growth inhibition upon DTX-Poligel exposure. A. Within each time point: # different from 93 μM (p<0.05); † different from 186 μM (p<0.05); § different from 372 μM (p<0.05). Within each concentration: * differences between exposure times (p<0.05). Data are represented as mean ± SEM (n=3). B. * differences among groups (p<0.05). Data are expressed as average tumor growth inhibition relative to tumor growth in non-treated control mice ± SEM (n=6/group).
3.4.2 SKOV3-luc tumor growth inhibition due to continuous and intermittent IP DTX therapy

The antitumor efficacy of continuous and intermittent IP DTX therapy was evaluated in vivo using the SKOV3-luc human ovarian cancer xenograft model in SCID mice. Bioluminescence imaging, an established method of efficacy assessment, was used to measure changes in tumor burden over time (317, 321). To confirm that this method accurately reflects tumor cell number, SKOV3-luc cells were treated with a range of DTX concentrations, and bioluminescence signal was correlated with cell viability as measured by the MTT assay. A strong linear correlation was observed ($R^2 = 0.93$). In addition, IC$_{50}$ studies showed no difference in DTX chemosensitivity between native and transfected SKOV3 cells (data not shown). Mice were treated with either continuous DTX via the DTX-PoLigel, or intermittently with Taxotere®, such that the cumulative DTX dose during the study period was identical. It is important to note that the weekly 8mg/kg dose of Taxotere® used is subtherapeutic, and it was used to match the 8mg/kg dose released weekly from 32mg/kg of DTX-PoLigel. At the end of the study, the average tumor growth in animals treated with intermittent DTX was over 7 times greater than in mice treated with continuous DTX (Fig. 3.2). Drug-free PoLigel had no effect on antitumor efficacy, as tumor progression was not different from control. The diversion in antitumor efficacy between the two treatment strategies occurred within the second cycle of chemotherapy.
Figure 3.2: *In vivo* efficacy of continuous and intermittent IP DTX in a SKOV3-luc xenograft model assessed by bioluminescence. Continuous therapy: DTX-PoLi\textsubscript{gel} on day 7 (32mg/kg); intermittent therapy: weekly Taxotere\textsuperscript{®} (8mg/kg) indicated by arrows. Significant differences are indicated by * (p<0.05). Data are expressed as mean % change in bioluminescence from day 7 within each group ± SEM (n=12/group on days 7 and 14, n=8/group on day 21, n=4/group on day 24).
3.4.3  *In vivo* tumor apoptosis, proliferation and angiogenesis during continuous and intermittent IP DTX therapy

Mechanisms leading to differences in efficacy between continuous and intermittent chemotherapy were investigated by immunohistochemistry to quantify tumor cell death, proliferation and angiogenesis. These parameters were analyzed in SKOV3-luc tumors extracted over time from mice treated with continuous (DTX-PoLiGal) and intermittent DTX. Differences in these factors between continuous and intermittently treated mice become apparent on day 21 (Fig. 3.3), which is also when tumor progression curves diverge significantly (Fig. 3.2).
Figure 3.3: Changes in tumor cell death, proliferation and angiogenesis over time following continuous or intermittent DTX therapy, illustrated by indices of Casp3 (A), TUNEL (B), CD31 (C) and Ki67 (D). Differences among groups at each timepoint are indicated by * (p<0.05). Data are represented as mean ± SEM (n=4/group).
Tumor cell apoptosis, measured by Casp3 activity, was 5 times greater on day 21 and 6 times greater on day 24 due to continuous therapy compared to intermittent therapy (Fig. 3.3A, 3.4). In fact, intermittent DTX led to 2-2.5 times less Casp3 activity than no treatment on days 21 and 24, which may suggest the development of drug resistance. TUNEL analysis revealed slightly greater cell death in intermittently-treated tumors on day 21 when compared to non-treated controls; however, no difference was detected on day 24 (Fig. 3.3B). Continuous DTX leads to 2.8 and 1.5 times greater cell death than intermittent DTX on days 21 and 24, respectively, although differences are not as pronounced as seen with apoptosis. The TUNEL assay does not distinguish between necrotic and apoptotic cells (322), hence these results suggest that intermittent DTX may lead to a greater number of necrotic cells than apoptotic ones, although both Casp3 and TUNEL analyses indicate greater cell death as a result of continuous chemotherapy.
Figure 3.4: Representative tumor sections following immunohistochemistry, illustrating the effects of intermittent and continuous DTX treatment on tumor apoptosis (Casp3), angiogenesis (CD31) and proliferation (Ki67) on day 24 post-inoculation (5μm sections at 100x magnification; scale bar, 100μm).
Indices of the endothelial cell marker CD31 were measured to assess angiogenesis, which did not increase as a result of continuous chemotherapy. Intermittent DTX caused a considerable increase in angiogenesis from day 21 to day 24 (Fig. 3.3C, 3.4), and at those times levels were identical to controls, indicating that intermittent therapy has no effect in angiogenesis inhibition. Tumor cell proliferation, quantified by Ki67 activity, decreases during continuous DTX exposure and progressively increases with intermittent therapy, surpassing non-treated controls 1.5-fold by day 24 (Fig. 3.3D, 3.4). This increase in tumor cell proliferation as a result of intermittent therapy was further explored in vitro.

3.4.4 In vitro cell death and proliferation during continuous and intermittent DTX exposure

Once changes to tumor biology after each treatment cycle were determined, we sought to understand changes in cell death and proliferation between treatment cycles in vitro by performing clonogenic assays immediately before and after each treatment cycle. Striking differences in SKOV3-luc cell proliferation resulted from the two treatment approaches (Fig. 3.5A). Cells treated with continuous DTX lost their clonogenic potential within the first treatment cycle. Intermittent DTX treatment led to a decrease in the number of colony-forming SKOV3-luc cells immediately after drug exposure (i.e. day 1), however cells quickly recovered and the number of clonogenic cells approached pre-treatment levels on day 3. Proliferation was reduced again upon drug exposure, and accelerated again during the treatment-free period. Accelerated proliferation after each treatment was evident in every cycle. In contrast, the effects of intermittent and continuous DTX on ID8 cell proliferation were identical (Fig. 3.5B). The number of colony-forming ID8 cells decreased steadily upon both treatment strategies, and proliferative potential was lost after two treatment cycles.
Figure 3.5: Changes in SKOV3-luc (A) and ID8 (B) cell proliferation during intermittent and continuous DTX therapy. Differences are indicated by * (p<0.05). Arrows indicate 12-hour intermittent DTX administration. Data are represented as mean ± SEM (n=3).
In vitro cell death was examined within one treatment cycle. Continuous DTX treatment resulted in a steady decline in SKOV3-luc cell viability over time (data not shown). Intermittent treatment induced the same amount of cell death as continuous treatment on day 1, immediately after intermittent drug exposure. However, during the treatment-free period, cell viability increased following intermittent DTX exposure. At the end of the treatment cycle, continuous DTX exposure resulted in 37% greater cell death than intermittent exposure. Similar to results obtained from in vitro proliferation studies, no significant difference in ID8 cell death resulted from the two treatment strategies (data not shown). In addition, there was no increase in cell death over time due to intermittent exposure, and only a slight decrease in cell viability was observed on day 3 of continuous therapy.

3.4.5 ID8 ascites inhibition during continuous and intermittent IP DTX therapy

In addition to studies in the SKOV3-luc xenograft model, the antitumor efficacy of continuous and intermittent IP DTX therapy was also evaluated in vivo using the ID8 syngeneic murine ovarian cancer model in C57Bl6 mice. Three main differences exist from the SKOV3-luc model: (1) ID8 is a slower growing, more chemosensitive cell line; (2) C57Bl6 host mice have an intact immune system; and (3) ID8 is a tumor model characterized by large amounts of ascites fluid formation, whereas SKOV3 cells mainly form large solid tumors. Disease progression was assessed throughout the treatment period by abdominal girth measurements and ascites fluid volume, established methods for assessing efficacy in tumor models characterized by ascites fluid accumulation (323). As described above, in vitro experiments showed no difference between the two different strategies in ID8 cell death and proliferation. To our surprise, continuous DTX therapy significantly deterred ascites fluid formation as assessed by abdominal girth, which became evident during the second treatment cycle, whereas intermittent therapy showed no efficacy (Fig. 3.6A). At the end of the 35-day study period, or 21 days post-treatment initiation, the volume of ascites fluid in mice treated with continuous DTX was reduced by more
than 15 times compared to non-treated mice and 10 times compared to intermittently treated mice (Fig. 3.6B). Although there is an apparent decrease in ascites volume due to intermittent therapy when compared to no treatment, this difference is not statistically significant. Mice treated with intermittent DTX and non-treated control mice exhibited anemia during the last treatment cycle, whereas mice treated continuously did not exhibit this condition.
Figure 3.6: *In vivo* efficacy of continuous and intermittent IP DTX in the ID8 syngeneic ovarian cancer model assessed by (A) abdominal growth and (B) post-mortem ascites volume. A. Continuous therapy: DTX-PoLi\text{gel} on day 14 (32mg/kg); intermittent therapy: weekly Taxotere® (8mg/kg) indicated by arrows. Differences between treatments are indicated by * (p<0.05). † indicates significant difference from both control and intermittent groups (p<0.05). B. Ascites volume on day 35. Differences between treatments are indicated by * (p<0.001). Data are expressed as mean ± SEM (n=10/group).
3.5 Discussion

Intermittent chemotherapy at the MTD, the approach currently used in the treatment of advanced ovarian cancer, has been highly unsuccessful. Studies have shown the benefits of shortening treatment-free periods in between cycles of chemotherapy by administering lower doses more frequently in a regimen termed metronomic therapy. Pre-clinical models and clinical trials have shown that metronomic administration of cytotoxic agents inhibits tumor growth to a greater extent than intermittent regimens administered at the MTD followed by long treatment-free periods (195, 319, 324-326). For the first time, we have completely eliminated treatment-free periods by administering DTX continuously via an injectable polymer-lipid implant formulation. We show here that continuous DTX is therapeutically beneficial over intermittent administration, and is efficacious against SKOV3-luc and ID8 ovarian cancer models in vitro and in vivo, with a dose-dependent decrease in tumor burden observed with increasing doses. During all animal studies, no signs of drug-related toxicities were observed, including peritonitis. Antitumor efficacy between intermittent and continuous DTX therapy was assessed in mice bearing SKOV3-luc xenografts in situ via bioluminescence imaging. Mice in the continuous treatment group experienced a slight tumor growth during the first three days of therapy, after which tumor volume remained stable, while tumor growth in intermittently treated and non-treated animals increased gradually. Our results show that continuous DTX administration led to seven-fold greater tumor inhibition than intermittent DTX.

Frequent low-doses of cytotoxic agents are believed to exert their antitumor activity primarily by inhibiting the formation of new vasculature in the tumor microenvironment (195). Angiogenesis promotes the recovery of tumors following cytotoxic attacks, and it provides adequate nutritional and oxygen supply essential for tumor proliferation and metastasis (191). In fact, a tumor’s angiogenic potential is directly correlated with poor prognosis. Metronomic taxane administration has been shown to inhibit neoangiogenesis while normalizing preexisting
vessels to reduce leakiness, and DTX administered in this manner has shown particularly high potency (327, 328). Similarly, our CD31 labeling results indicate that little or no neoangiogenesis occurred in tumors treated continuously, while intermittent treatment led to a steady increase in CD31 labeling that was at par with levels in non-treated controls. This may have been due not only to the intermittent MTD regimen, but also due to the vehicle of Taxotere®, PS80, which has been shown to nullify the anti-angiogenic activity of DTX (201).

Our results demonstrate that continuous DTX therapy affects the tumor microenvironment by targeting vascular endothelial cells, as metronomic DTX does, and also targets tumor cells by inducing high levels of apoptosis and reducing proliferation. These could be direct effects on tumor cells, collateral effects caused by the lack of angiogenesis progression which deprives tumors of proper nutrition and oxygenation, or a combination of both. Overall, DTX administered continuously leads to greater SKOV3-luc cell death both in vitro and in vivo than intermittent DTX. In vivo, we have shown that continuous administration of DTX induces considerably higher levels of apoptosis on days 21 and 24 than high intermittent doses, as shown by caspase-3 activity. Interestingly, tumors of non-treated mice showed a greater apoptotic index than intermittently treated tumors, which may suggest the development of DTX resistance during intermittent therapy, a possibility that will be addressed in future studies. Apoptosis is beneficial for tumor volume reduction and aids drug penetration into tumors by decreasing tumor cell density through cell shrinkage, which expands the tumor interstitium (329). In fact, taxane penetration into tumors is dependent on the duration of drug exposure (330). As with caspase-3, TUNEL labeling was higher in continuously treated tumors on days 21 and 24 than in intermittently treated tumors, although TUNEL labeling was higher than caspase-3 labeling due to intermittent treatment. Studies have shown that DNA fragmentation, the phenomenon detected by TUNEL labeling, is not always specific to apoptosis, and happens at early stages of necrosis (331). Therefore, since caspase-3 activity levels are very low in intermittently treated tumors, the slightly higher labeling by TUNEL may indicate necrosis. In
support of this, cytotoxic drugs have been shown to induce cancer cell apoptosis if administered at low doses but induce necrosis at high doses (329, 332).

In addition to the benefits of long drug exposure provided by our treatment regimen, cytotoxic agents more effectively target tumor cells if the treatment-free period is reduced or, in this case, eliminated altogether (209). In both chemotherapy and radiotherapy, local tumor control and survival are significantly improved when the total dose is given in a short period of time, thereby decreasing the length of treatment-free periods (207, 209, 317, 333). Cytotoxic doses are achieved immediately upon administration; however, levels decrease rapidly within a few hours, allowing surviving cells to re-enter the cell cycle, proliferate and repopulate the tumor (136, 208). The rate of tumor cell proliferation has been shown to accelerate upon each treatment cycle (136, 207-209). Fittingly, our results show that intermittent DTX results in greater tumor proliferation than continuous DTX and non-treated controls, suggesting accelerated proliferation as a result of intermittent dosing. Closer examination in vitro revealed that, at every treatment cycle, the number of colony-forming SKOV3-luc cells decreases immediately upon intermittent dosing, followed by a sharp increase that is only slowed by another intermittent dose. Continuous DTX completely eliminates the clonogenic ability of SKOV3-luc cells within the first treatment cycle.

Interestingly, in vitro studies of cell death and proliferation in ID8 cells revealed no differences between intermittent and continuous DTX exposure. This is likely due to the intrinsic differences between ID8 and SKOV3-luc cells. ID8 cells are derived from the mouse ovary epithelium, and were immortalized in vitro. Thus, these cells were not exposed to any treatment. SKOV3 cells are derived from a previously treated patient, and they lack p53 activity and are, therefore, more chemoresistant (209). The IC$_{50}$ of DTX in ID8 cells is over one hundred times lower than in SKOV3 cells. Our results suggest that the benefits of continuous chemotherapy in affecting tumor cells directly may be more applicable to more chemoresistant tumors. On the
other hand, the effects of the two different treatment regimens on the tumor microenvironment must be considered. In vivo, continuous DTX resulted in substantially greater therapeutic benefit over intermittent DTX in mice bearing ID8 xenografts. Since our in vitro results indicate no differences in the biology of tumor cells treated with the two approaches, we suggest that differences in efficacy observed in vivo are a result of changes to the microenvironment. The ID8 xenograft model is mostly characterized by a large accumulation of ascites fluid. The permeability of vasculature contributes greatly to the formation of ascites fluid, which facilitates dissemination of malignant cells throughout the peritoneal cavity. VEGF plays a major role in increasing the permeability of existing vasculature and has been proven essential for the ascites formation process in ovarian cancer (191). In vitro studies have demonstrated the ability of taxanes to decrease VEGF expression (334). Our results show that continuous DTX deterred ascites fluid accumulation, whereas intermittent DTX showed no effect in the ID8 xenograft model. It is plausible that although continuous and intermittent DTX induce the same effects on ID8 cells, as shown in vitro, the impact on angiogenesis differs greatly, and continuous DTX targets VEGF to an extent not possible in intermittent therapy, thereby significantly decreasing ascites fluid accumulation. This theory will be addressed in future studies.
3.6 Conclusion
In summary, we have shown for the first time that continuous DTX therapy is superiorly efficacious to intermittent therapy in ovarian cancer at the same cumulative DTX dose. In less chemosensitive disease, such as SKOV3-luc xenografts, greater antitumor efficacy by continuous chemotherapy is a result of changes to the tumor cells themselves, leading to slower proliferation and greater cell death, and changes to the tumor microenvironment by inhibiting neoangiogenesis. In more chemosensitive disease, such as ID8 xenografts, continuous and intermittent DTX equally increase tumor cell death and decrease proliferation, although it is likely that continuous DTX impacts angiogenesis, inhibiting ascites fluid accumulation and resulting in greater efficacy. Future studies will examine the effects of continuous DTX therapy on neoangiogenesis. Since intermittent DTX induced less apoptosis than observed in non-treated tumors, we will also investigate the development of DTX resistance as a result of intermittent and continuous therapy. Overall, our results provide a conceptual framework for eliminating treatment-free periods and increasing tumor drug exposure through continuous chemotherapy in the treatment of ovarian cancer. Clinically, this can be achieved by administering a delivery system such as the one described herein during debulking surgery. Although the present formulation of DTX-PoLi\textsubscript{gel} releases its drug load over a period of one month, material ratios could be modified to extend drug release over several months.

3.7 Acknowledgements
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Chapter 4

Chemotherapy Dosing Schedule Influences Drug Resistance Development in Ovarian Cancer


Author Contributions: De Souza R performed all experiments and data analysis, and wrote the manuscript. Efficacy studies were conducted in conjunction with Zahedi P, who also formulated the PoLi$_g$el and synthesized water-soluble chitosan. Badame RM assisted with a few protein extractions and Western blots.
4.1 Abstract

Drug resistance leads to chemotherapy failure and is responsible for the great majority of patient deaths with metastatic, late-stage ovarian cancer. The present study addressed whether changes in chemotherapy dosing schedule affect the development, further worsening, or circumvention of drug resistance in chemosensitive and chemoresistant ovarian cancer. SCID mice bearing HeyA8 and HeyA8-MDR xenografts were treated with DTX intermittently (1x/week or 3x/week) or continuously for 21 days. Tumor mRNA expression of genes implicated in DTX resistance was measured by RT-qPCR. Analyzed genes included those encoding for the drug efflux transporters mdr1 and mrp7 and molecules that interfere with or overcome the effects of DTX, including β-tubulinIII, actinin4, stathmin1, bcl2, rpn2, thioredoxin and akt2. In both models, continuous DTX resulted in greater antitumor efficacy than 1x/week or 3x/week dosing, and did not induce upregulation of any analyzed genes. Once weekly dosing caused upregulation of various drug resistance related genes, especially in chemoresistant xenografts. More frequent, 3x/week dosing diminished this effect, although levels of various genes were higher relative to continuous chemotherapy. Drug efflux transporter expression was further examined by Western blotting, confirming that intermittent, but not continuous DTX induced significant upregulation. Overall, our results show that the presence and length of treatment-free intervals contribute to drug resistance development. Elimination of these intervals by continuous dosing resulted in superior antitumor efficacy and prevented drug resistance induction in chemosensitive and chemoresistant disease. These results encourage the clinical implementation of continuous chemotherapy to overcome and/or prevent drug resistance in newly diagnosed and recurrent, refractory ovarian cancer.
4.2 Introduction

The development of drug resistance is the leading cause of chemotherapy failure in the treatment of cancer (335). Patients initially respond well to chemotherapy; however, cancer cells have significant plasticity (179). Multiple chemotherapy cycles have been shown to select for tumor cells that are inherently resistant or that have developed resistance over the course of treatment (176, 180), and eventually, the disease becomes incurable. Ovarian cancer is a prime example of such a scenario, with drug resistance causing 90% of patient deaths with metastatic late-stage disease (176-178). Since ovarian cancer is asymptomatic until late stages, it is essential to identify strategies to prevent and/or overcome drug resistance to maximize chemotherapy efficacy so that better outcomes can be achieved.

Akin to many solid tumors, ovarian cancer treatment involves debulking surgery followed by cycles of chemotherapy administered in an intermittent fashion (336). Systemic exposure to high doses of chemotherapeutics is followed by a 3-4 week treatment-free interval to allow for recovery of healthy tissues (336). It has been suggested that short bursts of chemotherapeutics followed by long intervals impose a selective pressure on cancer cells (176, 337). This approach only eliminates vulnerable cells and favours resistant cells which repopulate the tumor at progressively faster rates after each treatment (135, 136), resulting in the development of drug resistance. We have previously developed an injectable, biocompatible polymer-lipid implant (DTX-PoLigel) capable of continuous, localized delivery of the chemotherapeutic drug DTX (131, 314). We have shown that continuous drug exposure results in greater antitumor efficacy than intermittent administration in ovarian cancer xenografts, due to greater tumor cell kill and reduced proliferation and angiogenesis (338). Interestingly, throughout the course of treatment, we observed that intermittently treated tumors displayed equal or less cell apoptosis compared to non-treated controls, suggesting the development of drug resistance and increased survival abilities. Previous work suggests that prolonging drug exposure or shortening treatment-free
intervals has the potential to circumvent the development of, or overcome, drug resistance (227). Thus, the present study sought to investigate whether the lack of drug resistance development is one of the mechanisms responsible for enhanced efficacy observed with continuous chemotherapy. Clinical trials have shown the survival benefits of shortening the length of intervals between chemotherapy treatments from three weeks to one week in ovarian cancer patients (148, 339, 340). As well, low-dose prolonged exposure of chemotherapeutics, especially those that are cell-cycle specific such as taxanes, has been shown to increase tumor cell kill (338, 341, 342).

Drug resistance can arise from both tumor microenvironmental and molecular factors. Issues such as hypoxic regions, irregular blood flow and supply, the extracellular matrix, high density of cells within a tumor, and high interstitial fluid pressure can create pharmacological sanctuaries, or physical barriers through which chemotherapeutics cannot properly diffuse, leading to limited tissue penetration (179, 180). Alternatively, drug delivery to tumor cells can be hindered by upregulation of cell membrane drug efflux transporters. Members of the adenosine triphosphate-binding cassette (ABC) transporter superfamily have been associated with drug resistance by decreasing the intracellular accumulation of hydrophobic chemotherapeutics (184). Of these, the product of the multidrug resistance 1 (mdr1) gene, P-gp, is one of the key molecules leading to cancer multidrug resistance (343). Overexpression of mdr1 has been shown in refractory ovarian cancer tumor cells (344). P-gp is capable of transporting a variety of substrates ranging in size from 300 to 2,000 Da (184). Cancer cells exhibiting upregulation of P-gp become resistant to multiple structurally and mechanistically unrelated chemotherapeutic agents, and are classified as multidrug resistant (343). Since intracellular drug accumulation is the basic requirement for chemotherapeutics to exert their effects, the upregulation of drug efflux transporters that hinder the acquisition of proper intracellular drug levels is of key importance in the development of drug resistance (180, 335). In addition, other molecular changes that interfere with the drug’s mechanism of action are also important, such as
modifications in the drug target, evasion of apoptosis and activation of survival pathways (180, 183).

Studies described herein address the effects of chemotherapy dosing schedule modifications on development, further worsening, or circumvention of drug resistance in chemosensitive and chemoresistant xenograft models of ovarian cancer, which reflects clinical scenarios of both newly diagnosed and recurrent disease. This was done by examining changes in the expression of genes that have been implicated in DTX or taxane resistance. Tumor cells resistant to DTX can exhibit an increased expression of P-gp (coded for by mdr1) and MRP7, the only two known ABC transporters capable of transporting this drug (216). The overexpression of mrp7 has been demonstrated in taxane-resistant patient tumors such as lung cancer (345). Since DTX acts by stabilizing microtubules via binding to β-tubulin subunits, the abnormal expression of certain β-tubulin isotypes or proteins such as stathmin1 hinders drug binding to its target (183, 219). The upregulation of molecules that promote survival or inhibit apoptosis even in the presence of cytotoxic agents, such as actinin4, Akt2, thioredoxin and Bcl2, have also been suggested to contribute to taxane or DTX resistance (220, 221, 224, 346). The expression of these genes was examined in xenografts extracted from SCID mice treated with continuous or intermittent (1x/week or 3x/week) DTX.
4.3  Materials and methods

4.3.1  Cell lines

The human ovarian cancer cell line HeyA8 and its multidrug resistant equivalent HeyA8-MDR were purchased from The University of Texas MD Anderson Cancer Center (Houston, TX; no authentication of cell lines was done by the authors). The HeyA8-MDR cell line was established by culturing HeyA8 cells in PTX-containing medium until the cells became resistant to 500ng/mL PTX (239). Cells were maintained in RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 mg/mL streptomycin). Cells were grown in monolayer at 37°C/5% CO₂ and 90% relative humidity.

4.3.2  Xenograft establishment and treatment groups

Animal studies were approved by the University of Toronto Animal Care Committee and adhered to these guidelines and those of the Canadian Council on Animal Care. SCID, 6-8-week-old female mice (Charles River) were inoculated IP with 1x10⁶ HeyA8 or HeyA8-MDR cells suspended in 200µL sterile PBS (pH 7.6). After 7 days, mice were divided into groups (n=6/group) for treatment initiation. Continuous therapy consisted of one IP injection of DTX-PoLi₉gel (32mg/kg), prepared as previously described (131), which releases 8mg/kg DTX weekly. Intermittent therapy consisted of either one or three IP injections of Taxotere® (Sanofi Aventis) weekly, so that mice in both groups received 8mg/kg total DTX per week. Non-treated controls received one weekly IP injection of sterile saline. Mice were monitored daily, and endpoints requiring humane euthanasia included weight loss in excess of 20%, excessive muscle wasting according to the “body conditioning scoring system” (318), severe abdominal distension, inactivity and hypothermia. All mice were sacrificed 21 days after treatment initiation, the time at which control mice reached the outlined endpoints. Tumors were weighed for antitumor efficacy evaluation and frozen in liquid nitrogen for subsequent gene and protein expression analyses.
4.3.3 RT-qPCR assessment of drug resistance related gene mRNA expression

Total mRNA was extracted from tumors using TRIzol reagent (Invitrogen) as per manufacturer’s protocol, and was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). After DNase I (Invitrogen) treatment, single-stranded cDNA was synthesized from 2 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer’s instructions. RT-qPCR reactions were performed by incubating 20 ng of cDNA with Power SYBR Green PCR Master Mix (Applied Biosystems) and primers specific for each transcript (Table 4.1), which were designed using NCBI’s Primer Designing tool. Amplification was performed on an Applied Biosystems 7900HT instrument equipped with a 384-well reaction block, using SYBR green chemistry. Samples were run in triplicates. mRNA levels were calculated using a standard curve method, were normalized to those of cyclophilin, and are expressed as percentages of non-treated controls.
Table 4.1: Primer sequences used for qRT-PCR. Sequences were designed using the NCBI Primer Designing Tool.

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4.3.4 Western Blot analysis of membrane efflux transporter expression

For analysis of membrane transporter expression, membrane protein was extracted by homogenizing 300 mg of tumor tissue in lysis buffer composed of 1x RIPA buffer (Cell Signaling Technology) with 4 μL/mL protease inhibitors (Sigma Aldrich) and 25 μL/mL 200mM PMSF, which was then centrifuged for 20 minutes at 2,000 g. The resulting pellet was re-homogenized in lysis buffer, followed by another 2,000 g centrifugation for 20 minutes. The total supernatant was centrifuged at 100,000 g for 60 minutes. The membrane protein pellet was washed and re-suspended in 0.5x lysis buffer by sonication. Protein samples were quantified using the Bradford assay. Membrane protein (20 μg for P-gp blotting, 10 μg for MRP7 blotting) samples were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), which was blocked with 5% skim milk powder in Tris-buffered saline for 1 hour at room temperature. Membranes were incubated with C-219 (anti-P-gp; 1:500; Abcam) or M71-3 (anti-MRP7; 1:1000; Abcam) primary antibodies overnight at 4°C, followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; Jackson ImmunoResearch Laboratories) and molecular weight markers (1:6000; Bio-Rad Laboratories). Bands were detected using an ECL Advance Western Blotting Detection Kit (GE Healthcare) and visualized using a FluorChem Imager (Alpha Innotech). Alpha Ease FC imaging software (Alpha Innotech) was used to quantify bands, which were normalized to AC-15 (anti-β-actin; 1:10000; Sigma-Aldrich).

4.3.5 Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were done using Statistical Package for the Social Sciences (SPSS, version 16.0). For comparisons between groups, one-way analysis of variance (ANOVA) was used and multiple comparisons with Bonferroni correction were performed upon statistical significance as determined by ANOVA. Significance was assigned at p < 0.05.
4.4 Results

4.4.1 Impact of treatment-free intervals on the antitumor activity of DTX in chemosensitive and chemoresistant ovarian tumors

To determine whether the reduction or elimination of treatment-free intervals improves antitumor efficacy in both chemosensitive and chemoresistant disease, SCID mice bearing HeyA8 or HeyA8-MDR xenografts were treated with DTX 1x/week, 3x/week, or continuously for a period of 21 days. The mRNA expression of \textit{mdr1} and \textit{mrp7} in HeyA8-MDR cells is 3.1 and 3.3 times greater than in HeyA8 cells, respectively (347). In addition, the DTX IC	extsubscript{50} values of HeyA8-MDR cells were shown to be 200 times greater than in HeyA8 cells (347). The average tumor burden in non-treated mice bearing HeyA8 (1.59±0.11g) and HeyA8-MDR (1.60±0.15g) xenografts was significantly greater than in mice treated continuously, 3x/week and 1x/week (HeyA8: 0.10±0.03g, 0.27±0.04g, 0.39±0.08g, respectively; HeyA8-MDR: 0.38±0.03g, 1.25±0.09g, 1.05±0.23g, respectively). In both HeyA8 and HeyA8-MDR models, continuous chemotherapy resulted in greater antitumor efficacy (93.7±2.1% and 76.4±2.0% tumor burden reduction relative to control, respectively) than intermittent therapy (Fig. 4.1). The presence of treatment-free intervals limited the antitumor efficacy of DTX irrespective of interval length, as no significant difference was observed between 1x/week and 3x/week treatments in both chemosensitive (75.3±5.3% vs. 73.7±4.0% reduction) and chemoresistant (34.6±14.1% vs. 37.8±3.9% reduction) disease (Fig. 4.1).
Figure 4.1: Antitumor efficacy of intermittent (Taxotere® 1x/week or 3x/week) and continuous (DTX-PoLigel) IP DTX chemotherapy in HeyA8 (A) and HeyA8-MDR (B) human ovarian cancer xenografts in SCID mice (DTX dose in all groups: 8mg/kg/week). Data represent mean tumor burden on day 21 post-treatment initiation relative to non-treated controls ± SEM (n=6/group). * indicates significant differences between treatment groups (p<0.05). Tumor burden in all treatment groups is significantly lower than in non-treated controls (p<0.05).
4.4.2 Expression of drug resistance related genes in chemosensitive (HeyA8) tumors following intermittent and continuous DTX chemotherapy

To assess the consequences of treatment-free intervals on drug resistance development in treatment-naïve tumors, the mRNA expression of key DTX resistance genes was measured by RT-qPCR in HeyA8 xenografts extracted from mice treated 1x/week, 3x/week, or continuously for 21 days. Intermittent, 1x/week DTX treatment led to significant upregulation of $mdr1$ (593.0±170.1% of control), while there was no difference in tumors treated 3x/week or continuously relative to controls (Fig. 4.2A). Intermittent treatment 1x/week also led to upregulation of $mrp7$, $actinin4$ and $bcl2$ relative to controls (168.6±32.8%, 255.7±49.8% and 224.1±15.2%, respectively), and expression levels of $mrp7$, β- $tubulin$ III, $actinin4$, $stathmin1$, and $bcl2$ were higher than in tumors treated continuously. As compared to controls, administration of DTX 3x/week only induced upregulation of $bcl2$ (182.7±33.1%), although levels of $stathmin1$ were higher than in tumors treated continuously. Interestingly, mRNA levels resulting from 3x/week DTX were not significantly lower than levels in tumors treated 1x/week, with the exception of $actinin4$. Continuous DTX did not induce upregulation of any analyzed genes; in fact, a downregulation of $mrp7$, β- $tubulin$ III and $stathmin1$ was observed (63.2±6.8%, 65.6±7.0%, and 54.3±10.6% of control, respectively) (Fig. 4.2B). No change in $rpn2$, thioredoxin and $akt2$ resulted from any of the three dosing schedules.
Figure 4.2. Effect of chemotherapy administration schedule on mRNA expression of *mdr1* (A) and other genes (B) implicated in DTX resistance in HeyA8 xenografts, as measured by qRT-PCR. mRNA concentrations were normalized to *cyclophilin*. Data represent mean normalized mRNA concentrations as percentages of control values ± SEM (n=4-6/group). * indicates significant differences between treatment groups (p<0.05).
4.4.3 Expression of drug resistance related genes in chemoresistant (HeyA8-MDR) tumors following intermittent and continuous DTX chemotherapy

The mRNA expression of DTX resistance genes was measured by RT-qPCR in HeyA8-MDR xenografts from mice treated 1x/week, 3x/week, or continuously for 21 days, to determine whether the presence and length of treatment-free intervals further aggravate drug resistance in already chemoresistant disease. A remarkable increase of 3,069.9±922.4% in *mdr1* expression resulted from 1x/week DTX treatment relative to control (Fig. 4.3A). Intermittent, 1x/week chemotherapy also induced upregulation of *mrp7*, *β-tubulin III*, *actinin4*, *rpn2*, *thioredoxin* and *akt2* (190.5±28.9%, 161.2±22.7%, 148.0±10.3%, 209.8±44.7%, 155.9±28.4% and 175.6±26.8% of control, respectively). In the case of all genes analyzed, mRNA levels were higher after 1x/week treatment than continuous treatment, with the exception of *bcl2*, for which levels did not change irrespective of treatment. A more frequent, 3x/week treatment schedule also led to upregulation of *mdr1*, with levels 1,575.3±94.3% greater than non-treated controls. This dosing schedule resulted in upregulation of *β-tubulin III* and *rpn2* relative to control (137.5±10.6% and 167.7±17.5%, respectively), which were also higher than levels in continuously-treated tumors. Similar to results obtained from HeyA8 xenografts, mRNA levels resulting from 3x/week DTX were not significantly lower than levels in tumors treated 1x/week, with the exception of *actinin4*. Relative to non-treated controls, mRNA levels of all genes in tumors of continuously treated mice did not increase, while *stathmin1* levels actually decreased (54.3±17.1% of control) (Fig. 4.3B).
Figure 4.3: Effect of chemotherapy administration schedule on mRNA expression of \textit{mdr1} (A) and other genes (B) implicated in DTX resistance in HeyA8-MDR xenografts, as measured by qRT-PCR. mRNA concentrations were normalized to \textit{cyclophilin}. Data represent mean normalized mRNA concentrations as percentages of control values ± SEM (n=4-6/group). * indicates significant differences between treatment groups (p<0.05).
4.4.4 Transporter expression in HeyA8 and HeyA8-MDR tumors following intermittent and continuous DTX chemotherapy

Since the ability of a drug to reach its intracellular target is crucial in determining the fate of cancer cells, the role of drug efflux transporters is of key importance in the development of drug resistance (180, 335). For this reason, the protein levels of P-gp and MRP7 in HeyA8 and HeyA8-MDR tumors were assessed by Western blotting after treatment with continuous or 1x/week intermittent DTX, to determine whether protein levels correlate with \textit{mdr1} and \textit{mrp7} mRNA levels. Intermittent, 1x/week treatment led to upregulation of P-gp in both HeyA8 and HeyA8-MDR (357.7±96.1% and 635.9±246.4% of control, respectively), while no induction resulted from continuous therapy (Fig. 4.4). A similar trend was seen with MRP7, as upregulation was seen upon intermittent, 1x/week chemotherapy in HeyA8 and HeyA8-MDR tumors (346.4±100.7% and 265.3±33.9% of control, respectively), and no change was observed upon continuous treatment (Fig. 4.5).
Figure 4.4: Effect of continuous and intermittent (1x/week) chemotherapy on P-gp transporter expression in HeyA8 (A,C) and HeyA8-MDR (B,D) tumors as assessed by Western blotting. Protein concentrations were normalized to β-actin. Data represent mean normalized P-gp protein concentrations as percentages of control values ± SEM (n=4-6/group). * indicates significant differences between groups (p<0.05).
Figure 4.5: Effect of continuous and intermittent (1x/week) chemotherapy on MRP7 transporter expression in HeyA8 (A,C) and HeyA8-MDR (B,D) tumors as assessed by Western blotting. Protein concentrations were normalized to β-actin. Data represent mean normalized MRP7 protein concentrations as percentages of control values ± SEM (n=4-6/group). * indicates significant differences between groups (p<0.05).
4.5 Discussion

Ovarian cancer is the number one killer of all gynaecologic malignancies (41). Current therapeutic approaches have a high failure rate, leading to a low 5-year survival rate of 25-35% (348). The development of drug resistance alone is responsible for the great majority of ovarian cancer deaths (178). For this reason, much effort has focused on overcoming drug resistance to achieve better therapeutic outcomes. One strategy is combination chemotherapy with agents that have different mechanisms of action and are structurally distinct; however, tumor cells respond to this approach by becoming multidrug resistant (180). Since P-gp, the product of mdr1, has been implicated in multidrug resistance of various cancers (343), another possible approach to overcome resistance is to use P-pg inhibitors to increase intracellular accumulation of chemotherapeutics that are substrates to this transporter (226). Although these agents have shown much promise in vitro, non-specific toxicities due to interference with clearance or metabolism of chemotherapeutics has thus far limited their clinical utility (226). In this study it was shown that drug resistance can be prevented and circumvented simply by modifying the schedule of chemotherapy administration.

Previous studies have shown that shortening the treatment-free intervals between chemotherapy treatments can enhance therapeutic efficacy (148, 339, 340). We have previously demonstrated that the complete elimination of these intervals through a continuous chemotherapy approach leads to a substantial improvement in ovarian cancer tumor suppression over intermittent administration (317, 338). Similarly, the present study highlights that continuous DTX therapy leads to significantly greater antitumor efficacy than intermittent administration in HeyA8 xenografts. The elimination of treatment-free intervals is able to circumvent drug resistant disease, as tumor burden in mice bearing HeyA8-MDR xenografts, which has an IC50 value for DTX 200 times greater than HeyA8 (347), was about 2.5-fold lower than in mice treated intermittently. This supports previous evidence that a change in treatment
schedule can improve efficacy in taxane-refractory disease (227, 228). Surprisingly, shortening treatment-free intervals by administering DTX 3x/week did not improve antitumor efficacy when compared to a 1x/week treatment schedule in both chemosensitive and chemoresistant tumor models, indicating that complete elimination of these intervals between doses is necessary for perceptible efficacy improvement.

It has been suggested that frequent administration of low-dose chemotherapeutics, such as the metronomic chemotherapy approach, targets mainly endothelial cells rather than cancer cells for an anti-angiogenic effect (349). Although we have shown that continuous chemotherapy does greatly decrease tumor angiogenesis (338), significant effects on tumor cells have also been shown, including inhibition of tumor cell repopulation (209, 338). Therefore, a focus on drug resistance development upon more frequent chemotherapy is warranted not only in endothelial cells, but especially tumor cells, which are highly heterogeneous and mutagenic (179). The present study addressed this issue by examining drug resistance development upon an intermittent, 1x/weekly DTX administration schedule, a more frequent, 3x/week schedule as used in preclinical metronomic chemotherapy studies (350), and a continuous schedule with no treatment-free intervals.

Overall, our results show substantial advantages of continuous chemotherapy in preventing drug resistance development in treatment-naïve disease, and inhibiting an increase in resistance in multidrug-resistant, refractory disease. After 21 days of continuous DTX therapy in both HeyA8 and HeyA8-MDR xenografts, no increase in mRNA expression was detected in any of the 9 genes analyzed relative to non-treated controls, including mdr1 which has been implicated in multidrug resistance of various solid tumors. On the other hand, intermittent 1x/week treatment resulted in an almost 6-fold upregulation in mdr1 mRNA expression in HeyA8 xenografts. This is consistent with previous work that shows upregulation of mdr1 upon intermittent therapy of ovarian cancer xenografts with PTX (337). This effect was further
pronounced in HeyA8-MDR tumors, with a 30-fold increase in \textit{mdr1} mRNA expression relative to control. This highlights the perils of intermittent chemotherapy in recurrent, refractory disease. This treatment strategy shows poor efficacy in recurrent disease (351); however, a greater concern may be the striking increase in \textit{mdr1}, which renders tumor cells resistant to several other chemotherapeutics, significantly hindering good prognosis. Intermittent therapy also led to an increase in \textit{rpn2} in HeyA8-MDR tumors, which contributes to P-gp-mediated DTX resistance, since it stabilizes the transporter in the cellular membrane via glycosylation (217). Knockdown of RPN2 by siRNA leads to reduced P-gp glycosylation, greater DTX retention within cancer cells, and higher drug sensitivity (217). The \textit{mrp7} gene, upregulated in both HeyA8 and HeyA8-MDR tumors, encodes for the only other known ABC transporter, in addition to P-gp, capable of taxane transport (216). MRP7 has the lowest degree of structural resemblance to other MRPs, and its overexpression conferred strongest resistance to DTX in a screen of various other anticancer agents (216). Achieving adequate intracellular drug levels is the most important requirement for therapeutic efficacy; thus, hindering this through overexpression of efflux transporters contributes greatly to drug resistance of cancer cells (180, 335). On both mRNA and protein levels, intermittent 1x/week chemotherapy led to an upregulation of P-gp and MRP7 efflux transporters, whereas continuous chemotherapy had no effect.

Intermittent 1x/week DTX administration also resulted in increased mRNA expression of other genes implicated in DTX and taxane resistance. DTX acts by binding to \(\beta\)-tubulin subunits to promote microtubule polymerization, stabilizing these so that depolymerisation cannot occur, leading to cell cycle arrest and subsequent cell death (183). Upregulation of the \(\beta\)-tubulinIII isoform, as observed clinically in taxane resistant ovarian cancer, causes DTX resistance by inhibiting taxane-induced microtubule assembly (183). DTX action on microtubules can also be hindered by stathmin1, a molecule found overexpressed in ovarian tumors (218). Stathmin1 stimulates microtubule depolymerisation and interferes with taxane binding to \(\beta\)-tubulin subunits (219). Thioredoxin also obstructs this process by interfering with the redox regulation of tubulin
cysteine residues, which microtubule assembly depends on (220). It has been shown that thioredoxin expression increases in tumors after DTX treatment (220). In chemoresistant (HeyA8-MDR) tumors, we found that intermittent 1x/week therapy leads to an upregulation of \( \beta\text{-tubulinIII} \), \textit{stathmin1} and \textit{thioredoxin} relative to controls and/or continuous chemotherapy, and an upregulation of \( \beta\text{-tubulinIII} \) and \textit{stathmin1} was observed in chemosensitive (HeyA8) tumors.

Resistance can also arise from increased activity of survival pathways. The serine/threonine kinase Akt2 is a member of the protein kinase AKT/PKB family that is repeatedly found overexpressed in 40% of EOCs and the one that most influences cell survival and proliferation in this type of cancer (221). In one study, downregulation of Akt2 by siRNA was sufficient to increase DTX sensitivity in cancer cells (222). Akt2 promotes DTX resistance by inactivating or inhibiting the transcription of pro-apoptotic factors, or by increasing transcription of pro-survival genes upon chemotherapeutic exposure (223). Actinin4 overexpression has also been shown to increase chemoresistance in ovarian cancer (224). This molecule influences the phosphorylation and activation of Akt2, indirectly contributing to DTX resistance in this manner (224). The anti-apoptotic protein Bcl-2 becomes deactivated by phosphorylation upon DTX treatment; thus, an overexpression of Bcl-2 would lead to the prevention of apoptosis and drug resistance (225). Relative to controls and/or continuous chemotherapy, we found that intermittent 1x/week therapy leads to an upregulation in chemoresistant (HeyA8-MDR) tumors of \textit{actinin4} and \textit{akt2}, and an upregulation in chemosensitive (HeyA8) tumors of \textit{actinin4} and \textit{bcl2}.

Although a decrease in the length of treatment-free intervals did not affect antitumor efficacy, increasing the treatment frequency to 3x/week slightly reduced the development of drug resistance as compared to 1x/week. In both chemosensitive HeyA8 and chemoresistant HeyA8-MDR xenografts, levels of DTX resistance-related genes upon 3x/week treatment were between levels resulting from continuous and 1x/week DTX. Nevertheless, drug resistance still
ensued from 3x/week treatment. This was especially the case in HeyA8-MDR xenografts, in which mdr1 expression was almost 16-fold greater than non-treated controls. An increased expression in other genes including β-tubulinIII, rpn2 and thioredoxin was also found in these tumors.

The present and previous work from our group have shown promising potential for the clinical application of continuous chemotherapy. The main concern of continuous drug exposure is toxicity to healthy tissues. However, we have shown a lack of systemic and local toxicity in mice treated in this manner (131). We have previously shown that continuous DTX did not cause myelosuppression in mice (352, 353), which is the most significant toxicity caused by DTX (354), as counts of red blood cells, platelets and leukocytes were within normal baseline values (data not shown). Neutropenia, the main adverse event associated with myelosuppression, also did not occur in healthy mice upon continuous DTX. While neutrophils were 50% lower than normal baseline upon intermittent DTX treatment, continuous treatment did not cause a decrease in neutrophils, which remained within the normal range (data not shown) (352, 353). This can likely be explained by the fact that very low doses of DTX are present at any given time due to continuous drug release, rather than immediate exposure to very high doses, as is the case with intermittent chemotherapy. Although further studies must be performed, our results to date indicate that continuous chemotherapy does not cause the expected toxicities. The benefits of this treatment strategy compounded with the observed lack of toxicity make the clinical implementation of continuous chemotherapy a realistic goal.
4.6 Conclusion
In summary, our results show that the presence and length of treatment-free intervals greatly contribute to the development of drug resistance. Intermittent 1x/week DTX administration led to an upregulation of various genes implicated in DTX resistance. Particularly high upregulation was observed for mdr1 (P-gp), which leads to multidrug resistance and ultimately, treatment failure (343). This effect was more pronounced in HeyA8-MDR xenografts, which reflect recurrent refractory disease. Reducing the interval length between each chemotherapy administration lessened the development of drug resistance particularly in HeyA8 tumors, although upregulation of certain DTX resistance-related genes still occurred mainly in HeyA8-MDR tumors, in which mdr1 was remarkably upregulated. Although the development of drug resistance was reduced upon more frequent administration, DTX treatment 1x/week or 3x/week yielded the same level of antitumor efficacy in both HeyA8 and HeyA8-MDR xenografts. The complete elimination of treatment-free intervals resulted in substantially greater antitumor efficacy in both chemo-sensitive and chemo-resistant xenografts, and did not induce upregulation of any of the examined genes related to DTX resistance. Since the development of drug resistance is a key cause of chemotherapy failure, the results presented herein support the clinical implementation of a continuous chemotherapy approach to prevent the development or increase in drug resistance in solid tumors such as ovarian, ultimately leading to improved therapeutic outcomes.

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Chapter 5

Continuous Chemotherapy Prolongs Survival and Delays Drug Resistance in Ovarian Cancer

This chapter will be submitted as a manuscript under the following title: “De Souza R, Zahedi P, Allen C, Piquette-Miller M. Continuous Chemotherapy Prolongs Survival and Delays Drug Resistance in Ovarian Cancer.”

Author Contributions: De Souza R performed all experiments and data analysis, and wrote the manuscript. Zahedi P formulated the PoLi Gel, synthesized water-soluble chitosan, and assisted with animal studies.
5.1 Abstract

Current ovarian cancer therapy leads to a 5-year survival rate of only 25-35%, largely due to the development of drug resistance. A continuous chemotherapy dosing schedule with DTX has been shown to result in greater antitumor efficacy than the intermittent approach currently used in the clinic. After a 21-day treatment period in a murine model of human ovarian cancer, continuous dosing did not result in increased drug resistance, whereas significant increases were observed upon intermittent therapy. The present study addressed whether long-term continuous chemotherapy lengthens survival and delays drug resistance in xenograft models of sensitive and resistant ovarian cancer. SCID mice bearing HeyA8 and HeyA8-MDR xenografts received DTX intermittently or continuously until endpoints were reached. Tumor expression levels of genes implicated in DTX resistance were measured by RT-qPCR, including genes encoding for drug efflux transporters mdr1 and mrp7 and genes that interfere with the cytotoxicity of DTX, including β-tubulinIII, actinin4, stathmin1, bcl2, rpn2, thioredoxin and akt2. Intermittent dosing resulted in a modest survival increase of 36% (8 days) and 10% (2 days) in sensitive and resistant models, respectively, whereas a striking 114% (25 days) and 95% (19 days) increase in survival was seen in mice treated with continuous DTX. The examined genes were found upregulated at the endpoint of continuous chemotherapy on day 47 (HeyA8) and day 39 (HeyA8-MDR) post-treatment initiation, and levels were comparable to those in intermittently treated tumors on day 30 (HeyA8) and day 22 (HeyA8-MDR). Continuous chemotherapy resulted in significantly prolonged survival and substantially delayed drug resistance. These results provide further support for the clinical implementation of continuous chemotherapy and provide a basis for future studies incorporating chemotherapy combinations in the continuous dosing setting in an effort to further delay drug resistance and enhance therapeutic outcomes.
5.2 Introduction

Ovarian cancer is one of the most lethal cancers to affect women (2). Due to difficulties in early diagnosis, about 80% of patients are diagnosed at late stages when the disease has metastasized throughout the peritoneal cavity (2, 4, 5). It is for this reason that improving upon current treatment strategies is vital in improving the management of late-stage disease. The standard treatment for ovarian cancer usually consists of debulking surgery and intermittent chemotherapy at MTDs every 3-4 weeks for 6 cycles (336). This 3-4 week interval between treatments allows for proliferating haematological precursor cells to repopulate and recover from the high cytotoxicity induced by MTD dosing (133). Vulnerable cancer cells are eliminated upon chemotherapy treatment; however, these treatment-free intervals allow surviving, resistant cells to repopulate the tumor (135, 136), resulting in the development of drug resistance. It is for this reason that although most patients achieve complete clinical response upon first line treatment, over 85% eventually relapse with drug resistant disease (2).

More frequent dosing schedules have been explored with the goal of increasing efficacy while decreasing systemic toxicity (137). Clinical trials have shown that shortening treatment-free intervals by weekly chemotherapy with taxane drugs provides superior therapeutic efficacy in ovarian cancer treatment (69, 140, 141, 143, 144). Metronomic chemotherapy consists of dosing multiple times per week, or even daily. Preclinical studies have shown the potential benefits of this approach, although these still must be established clinically (355). Although more frequent dosing seems promising, weekly and metronomic chemotherapy approaches still include treatment-free intervals. Alternatively, eliminating the treatment-free intervals through continuous chemotherapy is a promising strategy.

The elimination of treatment-free intervals was previously assessed using an injectable biocompatible polymer-lipid implant (DTX-PoLi gel) capable of continuous, localized delivery of the taxane DTX (131, 338, 356). When compared to intermittent administration, continuous drug
exposure resulted in greater antitumor efficacy, greater tumor cell kill, reduced proliferation and angiogenesis in murine xenograft models of ovarian cancer (338). Increasing the extent of drug exposure or decreasing the length of treatment-free intervals can potentially prevent or overcome drug resistance (227). In addition, low-dose prolonged chemotherapy of drugs that are cell-cycle specific such as taxanes has been shown to increase antitumor activity in various studies (338, 341, 342).

We have recently examined the impact of chemotherapy dosing schedule on levels of drug resistance in chemosensitive (HeyA8 cells) and chemoresistant (HeyA8-MDR cells) xenograft models of ovarian cancer, which modelled newly diagnosed and recurrent disease, respectively (134). Antitumor efficacy in both models was substantially greater upon continuous DTX treatment than intermittent dosing. After 21 days of treatment, intermittent dosing caused upregulation of various DTX-related genes in both models, whereas gene expression levels did not differ between continuously treated and control tumors (134). A more frequent dosing schedule of three times per week treatment yielded the same antitumor efficacy as once weekly treatment and produced a similar but less pronounced impact on drug resistance. This established that the presence and length of treatment-free periods contribute to drug resistance development, which can only be avoided by the complete elimination of treatment-free periods as is the case in continuous therapy.

The aforementioned study was terminated 21 days post-treatment initiation as control mice had reached the predetermined ethical endpoints at that time. To allow for direct comparisons between treatments, all groups were terminated simultaneously. Since the level of drug resistance did not increase after 21 days of continuous treatment, the present study seeks to investigate whether long-term continuous chemotherapy eventually leads to tumor growth and an increase in drug resistance. This was examined by treating SCID mice bearing HeyA8 and HeyA8-MDR xenografts with continuous and intermittent DTX, and allowing each group to
continue until each treatment group reached the predetermined ethical endpoints. Tumors were then analyzed for expression of genes implicated in DTX resistance.
5.3 Materials and Methods

5.3.1 Cell lines

The human ovarian cancer cell line HeyA8 and its taxane resistant equivalent HeyA8-MDR were purchased from The University of Texas MD Anderson Cancer Center (Houston, TX). Cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin G and 100 mg/mL streptomycin), and were grown in monolayer at 37°C/5% CO₂ and 90% relative humidity.

5.3.2 Xenograft establishment and DTX treatment

Animal studies were approved by the University of Toronto Animal Care Committee and the study protocol adhered to these guidelines and those of the Canadian Council on Animal Care. Female 6-8 week-old SCID mice (Charles River) were inoculated intraperitoneally (IP) with 1x10⁶ HeyA8 or HeyA8-MDR cells suspended in 200µL sterile PBS (pH 7.6). Tumor establishment was allowed for 7 days, after which mice were placed into treatment groups (n=6/group). Continuous therapy was administered via one IP injection of DTX-PoLi₉gel (32mg/kg), prepared as previously described (131), which releases a DTX dose of 8mg/kg/week. Since the DTX-PoLi₉gel formulation depletes its drug load after 28 days, another IP injection of this formulation was administered 28 days after the first injection. Intermittent therapy consisted of one weekly IP injection of Taxotere® (Sanofi Aventis) such that both treatment groups received a total weekly DTX dose of 8mg/kg/week. Non-treated controls received one weekly IP injection of sterile saline. Mice were monitored daily, and endpoints requiring humane euthanasia included excessive muscle wasting according to the “body conditioning scoring system” which was assessed as previously described (318), weight loss in excess of 20%, hypothermia, severe abdominal distension, dehydration, inability to groom, and/or inactivity. 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. Within each group, mice were sacrificed concurrently once any of these endpoints was reached by at least 50% of animals in that group. Tumors were weighed and frozen in liquid nitrogen for subsequent gene expression analysis.

5.3.3 RT-qPCR assessment of mRNA expression

The TRIzol reagent (Invitrogen) was used to extract total mRNA from tumors following manufacturer’s instructions, which was quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Total mRNA was treated with DNase I (Invitrogen), followed by single-stranded cDNA synthesis from 2 μg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer’s protocol. NCBI’s Primer Designing tool was used to design primer sequences specific for each transcript as previously described (134). RT-qPCR reactions were performed by incubating primers with 20 ng of cDNA and Power SYBR Green PCR Master Mix (Applied Biosystems). An Applied Biosystems 7900HT instrument equipped with a 384-well reaction block was used for amplification, using SYBR green chemistry. Samples were run in triplicates. mRNA levels were calculated using a standard curve method, were normalized to levels of cyclophilin, and are expressed as percentages of non-treated controls.

5.3.4 Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 16.0). For comparisons between groups, one-way analysis of variance (ANOVA) was used and multiple comparisons with Bonferroni correction were performed upon statistical significance as determined by ANOVA. Significance was assigned at p < 0.05.
5.4 Results

5.4.1 Survival assessment of continuous and intermittent chemotherapy in chemosensitive (HeyA8) and chemoresistant (HeyA8-MDR) xenograft models

To determine whether long-term continuous chemotherapy enhances survival, SCID mice bearing HeyA8 or HeyA8-MDR xenografts were treated with DTX intermittently (once weekly) or continuously until at least 50% of mice within each group reached any of the predetermined ethical endpoints. Mice in all groups showed signs of excessive muscle wasting, inactivity, dehydration and abdominal distension at the time of termination of each individual group. Since each group was sacrificed when 3 mice out of 6 reached pre-determined endpoints, survival times may not reflect true survival curves that may have resulted from observing each individual mouse to the endpoint. Control mice bearing HeyA8 tumors were sacrificed on day 22. Intermittent treatment slightly prolonged survival to day 30 post-treatment initiation, while continuously treated mice did not reach endpoints until day 47. A similar trend was observed in mice bearing HeyA8-MDR xenografts. Non-treated mice reached the predetermined endpoints on day 20. In this chemoresistant model, intermittent treatment led to a fairly small survival advantage, with mice reaching endpoints on day 22 post-treatment initiation. Continuous chemotherapy, on the other hand, extended survival of mice to day 39. At the final timepoint, average tumor burden in continuously treated, intermittently treated, and control mice reached statistically equivalent levels in HeyA8 (0.95±0.13g, 1.10±0.16g and 1.10±0.11g, respectively; Fig. 5.1A) and in HeyA8-MDR (1.11±0.12g, 1.93±0.35g and 1.90±0.42g, respectively; Fig. 5.1B) xenografts, although this only occurred much later upon continuous treatment. No differences were observed between treatment groups in terms of tumor appearance and distribution throughout the peritoneal cavity. In general, mice presented with one large solid tumor mass near the site of cell inoculation and small tumors spread throughout the peritoneal cavity. Ascites fluid was not present in any of the mice.
Figure 5.1: Endpoint tumor weight measurements upon intermittent (Taxotere® once weekly) and continuous (DTX-PoLigel) IP DTX chemotherapy in HeyA8 (A) and HeyA8-MDR (B) human ovarian cancer xenografts in SCID mice (total DTX dose in both groups: 8mg/kg/week). Data represent mean tumor burden ± SEM (n=6/group).
5.4.2 Expression of DTX resistance genes in chemosensitive (HeyA8) tumors upon long-term intermittent and continuous chemotherapy

To establish whether long-term continuous chemotherapy eventually leads to the development of drug resistance in chemosensitive disease, the mRNA expression of genes implicated in DTX resistance was measured by RT-qPCR in HeyA8 xenografts extracted from mice treated intermittently or continuously. Consistent with previous results, considerable upregulation of \textit{mdr1} was observed after 30 days of intermittent treatment (430.4±151.5% of control) (Fig. 5.2A). After 47 days of continuous treatment, upregulation of \textit{mdr1} ultimately occurred (335.8±120.1% of control), approaching levels observed after 30 days of intermittent DTX. While only \textit{mdr1}, \textit{mrp7} (149.3±11.2% of control) and \textit{stathmin1} (190.6±23.8% of control) were significantly upregulated as a result of continuous treatment relative to controls, a trend towards increased expression can be observed for most analyzed genes (Fig. 5.2B).
Figure 5.2: Expression levels of *mdr1* (A) and other genes (B) implicated in DTX resistance in HeyA8 xenografts after 30 days of intermittent and 47 days of continuous DTX, as measured by qRT-PCR. mRNA concentrations were normalized to *cyclophilin*. Data represent mean normalized mRNA concentrations as percentages of control values ± SEM (n=6/group). * indicates significant differences between groups (p<0.05).
5.4.3 Expression of DTX resistance genes in chemoresistant (HeyA8-MDR) tumors upon long-term intermittent and continuous chemotherapy

To assess whether drug resistance in chemoresistant disease is altered after long-term continuous chemotherapy, the mRNA expression of genes implicated in DTX resistance was measured by RT-qPCR in HeyA8-MDR xenografts extracted from mice treated intermittently or continuously. After only 22 days of intermittent treatment, *mdr1* expression significantly increased by 1785.1±166.4% of control, a similar level observed after a much longer period of 39 days of continuous treatment (1821.8±310.6% of control) (Fig. 5.3A). The majority of analyzed genes were also found significantly upregulated after 39 days of continuous DTX (Fig. 5.3B), including *mrp7* (219.0±45.3% of control), *β-tubulinIII* (228.4±52.2% of control), *actinin4* (217.8±52.3% of control), *bcl2* (225.1±31.9% of control), *rpn2* (304.4±69.3% of control) and *thioredoxin* (247.9±54.9% of control). Most of these genes were also found upregulated after 22 days of intermittent chemotherapy, with the exception of *bcl2* and *thioredoxin*. Although upregulation of *stathmin1* and *akt2* is not statistically significant, a trend towards increased expression can be observed.
Figure 5.3: Expression levels of *mdr1* (A) and other genes (B) implicated in DTX resistance in HeyA8-MDR xenografts after 22 days of intermittent and 39 days of continuous DTX, as measured by qRT-PCR. mRNA concentrations were normalized to *cyclophilin*. Data represent mean normalized mRNA concentrations as percentages of control values ± SEM (n=6/group). * indicates significant differences between groups (p<0.05).
5.5 Discussion

The development of drug resistance in ovarian cancer is accountable for the majority of patient deaths (178) and is a great contributor to the low 5-year survival rate of 25-35% that results from current treatment strategies (348). Approaches that can avoid, delay or overcome drug resistance present a possible solution to this poor treatment outcome. Inhibitors of the multidrug efflux transporter P-gp have been explored, as this transporter leads to drug resistance in many solid tumors (226, 343). It has been shown that although the addition of a transporter inhibitor to continuous chemotherapy results in greater intracellular drug accumulation and tumor cell death, the effects of continuous chemotherapy alone approached these therapeutic benefits (347). Similarly, we have recently shown that changes in chemotherapy dosing schedule can have a significant impact on therapeutic efficacy in both drug sensitive and resistant ovarian cancer xenografts (134). Intermittent regimens that included treatment-free intervals, whether short or long, led to equally poor antitumor efficacy, whereas the elimination of these intervals by continuous dosing provided a substantial therapeutic advantage after 21 days of treatment. In the present study we show that, when compared to non-treated controls, intermittent DTX resulted in a modest increase in survival of 36% and 10% in sensitive and resistant models, respectively, whereas approximately 2-fold longer survival times were seen in continuous DTX treatment groups. Thus, continuous DTX resulted in substantially greater survival times than intermittent therapy in both sensitive and resistant xenografts. These results show that a simple change in dosing schedule without modifying the total cumulative dose can lead to striking survival benefits, and this is especially the case in drug resistant disease.

Our previous study shows that, after 21 days, significant increases in expression levels of genes implicated in DTX resistance ensue upon intermittent, but not continuous treatment (134). Thus, we sought to determine whether long-term continuous chemotherapy eventually led to the development of drug resistance. When mice treated continuously showed signs of
excessive muscle wasting, inactivity, dehydration and abdominal distension and were sacrificed on day 47 (HeyA8) and day 39 (HeyA8-MDR), the expression of DTX resistance-related genes in tumors reached similar levels to those seen in the tumors of intermittently treated mice at the endpoints. The most significant increase was observed in the mdr1 gene which encodes for the transporter P-gp, implicated in multidrug resistance of various cancers (343). When compared to non-treated controls, mdr1 expression was about 3-fold and 4-fold greater in chemosensitive tumors treated with continuous and intermittent DTX, respectively, and about 18-fold greater in chemoresistant tumors after either treatment regimen.

A trend towards upregulation can also be observed for most genes in the chemosensitive model, although upregulation is not as striking as seen in the chemoresistant tumors. In addition to mdr1, mrp7 and stathmin1 were significantly upregulated in HeyA8 tumors after continuous chemotherapy. The mrp7 gene encodes for a different membrane efflux transporter that is capable of effluxing taxanes from cells (216). Stathmin1 causes DTX resistance by interfering with its mechanism of action. DTX induces cytotoxicity by binding to β-tubulin and stabilizing microtubules, inhibiting their depolymerisation (183). Stathmin1 interferes with this process by promoting microtubule depolymerisation and interfering with DTX binding to β-tubulin (219). Upregulation of these genes was also observed in HeyA8-MDR xenografts, although it was not statistically significant in the case of stathmin1. In addition, the majority of the other analyzed genes were significantly upregulated in chemoresistant HeyA8-MDR tumors after 39 days of continuous chemotherapy. These include rpn2, β-tubulinIII, thioredoxin, actinin4 and bcl2. Increased expression of rpn2 plays a role in P-gp-mediated resistance, as it stabilizes P-gp in the cell membrane (217). DTX cannot bind to the β-tubulinIII isoform of β-tubulin, and its overexpression does not allow this drug to exert its action (183). Thioredoxin interferes with redox processes that are essential for microtubule assembly (220). Both actinin4 and Bcl-2 are proteins that enhance cell survival capabilities when upregulated, even in the presence of DTX (224, 225). Although levels of akt2 are not statistically different from controls, a trend towards
increased expression exists. Akt2, a protein found overexpressed in 40% of epithelial ovarian cancers, is one of the key molecules that promote survival and proliferation upon exposure to DTX (221-223, 357).
5.6 Conclusion

In summary, results presented herein show that, although levels of drug resistance eventually increase as a result of long-term continuous chemotherapy, the elimination of treatment-free intervals considerably prolongs survival and delays the development or further worsening of drug resistance in chemosensitive and chemoresistant ovarian cancer, respectively. To date, our studies have focused on taxane monotherapy since taxanes are cell cycle specific agents that should benefit from continuous drug exposure. Future studies should examine the impact of combination therapy with agents that have different mechanisms of action in an effort to overcome or delay drug resistance (180). In fact, taxanes are generally administered in combination with platinum agents (59). The prevention or delay of drug resistance development is a fundamental strategy in successful ovarian cancer management, as drug resistance is the main cause of chemotherapy failure. The results presented here and in previous studies support continuous chemotherapy as a strategy to significantly prolong survival by delaying the development or further worsening of drug resistance in ovarian cancer.

5.7 Acknowledgements

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Chapter 6

Thesis Conclusions and Future Research Directions

6.1 Overall Thesis Conclusions

This thesis has investigated the complete elimination of treatment-free intervals between chemotherapy cycles in terms of therapeutic efficacy and tumor responsiveness in models of ovarian cancer. The delivery of localized DTX on a continuous basis was achieved using an injectable polymer-lipid implantable delivery system, PoLi\textsubscript{gel}. This injectable formulation is an improvement over the previously developed PoLi\textsubscript{film} due to ease of administration without the need for surgical placement or removal. The \textit{in vitro} and \textit{in vivo} biocompatibility and biodegradability of the PoLi\textsubscript{gel} formulated with LA, rather than LCl, was established over the four week time period during which the DTX-loaded formulation releases its drug load (Chapter 2). We did not detect any evidence of local or systemic toxicities after injection of the blend, as evidenced by normal architecture of peritoneal tissues, interleukin-6 levels, weight changes, and physical and behavioural patterns of animals. These results established the biocompatibility of PoLi\textsubscript{gel}-LA, and this formulation was carried forward in future studies.

Continuous, localized DTX therapy was then investigated using the DTX-PoLi\textsubscript{gel} formulation (Chapter 3). The elimination of treatment-free intervals resulted in superior efficacy when compared to conventional, intermittent localized therapy with the clinically available DTX formulation Taxotere in ovarian cancer at the same cumulative DTX dose. As compared to intermittent therapy, continuous chemotherapy was shown to decrease tumor cell proliferation rates, increase tumor cell death, and inhibit angiogenesis in SKOV3-luc xenografts. Continuous therapy also led to greater antitumor efficacy in the ID8 model, although continuous and
intermittent DTX equally increased tumor cell death and decreased proliferation rates in this model. High efficacy upon continuous administration of DTX in the ID8 model, which is characterized by large ascites fluid volume, was likely a result of the anti-angiogenic effect of continuous chemotherapy, since anti-angiogenesis normalizes blood vessels and reduces vessel leakiness, thereby reducing ascites accumulation. These results show that eliminating treatment-free intervals and increasing tumor drug exposure through continuous chemotherapy is a beneficial strategy in the treatment of ovarian cancer.

The impact of the presence and length of treatment-free intervals on drug resistance was examined in chemosensitive (HeyA8) and chemoresistant (HeyA8-MDR) xenograft models of ovarian cancer after a 21-day period of DTX administration 1x/week, 3x/week or continuously (Chapter 4). These studies examined the expression levels of a large number of genes implicated in DTX resistance. This was important for robustness, as some studies on drug resistance focus solely on one or few genes. Intermittent 1x/week DTX therapy resulted in upregulation of various genes implicated in DTX resistance in HeyA8 tumors, especially mdr1 (P-gp), which leads to multidrug resistance and ultimately, treatment failure. These observations were more prominent in HeyA8-MDR xenografts, which reflect recurrent, resistant disease. Reducing the interval length between each chemotherapy administration by administering DTX 3x/week lessened the development of drug resistance in HeyA8 tumors. Upregulation of certain DTX resistance-related genes still occurred mainly in HeyA8-MDR tumors, in which mdr1 was remarkably upregulated upon 3x/week treatment. Interestingly, DTX administration 1x/week and 3x/week resulted in the same level of antitumor efficacy in both models. On the other hand, complete elimination of treatment-free intervals resulted in substantially greater antitumor efficacy in both models, and did not induce upregulation of any of the examined genes related to DTX resistance. The development of drug resistance is the main cause of chemotherapy failure, and these results show that continuous chemotherapy is a promising approach to delay the
development or increase of drug resistance in chemosensitive and chemoresistant ovarian cancer, respectively.

All studies to this point were terminated at the time when non-treated control animals reached the pre-determined ethical endpoints. Therefore, long-term continuous chemotherapy was investigated to determine its survival advantage and its impact on drug resistance (Chapter 5) SCID mice bearing HeyA8 and HeyA8-MDR xenografts were treated with intermittent (1x/week) or continuous DTX, and in each individual group were simultaneously sacrificed when endpoints were reached by 50% of animals within each group. Mice bearing HeyA8 xenografts survived to day 47, day 30 and day 22 with continuous DTX, intermittent DTX, and no treatment, respectively. Mice bearing HeyA8-MDR xenografts survived to day 39, day 22 and day 20 with continuous DTX, intermittent DTX, and no treatment, respectively. It is important to note that, since each group was sacrificed when 3 mice out of 6 reached pre-determined endpoints, survival times do not reflect true survival curves that would have resulted from observing each individual mouse to the endpoint. Continuous chemotherapy considerably improved survival in both models when compared to intermittent therapy, although drug resistance levels were found increased at the endpoint, as evidenced by an increase in expression levels of genes implicated in DTX resistance. These results establish a framework for combination continuous chemotherapy to further prolong survival and delay drug resistance.

Overall, we have shown for the first time the therapeutic advantages of DTX administration on a continuous basis in multiple IP murine models of advanced epithelial ovarian cancer. Complete elimination of treatment-free intervals leads to substantial increases in antitumor efficacy in these models while notably delaying the development of drug resistance in tumors. Tremendous effort has been placed on improving current treatment strategies over the past decade; unfortunately, survival rates remain alarmingly low. Results presented in this thesis contribute to the knowledge-base of ovarian cancer therapeutic strategies, in showing
that a simple change in chemotherapy dosing schedule can largely impact therapeutic outcome, even in aggressive, chemoresistant disease, and on the delay of drug resistance. This concept encourages the exploration of modified ways to administer existing chemotherapeutics, rather than focusing only on developing novel therapies, in an effort to yield survival improvements in ovarian cancer patients.

### 6.2 Future Research Directions

Studies described in this thesis focused on monotherapy with the taxane DTX, since taxanes are cell cycle specific agents that should benefit from prolonged drug exposure. We have shown that, although continuous DTX monotherapy significantly delays drug resistance at the cellular level, upregulation of genes implicated in DTX resistance eventually occurs. Monotherapy is rarely used clinically, and combination of taxanes and platinum agents have proven highly efficacious. This warrants future studies to assess the combination of taxanes and platinum agents in the continuous chemotherapy setting. Before this can be considered, it will be important to establish whether continuous administration of platinum agents provides a benefit.

Platinum agents are not classified as cell-cycle specific, and a continuous dosing schedule may not yield significant advantages over the currently used intermittent approach (139). However, one may argue that since these drugs can act in all phases of the cell cycle, more frequent exposure can ensure a greater degree of cell kill while shorter treatment-free intervals allow less time for surviving cells to repopulate the tumor (140, 141, 358). Since carboplatin is well-tolerated, its daily administration has been assessed in various clinical studies with high success rates in combination with radiotherapy (359-361). No significant toxicities were reported in these studies. Weekly platinum and taxane combinations have been successful. A recent phase I trial of biweekly carboplatin and PTX resulted in a high response rate in untreated late-stage ovarian cancer (362). A high incidence of neurotoxicity, likely a
result of PTX, led the authors to not recommend this regimen. Weekly carboplatin and PTX, on the other hand, has shown high efficacy in ovarian cancer (363), and was tolerated. Since a lower dose is administered at a given time as opposed to the maximum tolerable dose, less toxicity results from weekly treatment (137, 141). This regimen results in high response rates even in heavily pre-treated and platinum-resistant disease (140). The combination of carboplatin and DTX seems to be a promising combination, as a high response rate of 67% was reported upon weekly carboplatin and DTX in patients with recurrent ovarian cancer (69). Studies have shown that this dosing regimen is so well tolerated that it can be administered on an outpatient basis (364). The clinical evidence seems to support more frequent dosing of platinum agents. On this basis, studies investigating the potential benefits of continuous platinum therapy over the conventional intermittent approach are worth pursuing. If continuous therapy with platinum agents is deemed superior, combination continuous chemotherapy with taxanes such as DTX may be a promising approach in further increasing the therapeutic efficacy observed with DTX monotherapy.

Although platinum agents are not retained in the peritoneal cavity as extensively as taxanes, IP delivery of these agents can still benefit from higher drug concentrations while the drug is still in the peritoneal cavity (365). A study has shown that while the peritoneal concentration of carboplatin was 17 times greater than in the plasma, the amount of drug in the systemic compartment 24 hours post-IP delivery was the same as with i.v. delivery (366). Thus, ovarian tumors treated with IP carboplatin can benefit from local and systemic drug levels. A meta-analysis of 6 randomized clinical trials found a highly significant advantage to IP cisplatin when compared to i.v. in terms of progression-free and overall survival in patients with late-stage ovarian cancer (80). Preclinical studies show that resistance to platinum agents can be overcome by exposing cells to higher concentrations of these drugs; hence, IP chemotherapy has the potential to overcome a modest level of platinum resistance (73).
The combination of DTX and carboplatin as first-line therapy in ovarian cancer has shown similar efficacy to PTX and carboplatin, with significantly less neurotoxicity, an irreversible toxicity, and greater quality of life (61, 70). Future studies should investigate whether the addition of carboplatin to continuous DTX therapy can further increase antitumor efficacy, improve tumor responsiveness to therapy, further delay drug resistance intensification, and extend survival in models of ovarian cancer. Even if continuous carboplatin chemotherapy is shown to not provide a therapeutic advantage over intermittent carboplatin, future studies should investigate the addition of intermittent carboplatin to continuous DTX, as the benefits of combination therapy to improve therapeutic efficacy and overcome resistant disease are widely accepted in the clinic.

Another promising direction is to investigate the combination of continuous chemotherapy with a targeted anti-angiogenic agent. Bevacizumab is an anti-VEGF monoclonal antibody that holds promise in the treatment of ovarian cancer (190). Monotherapy with bevacizumab has shown limited activity, with low response rates of 15-20%; however, this drug provides significant clinical beneficial when used in combination with chemotherapeutic agents, with response rates of up to 80% (190, 367). This has been repeatedly attributed to improved delivery of chemotherapeutics upon vessel normalization caused by anti-angiogenesis (190, 368-371). A recent study confirmed greater amounts of PTX in tumors upon combination with bevacizumab than with PTX alone, and vascular permeability in tumors was decreased (372). Interestingly, levels in the liver were not different between treatment groups, showing that bevacizumab only increases PTX concentrations in tumors and not in healthy tissues (372). This trend was also shown in other solid tumor types (373). In addition, vessel normalization improves oxygenation of quiescent cells, which can then enter the cell cycle and become vulnerable to cytotoxic agents (185). A phase II study showed a high overall response rate of 80% when bevacizumab was used in combination with carboplatin and PTX for primary treatment of ovarian cancer, with no incidence of bowel perforation (374). Bowel perforations
are a concern when bevacizumab is used, with an overall risk of 5-11% (367, 375). Selecting for patients that have been optimally debulked and have minimal or no residual disease on the bowels, such as was done in this study, may prevent this from occurring (374, 375).

Studies have also shown that the addition of bevacizumab to frequent chemotherapy dosing, such as weekly PTX, significantly increases therapeutic efficacy in ovarian cancer when compared to weekly chemotherapy alone (367, 376). 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 (367). An ongoing phase III trial by the Gynecologic Oncology Group, GOG 252, to further assess IP versus i.v. chemotherapy in ovarian cancer has added bevacizumab to all arms of the study (377). The combination of anti-angiogenic agents and other cytotoxic agents has also been deemed beneficial in various other types of solid tumors (371, 373, 378-391). Microtubule-disrupting agents such as the taxanes have anti-angiogenic activity (141). In fact, endothelial cells have a 10- to 100-fold greater sensitivity to taxanes compared to tumor cells (141). DTX is ten times more aggressive at targeting endothelial cells and preventing capillary sprouting than PTX (196, 197). More frequent dosing of chemotherapeutics has an anti-angiogenic effect, which could partly explain why this approach is efficacious in disease resistant to the 3-weekly dosing schedule (141). A more frequent dosing schedule, termed metronomic chemotherapy, also has an anti-angiogenic effect, and combination with an anti-angiogenic agent further enhances this effect (355).

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 continuous chemotherapy regimen is beneficial. It is possible that continuous chemotherapy provides enough anti-angiogenic activity that the inclusion of this agent may prove to be redundant. The extent of taxane penetration and vessel normalization should be investigated upon combination with bevacizumab and with taxane therapy alone.
References


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alone or with concomitant daily low-dose carboplatin in locally advanced, unresectable head and neck cancer: definitive results of a phase III study with a follow-up period of up to ten years. Tumori. 2010;96:246-53.


Appendix I: Analysis of disease burden distribution

Note: This Appendix is complementary to studies presented in Chapter 3.

Appendix I Figure 1: Analysis method used to assess pattern of disease burden over time in SCID mice upon continuous or intermittent DTX treatment (n=4/group). Red grid represents the region of interest used to quantify bioluminescence signal in each of the 6 designated quadrants. This allowed for the percent quantification of disease burden in sites proximal and distal to the right bottom quadrant, the site of SKOV3-luc cell inoculation and drug treatment administration.
Appendix I Figure 2: Pictorial representation of disease abundance at each peritoneal quadrant of SCID mice upon intermittent, continuous or no DTX treatment on Days 7, 10 and 14, represented as a mean percentage of the total bioluminescence in all six quadrants (n=4/group). Treatment began on Day 7 post-inoculation with SKOV3-luc cells.
Appendix I Figure 3: Pictorial representation of disease abundance at each peritoneal quadrant of SCID mice upon intermittent, continuous or no DTX treatment on Days 17, 21 and 24, represented as a mean percentage of the total bioluminescence in all six quadrants (n=4/group). Treatment began on Day 7 post-inoculation with SKOV3-luc cells.
Appendix I Figure 4: Disease burden distribution in six quadrants of the peritoneal cavity in control non-treated SCID mice bearing SKOV3-luc tumors (n=4). Data are presented as the average percent bioluminescence in each quadrant relative to the total bioluminescence in all quadrants ± SEM. * represent significant differences between quadrants (p<0.05).
Appendix I Figure 5: Disease burden distribution in six quadrants of the peritoneal cavity in SCID mice bearing SKOV3-luc tumors treated with continuous DTX at a total weekly dose of 8mg/kg (n=4). Data are presented as the average percent bioluminescence in each quadrant relative to the total bioluminescence in all quadrants ± SEM. * represent significant differences between quadrants (p<0.05).
Appendix I Figure 6: Disease burden distribution in six quadrants of the peritoneal cavity in SCID mice bearing SKOV3-luc tumors treated with intermittent DTX on days 7, 14 and 21 at a total weekly dose of 8mg/kg (n=4). Data are presented as the average percent bioluminescence in each quadrant relative to the total bioluminescence in all quadrants ± SEM. * represent significant differences between quadrants (p<0.05).
Appendix I Figure 7: Disease burden distribution on days 7 and 10 in SCID mice bearing SKOV3-luc tumors treated with intermittent or continuous DTX, both at a total weekly dose of 8mg/kg, or no DTX treatment (n=4). Data are presented as the average absolute bioluminescence signal in each quadrant ± SEM.
Appendix I Figure 8: Disease burden distribution on days 14 and 17 in SCID mice bearing SKOV3-luc tumors treated with intermittent or continuous DTX, both at a total weekly dose of 8mg/kg, or no DTX treatment (n=4). Data are presented as the average absolute bioluminescence signal in each quadrant ± SEM.
Appendix I Figure 9: Disease burden distribution on day 21 in SCID mice bearing SKOV3-luc tumors treated with intermittent or continuous DTX, both at a total weekly dose of 8mg/kg, or no DTX treatment (n=4). Data are presented as the average absolute bioluminescence signal in each quadrant ± SEM.
Appendix I Figure 10: Disease burden distribution on day 24 in SCID mice bearing SKOV3-luc tumors treated with intermittent or continuous DTX, both at a total weekly dose of 8mg/kg, or no DTX treatment (n=4). Data are presented as the average absolute bioluminescence signal in each quadrant ± SEM.
Appendix II: Survival curves

Note: This Appendix is complementary to studies presented in Chapter 3.

Appendix Figure 1: Survival of SCID mice bearing SKOV3-luc xenografts upon intermittent and continuous DTX therapy, expressed as a percentage of the original number of animals at the start of the study (n=6/group). Mice were kept in the study until they reached any of the pre-determined ethical endpoints. Intermittent treatment consisted of one weekly IP injection of Taxotere® at the timepoints indicated by black arrows. Continuous treatment consisted of one group receiving a single IP injection of DTX-PoLi\textsubscript{gel} on day 7 (1x PoLi) and a second group receiving an additional IP DTX-PoLi\textsubscript{gel} injection on day 28 as indicated by a grey arrow, the point at which the DTX load is depleted from the PoLi\textsubscript{gel} formulation. In all groups, the cumulative weekly DTX dose was 8mg/kg.
Appendix Figure 2: Survival of C57Bl6 mice bearing ID8 ascites upon intermittent and continuous DTX therapy, expressed as a percentage of the original number of animals at the start of the study (n=6/group). Mice were kept in the study until they reached any of the pre-determined ethical endpoints. Intermittent treatment consisted of one weekly IP injection of Taxotere® at the timepoints indicated by black arrows. Continuous treatment consisted of one group receiving a single IP injection of DTX-PoLi\textsubscript{gel} on day 14 (1x PoLi) and a second group receiving an additional IP DTX-PoLi\textsubscript{gel} injection on day 35 as indicated by a grey arrow, the point at which the DTX load is depleted from the PoLi\textsubscript{gel} formulation. In all groups, the cumulative weekly DTX dose was 8mg/kg.
Appendix III: Continuous DTX release from PoLi_{gel}

Note: This Appendix is complementary to studies presented in Chapter 3. Data presented here were obtained by Payam Zahedi and are part of a manuscript titled: “Zahedi P, Stewart J, De Souza R, Piquette-Miller M, Allen C. Sustained Intraperitoneal Chemotherapy for Ovarian Cancer Treatment Results in Favorable Drug Distribution at the Whole Body, Peritoneal and Intratumoral Levels.” This manuscript was submitted to Journal of Controlled Release.

**Appendix Figure 1**: Plasma DTX concentrations in SCID mice bearing SKOV3 tumors treated IP with continuous DTX via the PoLi_{gel} formulation, at a total DTX dose of 32 mg/kg (n = 4 per group). Treatment was initiated on Day 0, 7 days post-inoculation IP with SKOV3 cells. Constant plasma levels can be seen throughout the treatment period, which illustrates continuous DTX release from the PoLi_{gel}.
Appendix Figure 2: Plasma DTX concentrations in C57Bl/6 mice bearing ID8 ascites treated IP with continuous DTX via the PoLi\textsubscript{gel} formulation, at a total DTX dose of 32 mg/kg (n = 4 per group). Treatment was initiated on Day 0, 14 days post-inoculation IP with SKOV3 cells. Constant plasma levels can be seen throughout the treatment period, which illustrates continuous DTX release from the PoLi\textsubscript{gel}.
Appendix IV: Supplemental PoLi\textsubscript{gel} cytotoxicity curves

Note: This Appendix is complementary to studies presented in Chapter 2.

Appendix Figure 1: Cytotoxicity evaluation of PoLi\textsubscript{gel-LA} and PoLi\textsubscript{gel-LCl} blends in L929 cells. These curves were used to calculate IC\textsubscript{50} values presented in section 2.4.1.
Appendix Figure 2: Cytotoxicity evaluation of PoLgel-LA and PoLgel-LCl blends in HeLa cells. These curves were used to calculate IC$_{50}$ values presented in section 2.4.1.
1. Assessment of hepatotoxicity and systemic inflammation

(An expansion of methods described in section 2.3.4):

ALT activity in serum was measured as an assessment of hepatotoxicity using an ALT Reagent Kit (Thermo Electron Corporation, Australia) as per instructions outlined by the manufacturer. Levels of circulating IL-6 were measured as an assessment of systemic inflammation using a mouse IL-6 ELISA kit (BD Biosciences, USA) as per manufacturer's protocol.

2. Immunohistochemistry staining protocol

(An expansion of methods described in section 3.3.7):

Formalin-fixed paraffin-embedded sections (4μm) were dewaxed in 5 changes of xylene and brought down to water through graded alcohols. Heat induced epitope retrieval was performed before immunostaining for cleaved caspase-3 and Ki67 by microwaving tissue sections in 10 mM citrate buffer at pH 6.0 inside a microwavable pressure cooker. After pressure build-up, boiling was maintained for 3 minutes. The cooker was then removed from the microwave oven and incubated at room temperature for 20 minutes. Sections were transferred to warm water and rinsed in PBS. Prior to CD-31 immunostaining, sections were treated with 0.1% trypsin (Difco) in PBS (pH 7.6) for 15 minutes at 37°C for antigen retrieval. Endogenous peroxidase and biotin activities were blocked respectively using 3% hydrogen peroxide and avidin / biotin blocking kit (Lab Vision Cat# TA-015-BB). After blocking for 15 minutes with 10% normal serum from the species where the secondary antibody was obtained, sections were incubated with anti-cleaved caspase 3 (1:600, overnight incubation, Cell Signaling cat. # 9661), anti-Ki67 (1:300, 1-hour incubation, Lab Vision cat. # RM9106), or anti-CD-31 (1:50, overnight, Biocare
cat # CM303.B) primary antibody. Antibody conditions were previously optimized. This was followed with a biotinylated secondary (Vector labs) for 30 minutes and horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID labs.) for 30 minutes. After washing well in PBS, color development was done with freshly prepared NovaRed solution (Vector Labs). Finally, sections were counterstained lightly with Mayer’s Hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher). Reaction products appear red to reddish brown, and non-reactive nuclei appear blue.

3. TUNEL staining protocol

(An expansion of methods described in section 3.3.7):

Paraffin sections were dewaxed through changes of xylene and brought down to water through graded alcohols. Sections were treated with 1% pepsin (Sigma: P7125) in 0.01N HCl at pH 2.0. Endogenous peroxidase was blocked using 3% aqueous hydrogen peroxide and endogenous biotin activity was blocked using avidin/biotin blocking kit (Lab Vision Cat# TA-015-BB). Sections were treated with a solution of 100 mL 50 mM TRIS-HCl (pH 7.5), 0.1015 g 50 mM MgCl2.6H2O, 78mL 100 mM β-mercaptoethanol, and 0.005g 0.005% BSA for 5-10 minutes. Sections were incubated with biotin-nucleotide cocktail, composed of DNA Polymerase 1 Large (Klenow) Fragment (Promega), dATP, dCTP, dGTP (Promega), and Bio-11-dUTP (Enzo), in a water bath at 37°C for 1 to 1.5 hr and washed in PBS. Ultra Streptavidin Horseradish Peroxidase Labeling Reagent (ID Labs inc. cat.# BP2378) was then added for 30 minutes at room temperature, followed by washing in PBS. Nova Red solution (Vector Labs. Inc.) was used for colour development, and sections were counterstained with Mayer’s hematoxylin. Apoptotic or necrotic cells appear red, while normal nuclei appear blue.