Intraperitoneal, Continuous Carboplatin Delivery for the Treatment of Ovarian Cancer

by

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Abstract

Ovarian cancer remains the deadliest gynecologic malignancy. Current treatment has low efficacy in the long term, leading to low 5-year survival rates of 20-40%. Treatment-free periods between cycles of chemotherapy are accepted in standard treatment. These periods lead to accelerated tumor cell proliferation, angiogenesis and drug resistance development. Studies presented herein show advantages of continuous carboplatin dosing schedule over conventional intermittent regimen, both administered intraperitoneally. Continuous carboplatin therapy blocked acceleration of cell proliferation observed during treatment-free period of intermittent therapy. Moreover, continuous carboplatin led to 57% inhibition of SKOV3 tumors grown intraperitoneally in SCID mice, a significant advantage over the 33% tumor suppression observed with intermittent carboplatin. Immunohistochemical analysis revealed continuous carboplatin led to greater tumor cell death while suppressing tumor cell proliferation and angiogenesis when compared to intermittent administration. These results show that the suppression of tumor growth with carboplatin can be enhanced by the elimination of treatment-free periods.
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Abbreviations and Terms

AUC – Area under the curve
BRCA – Breast Cancer
DNA - Deoxyribonucleic acid
EOC – Epithelial Ovarian Cancer
FIGO - Federation of Obstetrics Gynecology
GOG – Gynecologic Oncology Group
HPLC - High Performance Liquid Chromatography
IC$_{50}$ - Half maximal inhibitory growth concentration
IP- Intraperitoneal
IV – Intravenous
MTD – Maximum tolerable dose
MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OS – Overall survival
PBS - Phosphate-buffered saline
PFS – Progression-free survival
SCID - Severe combined immunodeficient
S-phase- Synthesis phase
TDTM – Total dose treatment method
1 Introduction

1.1 The Origin and Effects of Cancer

The development of a living organism depends largely on the division and maintenance of cells; the basic unit of a living organism. The human body is actively producing, repairing and digesting somatic cells. An organism has a wide range of biologically protective mechanisms responsible for regulating the cell cycle; including those that detect and respond to mutations to our genetic material; deoxyribonucleic acid (DNA). Sporadically, the organism safe guards fail, resulting in cells which fail to follow the same cell cycle dynamics seen in healthy cells.

Cancer is broadly defined as the uncontrolled growth of cells having undergone a genetic mutation. Possible causes include the external environment, genetics and life style choices to name a few. According to the National Cancer Institute, the United States is estimated to diagnose 1,596,670 newly reported cases of cancer in 2011 alone. Moreover, estimates show that 571,950 people will succumb to the disease. The high mortality rate, however, does not reflect on all types of cancers equally. Different types of cancer are characterized based on the organ and tissue of origin. Ovarian cancer effects a mere 0.017% of women (Cragun), however, the chances of long term survival are marginal.

1.2 Ovarian cancer

Estimates show that less than 1% of women will be affected by ovarian cancer, however, out of the diagnosed women, the reported 5-year survival rate is 20–30% (Rubin, Randall et al. 1999). The low rate of survival is mainly attributed to late stage of diagnosis; 90% of ovarian cancer is detected at stages III – IV. Late stage diagnosis of ovarian cancer is a result of lack of
disease specific symptoms; mainly being abdominal discomfort and bloating (Rubin, Randall et al. 1999).

Ovarian cancer exists in three different histopathologic forms, each originating from different tissues of the ovaries (Holschneider and Berek 2000). Epithelial ovarian cancer (EOC) is reported to be the more frequent form, accounting for 90% of diagnosed cases. The other reported forms of ovarian cancer are gonadal–stromal and germ cell, each accounting for 6 and 4% of diagnosed cases, respectively (Holschneider and Berek 2000).

1.2.1 Epithelial Ovarian Cancer

Epithelial ovarian cancer is further divided into four different sub-types (Cloven, Kyshtoobayeva et al. 2004). Serous carcinoma accounts for 75% of the diagnosed cases; having a histological resemblance of epithelium of the fallopian tube. Serous carcinoma is further subdivided into high-grade and low-grade tumors (Landen Jr, Birrer et al. 2008). High-grade serous carcinoma is thought to originate from intraepithelial carcinoma of the fallopian tube and is classified as being rapidly reproducing, chemo-sensitive and having a poor survival prognosis for the patient (Landen Jr, Birrer et al. 2008). Low-grade serous carcinoma is thought to evolve from adenofibromas or borderline tumors. Low-grade serious carcinoma is reported to have a slow rate of proliferation, chemo-resistance and a better prognosis than that of high-grade serous carcinomas (Landen Jr, Birrer et al. 2008).

Endometrioid and mucinous subtypes of EOC resemble the endometrium and gastrointestinal epithelium respectively, and each account for 10% of the diagnosed cases (Metzger-Filho, Moulin et al.). Clear cell carcinoma accounts for 5% of the diagnosed cases (Metzger-Filho,
Moulin et al.). Similarly, clear cell carcinoma resembles the endometrium as endometrioid ovarian cancer. However, studies have shown certain lifestyle choices contribute to increased risk of one sub-type over the other: increased obesity and decreased smoking habits contribute to higher risk of clear cell carcinoma (Auersperg, Wong et al. 2001; Nagle, Olsen et al. 2008). Therefore, the two subtypes of EOC cannot be considered to be the same, as different risk factors influence each subtype of EOC differently.

The prevalence of epithelial ovarian cancer is generally thought to be unpredictable. However, genetic predisposition to epithelial ovarian cancer has been reported. It has been suggested that 90% of the hereditary patients are carriers of the mutated breast cancer1 (BRCA1) and/or BRCA2 genes (Antoniou, Pharoah et al. 2003). BRCA1 and BRCA2 are part of a family of genes known for their function of tumor suppression and regulation of cellular proliferation and the repair of DNA (Holschneider and Berek 2000). Germline mutations of BRCA1 and BRCA2 have been associated with a 20–60% and 10–35%, respectively, of increased risk of development of epithelial ovarian cancer (Antoniou, Pharoah et al. 2003).

1.3 Hypotheses of Origin of Epithelial Ovarian Cancer

As with most cancers, explicit causes of ovarian cancers have not been established. However, four different hypotheses have been developed that attempt to address causes and origins of epithelial ovarian cancer (Landen Jr, Birrer et al. 2008). The first hypothesis addresses the ovulation of the ovaries and is known as the incessant ovulation hypothesis. This theory suggests the shedding and subsequent healing of the ovaries’ surface during ovulation, may lead to cell mutations, explaining why older women, having gone through more menstrual cycles are more
prone to the malignancy (Fleming, Beaugie et al. 2006). As a consequence, women bearing more children, using oral contraceptives and other factors that decrease the number of ovulations are considered to be less prone to development of epithelial ovarian cancer. A second hypothesis leading to the development of EOC points to the over expression of gonadotropins and is known as the gonadotropin stimulation. Studies have shown the follicle-stimulating hormone and the luteinizing hormone to be responsible for stimulation of EOC, therefore, women with elevated levels of gonadotropins are more prone to EOC (Gadducci, Cosio et al. 2004). Another hypothesis known as the hormonal stimulation, and as the gonadotropin stimulation theory, it suggests the promotion of EOC with conditions that present elevated levels of androgens (Risch 1998). The last hypothesis is known as the inflammation theory, which suggests the inflammatory effects that follow each ovulation, promote susceptibility to mutation of the ovarian surface epithelium (Ness and Cottreau 1999). Due to the collective observations of the aforementioned symptoms of each of the theories, it is generally believed that the likely cause of EOC is due to a combination of the four proposed hypotheses (Landen Jr, Birrer et al. 2008).

1.4 Diagnosing and Staging

High rate of mortality associated with ovarian cancer is due to lack of specific symptoms and methods of detecting early stage EOC, where chances of survival are as high as 90% (Cragun). Prolonged periods of bloating and abdominal discomfort are first signs for women to check for presence of ovarian cancer (Goff, Mandel et al. 2007). Screening involves pelvic examinations, abdominal and/or pelvic ultrasounds, CT scans and measurements of CA 125 (Kinkel, Lu et al. 2005). Unfortunately these diagnostic tools are not sensitive enough for early stage disease.
A valuable tool of early detection of epithelial ovarian cancer is the detection and quantitation of ovarian cancer specific biomarkers. Clinical trials have identified CA125 (Bast Jr, Klug et al. 1984), prolactin (Jha, Farooq et al. 1991) and mesothelin (Scholler, Fu et al. 1999) as some of the potential candidates of detection of epithelial ovarian cancer. Of all established biomarkers, only CA 125 is detected at early stages of the malignancy. Unfortunately, CA 125 does not provide definite and exclusive diagnosis of ovarian cancer. Elevated levels of CA 125 are seen in 90 % of patients with late stage ovarian cancer, however, only 50 % of patients exhibit elevated levels of CA 125 at early stages of the disease (Gubbels, Claussen et al.). Additionally, elevated levels of CA 125 are seen in patients with pancreatic, breast, bladder, liver, and lung cancers (Niloff, Klug et al. 1984). Moreover, women undergoing their menstrual cycle and women carrying a child are exposed to higher levels of CA 125 (Niloff, Klug et al. 1984; Niloff, Knapp et al. 1984). Relying on elevated levels of CA 125 alone cannot bring to a conclusive diagnosis of ovarian cancer. However, the combination of ultrasonography and measurements of CA 125 are considered to be a reliable method of diagnosing ovarian cancer.

Staging of ovarian cancer occurs during a laparotomy (Bell, Petticrew et al. 1998). Guidelines set forth by the Federation of Obstetrics and Gynaecology (FIGO) stages ovarian cancer from stage I to stage IV. Stage I ovarian cancer is considered to be the least invasive, where the tumor is confined to one or both of the ovaries. Stage II ovarian cancer presents tumor metastasis beyond the ovaries reaching any of the organs residing within the pelvic region. Stage III ovarian cancer presents metastasis beyond the pelvis into the peritoneal cavity. Stage IV ovarian cancer presents metastasis beyond the peritoneal cavity (Kurtz, Tsimikas et al. 1999). It is thought that the local spread of tumors occurs through lateral growth, whereas, ascites mediate the metastasis to the
peritoneal cavity. Metastasis beyond the peritoneal cavity is mediated by lymphatic and systemic circulation (Lengyel).

1.5 Treatment

For many years, debulking surgery has been a key component to the treatment of ovarian cancer. Debulking surgery has evolved by increasing the mass of removed tumor through surgery. Chemotherapy on the other hand, has transformed quite drastically. Early treatment of ovarian cancer has evolved from the administration of a single agent cyclophosphamide or melphalan (Guarneri, Piacentini et al.). In the late 1970’s, the Gynecologic Oncology Group (GOG) ran a clinical trial which incorporated cisplatin into the standard of care of that time. Results of that study showed a significant increase in progression free survival in the treatment arm incorporating cisplatin (Omura, Blessing et al. 1986). Further clinical trials, namely the GOG 111, compared combination therapy of cisplatin and cyclophosphamide vs cisplatin and paclitaxel. The results of that study showed that combination of cisplatin-paclitaxel lead to a significant improvement in progression free survival (McGuire, Hoskins et al. 1996), which resulted in the replacement of cyclophosphamide with paclitaxel. In 2004, a cisplatin analogue, carboplatin, replaced cisplatin in the primary treatment of ovarian cancer due to a more favorable toxicity profile.

Presently, treatment of ovarian cancer consists of cytoreductive surgery, followed by chemotherapy in the form of intravenous (IV) infusions of combination carboplatin and paclitaxel at their maximum tolerable doses (MTD) administered every 3 weeks for a total of 6 cycles. Preclinical in vivo studies showed this treatment approach to lead to increased rates of tumor proliferation (Vassileva, Allen et al. 2008), hematologic and gastrointestinal toxicities (Lowenthal and Eaton 1996) and low dose of the agents at the disease sites (Tannock, Lee et al. 2002).
Drug free periods, in intermittent therapy, are required in between treatments cycles for recovery of healthy tissue (Metzger-Filho, Moulin et al.; Gasparini 2001). It is believed that intermittent administration of cytotoxic agents target more vulnerable cells, those that are closest to the capillaries and are less prone to drug resistance (Davis, Chapman et al. 2003). During drug free periods, it has been shown that remaining malignant cells re-enter the cell cycle at an accelerated rate of proliferation possessing higher potential for chemotherapeutic resistance (Wu and Tannock 2003).

IV administration of cytotoxic agents has dose limits due to systemic toxicity. Total drug administered intravenously has a limited potential of reaching the target site, hence only a fraction of the drug produces the intended cell kill. Therefore, therapeutic levels of the agents are time limited. Further, drugs are rapidly distributed into tissues and are subject to hepatobiliary and renal clearance prior to reaching the tumor site. The presence of drugs in serum may lead to irreversible protein binding thereby decreasing free drug concentrations (Jain 1997). Further, circulation in tumor capillaries does not follow the same pattern of blood flow as that of normal tissue (Vaupel, Kallinowski et al. 1989). Rapidly proliferating tumors are known to form leaky vasculature, which increases intratumoral pressure thereby limiting the depth and extent of tumor that can be reached via the circulatory system, hence limiting depth of penetration of cytotoxic agents (Tannock, Lee et al. 2002). Alternative methods of drug administration are needed to achieve better outcomes in ovarian cancer therapy.

Intraperitoneal (IP) chemotherapy involves the delivery of cytotoxic agents directly into the peritoneal cavity. Treatment of cancers bound to the peritoneal cavity can potentially benefit from drug delivery through the peritoneal cavity by increased drug exposure and reduced systemic toxicities.
1.6 IP Chemotherapy

Direct intraperitoneal administration of chemotherapy to patients with small volume advanced ovarian cancer has a number of advantages over IV chemotherapy mainly due to the fact that ovarian cancer metastasizes throughout the peritoneal cavity. Locoregional administration provides higher drug concentrations at the disease sites and lower systemic concentrations, thereby potentially increasing efficacy and decreasing systemic toxicity. Important factors to consider when choosing a candidate drug for IP therapy include effectiveness of the drug for the disease being treated, the extent of drug retention in the peritoneal cavity, and lack of peritoneal toxicity (Markman 2003). The use of platinum drugs for the treatment of ovarian cancer has been well established in the IV setting. IP administration of these drugs is promising, as the same 24-hour concentration of free platinum in serum is achievable when administered IV or IP (Miyagi, Fujiwara et al. 2005), while IP administration results in peritoneal concentrations 10-20-fold greater than in the systemic circulation (Markman, Rowinsky et al. 1992; Hofstra, Bos et al. 2002; Morgan, Doroshow et al. 2003; Hess, Benham-Hutchins et al. 2007; Markman 2009; Sugarbaker 2009). Phase III clinical trials have demonstrated substantial survival benefits of IP chemotherapy when compared to IV in ovarian cancer treatment using the platinum agent cisplatin (Alberts, Liu et al. 1996; Markman, Bundy et al. 2001; Armstrong, Bundy et al. 2006; Fujiwara, Aotani et al. 2011). Alberts et al, demonstrated overall survival of 41 months for the IV group and 49 months for the IP group. Markman et al, showed similar benefits of IP therapy where he reports progression-free survival of 27.9 months and 22.2 months as well as overall survival of 63.2 months and 52.2 months for IP and IV therapies respectively. Armstrong et al, has shown IP therapy to have a progression-free survival 23.8 months vs. 18.3 months IV and overall survival to be 66.9 months and 49.5 months IP and IV respectively. Further, a meta-analysis of 6 randomized clinical trials found a significant advantage of IP cisplatin when compared to IV in terms of progression-free and
overall survival in patients with late-stage ovarian cancer (Hess, Benham-Hutchins et al. 2007). Unfortunately, this regimen was associated with increased toxicity and low patient compliance possibly due to local cisplatin related toxicities in the IP treatment groups.

1.7 Carboplatin

Another platinum agent, carboplatin, has replaced cisplatin as the platinum agent of choice in the standard IV treatment of ovarian cancer (Guarneri, Piacentini et al.). While the two drugs show comparable therapeutic efficacy (Kavanagh and Nicaise 1989), carboplatin has a more favorable toxicity toxic profiles.

![Molecular structure of platinum analogues carboplatin and cisplatin.](image)

**Figure 1.** Molecular structure of platinum analogues carboplatin and cisplatin.

The cyclobutanedicarboxylate bidante ligand of carboplatin serves as a much slower leaving group than the chloride ligands of cisplatin. As a result, carboplatin has a considerably longer plasma half life and reduced renal toxicities (Roberts et al.). In terms of its potential to replace cisplatin in the IP setting, carboplatin has shown slower peritoneal clearance (Van Der Vijgh 1991) and reduced
peritoneal toxicity as compared to cisplatin (Bookman, Greer et al. 2003). A comparison of two separate clinical trials utilizing IP cisplatin (Armstrong, Bundy et al. 2006) and IP carboplatin (Kim, Paek et al.) reveal IP carboplatin to lead to reduced adverse events involving gastrointestinal, neurologic and metabolic toxicities in patients with stage III ovarian cancer. As a result, the Japanese Gynecologic Oncology Group has begun a phase II/III clinical trial designed to compare IP and IV administration of carboplatin (Fujiwara, Aotani et al.). In this study, patients will undergo debulking surgery followed by 6 cycles of IP carboplatin at MTD with 15-day drug-free periods in between treatment cycles, required for recovery of healthy tissue. The treatment-free periods, however, may allow tumor cells to adapt to treatment, potentially leading to accelerated tumor proliferation, drug resistance and other factors that could eventually result in loss of efficacy (Durand and Vanderbyl 1990; Brade and Tannock 2006; Vassileva, Allen et al. 2008).

1.7.1 Carboplatin Mechanism of Action

Carboplatin induces its cytotoxic effect by covalently binding to DNA strands thereby halting cell division (Reedijk and Lohman 1985). Mechanistic studies have illustrated that carboplatin exchanges its carboxyl ligands for an aqua ligand. The aquated platinum species further undergoes a second ligand exchange to have two aqua ligands (Arambula, Sessler et al. 2009). A third ligand exchange then occurs whereby the platinum metal center acts as an electrophile reacting with a lone pair of electrons on the N\textsuperscript{7} atom of the imidazole rings on guanine and adenine nucleobases (Fuertes, Alonso et al. 2003). This binding leads to both intra and interstrand cross-links with DNA (Fig. 2) leading to eventual cell death through apoptosis.
**Figure 2.** The binding of platinums to DNA fragments, forming intra- and interstrand adducts to DNA, eventually leading to apoptosis (Sat et al. 2010).
1.8 Alternative Chemotherapy Regimens

An alternative treatment regimen known as metronomic chemotherapy is the administration of cytotoxic agents on a more frequent basis (Hanahan, Bergers et al. 2000). Preclinical studies involving shortened durations of treatment-free periods and more prolonged exposure of taxanes at lower doses result in an anti-angiogenic effect (Browder, Butterfield et al. 2000), reduced rates of tumor repopulation (Davis and Tannock 2000; Brade and Tannock 2006) and a decrease in tumor burden greater than conventional treatment (De Souza, Zahedi et al. 2010).

Studies in murine xenografts of ovarian cancer have shown the complete eliminating drug-free periods by continuous chemotherapy of taxane drugs leads to a substantial increase in therapeutic efficacy when compared to conventional intermittent treatment (Vassileva, Grant et al. 2007; Vassileva, Moriyama et al. 2008; De Souza, Zahedi et al. 2010; De Souza, Zahedi et al. 2011). This enhanced efficacy was accredited to lower tumor repopulation, neoangiogenesis and drug resistance, yielding a greater amount of tumor cell apoptosis. Taxane drugs are cell-cycle specific agents (Escobar and Rose 2005) that should theoretically benefit the therapy from prolonged exposure, as this allows tumor cells to reach vulnerable cell cycle phases (El-Kareh, Labes et al. 2008).

Platinum agents are not considered cell-cycle specific, as these drugs can act during any phase of the cell cycle. However, extended exposure of platinum agents may still be beneficial by resulting in greater tumor cell kill, while the absence of drug-free periods in prolonged low dose chemotherapy may prevent surviving cells from repopulating the tumor (Cadron, Leunen et al. 2007; Sharma, Graham et al. 2009; Baird, Tan et al. 2010). In fact, weekly carboplatin administration has shown high efficacy in heavily pre-treated ovarian cancer, while toxicities were lower than 3-weekly carboplatin administration (Lokich 1999; Baird, Tan et al. 2010). A complete
elimination of drug free periods by providing continuous release of carboplatin may have the potential to further enhance efficacy as compared to therapies with drug free periods.

1.9 Animal Model of Ovarian Cancer

*In vivo* experiments to be presented have been based on a xenograft model of SKOV3 human ovarian cancer using severe combined immunodeficiency (SCID) female mice. A major characteristic of SCID mice is the lack of functional thymus and bone cells (Custer, Bosma et al. 1985). The absence of these white blood cells allows for cultivation of xenografts and the subsequent development of tumors.

SKOV3 cells originated from ascites fluids of an ovarian cancer patient. The SKOV3 cell line is of clear cell histology (Garson, Shaw et al. 2005), known to be highly resistant to standard chemotherapy (Pectasides, Fountzilas et al. 2006). Injection of SKOV3 cells in SCID mice produces xenografts with rapid doubling times. The SKOV3 murine model closely resembles ovarian cancer by producing multiple solid tumors along the peritoneal cavity.

Subcutaneous models of ovarian cancer provide the convenience of monitoring the progression of tumor development over time, without sacrificing the animal. However, the subcutaneous xenograft model does not have the ability to model metastasis, an important characteristic of ovarian cancer. In the present work, IP injections of SKOV3 cells presented high degree of metastasis with the presence of tumors at the injection site as well as more distal sites in the peritoneal cavity.
2 Rationale and Hypothesis

2.1 Rationale

In 2010, 21,800 women were diagnosed with ovarian cancer in the United States alone, with the majority succumbing to the disease. Lack of disease specific symptoms and inadequate screening techniques result in disease diagnosis at its late stages, resulting in a poor 5-year survival rate of 20-40% (Rubin, Randall et al. 1999). Current treatment consists of cytoreductive surgery followed by chemotherapy in the form of intravenous (IV) infusions of combination carboplatin and paclitaxel at their maximum tolerable doses (MTD) administered every 3 weeks for a total of 6 cycles. Preclinical animal studies with taxanes have demonstrated this chemotherapeutic approach to lead to increased rates of tumor proliferation (Vassileva, Allen et al. 2008) and low dose of the agents at the disease sites (Tannock, Lee et al. 2002). A possible alternative treatment method, potentially lacking the aforementioned issues, involves continuous, localized chemotherapy.

Preclinical models have shown prolonged low dose chemotherapeutic cyclophosphamide to produce an antiangiogenic effect in Lewis lung carcinoma (Browder, Butterfield et al. 2000). Browder et al. suggest the extended and low dosing of cyclophosphamide leads to inhibition of angiogenesis by means of sustained apoptosis of vascular endothelial cells. Further, clinical studies of oropharyngeal cancer show patients treated with chemotherapeutic 5-bromodeoxyuridine to exhibit a significantly higher rate of cell proliferation compared to untreated patients. Preclinical studies in a murine xenograft model of ovarian cancer demonstrate the complete elimination of drug-free periods with continuous chemotherapy results in a substantial increase in therapeutic efficacy of the taxanes compared to conventional intermittent treatment (Vassileva, Grant et al.
Post operative administration of chemotherapy to patients with small volume advanced ovarian cancer directly to the peritoneal cavity, via intraperitoneal (IP) chemotherapy, has a number of advantages over IV chemotherapy mainly due to the fact that ovarian cancer metastasizes throughout the peritoneal cavity. Locoregional administration provides higher drug concentrations at the disease sites and lower systemic concentrations, thereby potentially increasing efficacy and decreasing systemic toxicity (Miyagi, Fujiwara et al. 2005).

The use of platinum drugs for the treatment of ovarian cancer has been well established in the IV setting. Use of cisplatin in IP chemotherapy has shown an increase in efficacy over IV therapy; however, use of cisplatin caused toxicity. Alternatively, carboplatin shows a similar therapeutic outcome and lower toxicity compared to that of IP cisplatin. Further, carboplatin is known to be a more chemically stable platinum analogue, making it a more attractive candidate drug for prolonged drug delivery systems, where chemical drug stability is essential.

The present studies sought to assess IP continuous chemotherapy of carboplatin in a xenograft model of human ovarian cancer in mice. Efficacy of continuous IP carboplatin was compared to conventional, intermittent IP dosing in terms of suppression of tumor growth. The degree of tumor cell repopulation, angiogenesis and apoptosis were further examined in tumors treated continuously and intermittently.
2.2 Hypothesis

Continuous intraperitoneal delivery of carboplatin will lead to an increase in efficacy in treatment of ovarian cancer by suppressing tumor proliferation, increasing cell kill and decreasing angiogenesis, in comparison to intermittent therapy.

2.3 Objectives

1. Establish in-vitro release of carboplatin using Alzet osmotic pumps. Determine stability and total drug release over a period of 14 days.

2. Determine and compare in-vitro activity of carboplatin using sustained and intermittent exposure. Determine effects of continuous and intermittent therapy on SKOV3 proliferation.

3. Determine and compare efficacy of sustained and intermittent carboplatin in a murine xenograft model of human ovarian cancer. Establish impact on cell death, angiogenesis and proliferation rates after continuous or intermittent therapy.
3 Methods and Materials

Carboplatin used for *in vitro* studies, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-[2H]-tetrazolium bromide), sodium dodecyl sulphate and crystal violet were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Carboplatin used for *in vivo* injection (50 mg / 5 ml) was purchased from Shoppers Drug Mart (The Hospital for Sick Children, Ontario, Canada). Alzet micro-osmotic pumps (model 1002) were purchased from Durect Corporation (Cupertino, California, USA). RPMI 1640, phosphate-buffered saline (PBS), trypsin:EDTA, fetal bovine serum, streptomycin/penicillin were purchased from Invitrogen (Burlington, Ontario, Canada). The SKOV3 human ovarian adenocarcinoma cell line was acquired from the American Type Culture Collection (Rockville, Maryland, USA), and was maintained as previously described (Vassileva, Grant et al. 2007). HPLC grade acetonitrile was purchased from Caledon (Georgetown, Ontario Canada).

3.1 *In Vitro* Release of Carboplatin

To assess the *in vitro* release of carboplatin-loaded Alzet pumps, 10 mg of carboplatin was dissolved in 1 ml of PBS and loaded into the reservoir of the Alzet pump at a concentration of 10 mg/ml (n=3). The drug-loaded pumps were submerged in PBS and maintained at 37 °C. Following 24 hours of carboplatin release, old buffer was replaced with fresh carboplatin-free buffer; this protocol was followed for a period of 14 days. Samples were stored at 4 °C in light free conditions. At the conclusion of the study, aliquots of each sample were assayed for platinum content analysis using High Performance Liquid Chromatography (HPLC).
3.2 HPLC

Agilent Series 1100 HPLC (Agilent Technologies, Canada) equipped with a Waters 4.6 mm×150 mm column (Atlantis T3, 5 µm particle size) attached to a Waters 3.9×20 mm guard column (XTerra MS C18, 5 µm particle size), connected to the Agilent 35900E dual channel interface detector (Agilent Technologies, Canada). Signals were analyzed using ChemStation software (Agilent Technologies, Canada). The wavelength of detection for carboplatin was 227 nm. For in vitro release samples were run through a 90% H2O and 10% acetonitrile mobile phase, with an observed carboplatin retention time of 5.6 minutes. All samples and standards were run at a flow rate of 0.5 ml/min with an injection volume of 100 µl per sample.

3.3 Carboplatin Stability

To assess the long-term in vitro stability of carboplatin-loaded Alzet pumps under physiological conditions, carboplatin dissolved in PBS was loaded into the reservoir of the Alzet pumps at a concentration of 10 mg/ml (n=3). The drug-loaded pumps were submerged in PBS buffer and were maintained at a temperature of 37 °C. The Alzet pumps were placed into fresh drug-free buffer every 24 hours for a period of 14 days. Every second day, aliquots of buffer which contained released drug were analyzed using high performance liquid chromatography. Sample standards were prepared on the same day as sample analysis. Drug stability was established by comparison of sample peak areas, presence of new peaks and elution times to the peaks produced by the prepared standards.
3.4 In Vitro Cytotoxicity

To determine the half maximal inhibitory growth concentration (IC50) values and the cytotoxicity of prolonged carboplatin exposure in SKOV3 cells, these cells were seeded onto 96 well plates at 30,000 cells per well and were incubated overnight. Cells were exposed to a range of 50 μg/ml to 700 μg/ml of carboplatin in PBS for periods of 24, 48 and 72 hours (n=3). The MTT assay was used to establish cell viability.

3.5 In Vitro Proliferation Potential Assessment

To determine clonogenicity of SKOV3 cells upon continuous and intermittent carboplatin treatment, cells were seeded onto 6-well plates at 1x10^5 cells per well. After an overnight incubation period, cells were divided into three treatment groups (continuous carboplatin, intermittent carboplatin, and non-treated controls) and treated with a dose of the previously established 72-hour IC₅₀ of 81 μg/ml over a period of 3 days (total dose 24.3 μg), this treatment method was termed as the ‘total dose treatment method (TDTM)’. Continuous treatment consisted of exposure to carboplatin loaded media for the full duration of the study 9 days (total of 9 treatment cycles), with replacement of carboplatin loaded media every 24 hours. Every 24 hours, the continuous treatment group was exposed to fresh media with carboplatin at 8.1 μg in 0.3 ml (27 μg/ml) of media. Intermittent treatment consisted of 24.3 μg of carboplatin in 0.3 ml of media (81 μg/ml), exposed for 12 hours, followed by washing and exposure to carboplatin-free media for the remainder of the 3-day treatment period for a total of 3 treatment cycles. The cumulative carboplatin dose for continuous and intermittent treatment groups was 72.9 μg (81μg/ml; the equivalent of [72-hour IC₅₀] administered 3 times) for the entire duration for the study of 9 days. Non-treated control cells were exposed to drug-free media, which was replaced every 24 hours. On
days 0, 1, 3, 4, 6, 7 and 9, the clonogenic assay was performed following a method previously described (De Souza, Zahedi et al. 2010). Briefly, cells from each treatment were trypsinized, counted, plated in serial dilutions and incubated for 5 days (n=3). Cells were then fixed with methanol and stained with 1% crystal violet. Colonies containing a minimum of 50 cells were considered viable colonies and were counted using Image J, an image processing and analysis software. The number of clonogenic cells was calculated as [(cells counted at each time point) x (viable colonies / cells plated)].

Intermittent treatment group will be treated on 72-hour intervals with 12 hours of carboplatin exposure. Out of the 72 hours, 60 hours will serve as treatment free intervals.

**Total Dose Treatment Method (TDTM) Calculation**

**Continuous group:**

\[
\text{TDTM} = (9 \text{ treatment cycles of } 24 \text{ hours}) \times (8.1 \, \mu g \, \text{of carboplatin}) = 72.9 \, \mu g \, \text{of carboplatin}
\]

**Intermittent Group:**

\[
\text{TDTM} = (3 \text{ treatment cycles of } 12 \text{ hours}) \times (24.3 \, \mu g \, \text{of carboplatin}) = 72.9 \, \mu g \, \text{of carboplatin}
\]

Alternatively, a second experiment measuring the clonogenic potential of SKOV3 cells was established using the ‘**AUC treatment method**’. This method accounted for total exposure concentrations over time. Using this treatment scheme, the continuous treatment arm was exposed to 4.05 µg of carboplatin in 0.3 ml of media (13.5 µg/ml), every 24 hours (total of 6 treatment
cycles). If applying the AUC treatment method to the intermittent treatment group in the TDTM treatment scheme, the total AUC dose for both groups becomes equivalent.

**AUC Treatment Method Calculation:**

Intermittent AUC treatment was determined as follows:

$$
AUC = (3 \text{ treatment cycles}) \cdot \left[ \frac{(24.3 \mu g \text{ of carboplatin}) \cdot (12 \text{ hours})}{(0.3 \text{ ml of media})} \right] = 2,916 \frac{\mu g \cdot \text{hr}}{ml}
$$

Based on the above AUC determined for the intermittent treatment group, the concentration for continuous treatment group was calculated based on the following equation in order to have equivalent AUC:

$$
AUC = (9 \text{ treatment cycles}) \cdot \left[ \frac{(4.05 \mu g \text{ of carboplatin}) \cdot (24 \text{ hours})}{(0.3 \text{ ml of media})} \right] = 2,916 \frac{\mu g \cdot \text{hr}}{ml}
$$

**Efficacy Studies**

Six- to eight-week-old female severe combined immunodeficient (SCID) mice, purchased from Charles River (Wilmington, Massachusetts, USA), were used in these studies. All animal studies followed the guidelines of the University of Toronto Animal Health Care Committee and the Canadian Animal Care Council. On day 0, SCID mice were inoculated IP with $1 \times 10^7$ SKOV3 cells. On day 7, animals were divided into three groups (n=6/group). Mice in the continuous treatment group were implanted with one carboplatin-loaded (1 mg; 10mg/ml) Alzet pump via abdominal incision. Mice in the intermittent treatment group were implanted with one saline loaded Alzet pump via abdominal incision, followed by a bolus intraperitoneal injection of carboplatin (440 μg) which was repeated on day 14. The cumulative carboplatin dose was equal for both
continuous and intermittent groups. Non-treated control mice were implanted IP with one saline loaded Alzet pump via abdominal incision, and received an IP injection of saline which was repeated on day 14. Animals were monitored daily for signs of toxicity and endpoints requiring humane euthanasia, including muscle wasting (Ullman-Culleré and Foltz 1999), weight loss in excess of 20%, signs of peritonitis, inactivity and lack of grooming. On day 21 all animals were euthanized, tumors were collected, and suppression of tumor growth was calculated as\[\frac{\text{(mean tumor weight untreated)} - \text{(mean tumor weight treated)} \times 100\%}{\text{(mean tumor weight untreated)}}.\]

Tumors were further processed for immunohistochemistry, as described below.

3.5.1 **Dose Justification**

In conventional intravenous based therapy of patients with ovarian cancer, carboplatin is administered at 300-360 mg/m\(^2\). Alternatively, studies on long-term survival of patients treated with intraperitoneal carboplatin have reported treatment of greater than 400 mg/m\(^2\) to lead to long term survival of 51 months compared to 25 months if treated with less than 400 mg/m\(^2\) (Fujiwara, Sakuragi et al. 2003). In contrast to human dosing, the use of carboplatin in mice is generally seen used at a range of 60-100 mg/kg (180-300 mg/m\(^2\)) (Boven, Van der Vijgh et al. 1985; Kelland, Jones et al. 1992; Jandial, Messer et al. 2009).

Animal dose of the continuous and intermittent treatment groups has been based on the cumulative 14-day release of carboplatin using the Alzet pump; measured to be 880 µg of carboplatin. Further, obtained pharmaceutical grade Paraplatin (carboplatin for injection) used in the animal studies, is strictly manufactured in a single concentration of 10 mg/ml. Based on the listed parameters, the total dose of carboplatin administered to each treated animal is 48.9 mg/kg.
While the majority of studies have used single doses of only 60 mg/kg, there are studies that have used multiple cycles of lower doses of carboplatin which have proven to be efficacious (Lu and Yin 1994).

### 3.6 Immunohistochemistry

At the conclusion of the study on day 21, mice from continuous, intermittent and control treatment groups were sacrificed. The primary tumor was collected from each of the mice from each treatment group (6 mice per treatment group). The 18 tumors (6 tumors/group) were fixed in 10% buffered formalin. Four sections have been taken from the center of each tumor with one section being used for the staining of each marker (Casp-3, TUNEL, Ki67 and CD-31). All fixed tumor samples were submitted to the pathology research program laboratory, at the Toronto General Hospital (Toronto, Ontario) for processing. Further processing included de-waxing in xylene and passaging through graded alcohol. Sections were mounted onto microscope slides and immunostained for proliferation with the Ki67 monoclonal antibody. Markers of cell death, namely Casp3 and TUNEL were incubated with caspase3 and biotin-nucleotide cocktail and DNA polymerase 1 respectively. Marker of angiogenesis was immunostained with anti-CD-31 antibody. Slides were imaged at 20x magnification using a bright-field scanner which scans the entire section (ScanScope XT, Aperio Technologies Inc.). Positive staining from the entire section was analyzed and expressed as a percentage of positive signal to blank, using ImageScope software (Aperio Technologies Inc., Version 10) using the Positive Pixel Count algorithm. ImageScope is capable of high image resolution, having the capability to detect each individual pixel of a given image; providing accurate detection for each positive stain signal in a given tissue.
3.7 Statistical Analysis

The reported results (n≥3) have been expressed as mean ± standard error. Statistical analyses between two groups were obtained using the Student’s t test, statistical analysis of three groups were obtained using ANOVA with significance assigned at \( P < 0.05 \). Data were analyzed using Statistical Package for the Social Sciences Version 16.0 (SPSS Inc., USA).
4 Results

4.1 In Vitro Release and Stability of Carboplatin

The rate of release of carboplatin from the Alzet mini osmotic pumps and the drug’s stability was measured for 14 days in PBS buffer. Fig. 3 shows the cumulative release of carboplatin over the duration of the study. On average, the Alzet pump resulted in a cumulative release of $88 \pm 4.8\%$ (880 ± 48 µg) of the total loaded carboplatin over 14 days. The average release of carboplatin was measured to be $62.85 \pm 7.4$ µg per 24 hours. HPLC chromatograms of day 0 to day 14 samples did not differ in terms of elution times, and no extra peaks were present at later time points, evidencing that carboplatin remained stable within the Alzet pump under physiological conditions for the entirety of the study (Fig 4).

![Figure 3](image-url)

**Figure 3.** Cumulative *in vitro* release from carboplatin-loaded Alzet pumps (10 mg/ml) over a 14-day period. Points represent mean (n=3), bars indicate SEM.
Figure 4. HPLC chromatogram of carboplatin (A) Alzet released on day 14, and (B) standard sample of carboplatin prepared on day of analysis. Both chromatograms show an identical elution of carboplatin with similar solvent peaks.
4.2  *In Vitro* Carboplatin Cytotoxicity Assessment

Carboplatin cytotoxicity was assessed using the MTT assay in SKOV3 cells using escalating doses of carboplatin and increased exposure times. Carboplatin concentrations of 50, 100, 300 and 500 µg/ml resulted in time and dose-dependent cytotoxicity (Fig. 5). Once a relationship between cytotoxicity and prolonged carboplatin exposure was shown, the potential for modifications in dosing schedule was examined *in vitro*. IC$_{50}$ values were measured to be 427 ± 21 µg/ml, 155 ± 13 µg/ml and 81 ±13 µg/ml for 24, 48 and 72 hours (Fig. 6) respectively, with entire concentration range shown in Table 1.

![Figure 5](image.png)

*Figure 5. In vitro* SKOV3 cell growth inhibition upon carboplatin exposure. Columns represent mean (n=3), bars represent SEM. Within each time point: #, different from 50 µmol/L (P < 0.05); ‡, different from 100 µmol/L (P < 0.05); §, different from 300 µmol/L (P < 0.05). * indicates significant differences between exposure times, within each concentration (P < 0.05).
### Table 1. Effect of exposure time and concentration on the cytotoxicity of carboplatin in SKOV3 cells as assessed using the MTT assay; (A) 24 hours, (B) 48 hours and (C) 72 hours of carboplatin exposure. Viability represents the mean of (n=3), error measured as SEM.
Figure 6. Dose response curve of cell growth inhibition of SKOV3 cell after 72 hours of carboplatin exposure.
4.3 *In Vitro* Proliferation upon Continuous and Intermittent Carboplatin Exposure

The impact of carboplatin exposure times on SKOV3 cell proliferation was examined using clonogenic assays. The previously determined 72-hour carboplatin IC₅₀ concentration of 81 µg/ml was used to treat the continuous and intermittent treatment for a period of 3 days; the total cumulative dose for both treatment groups was 72.9 µg per well. During the first 24 hours of the study, cells in the continuous treatment group lost all clonogenic potential resulting absence of colony formation (Fig. 7A). In contrast, cells treated intermittently showed an increase in the number of clonogenic cells 24 hours after each treatment cycle (Fig. 7A). The percentage of clonogenic SKOV3 cells upon continuous treatment remained 0% for the entirety of the experiment. These results further showed the potential for continuous carboplatin treatment, which was then assessed *in vivo*.

Proliferation of SKOV3 cells has also been explored using AUC based dosing of carboplatin. The resulting clonogenic potential of the continuous treatment group began losing clonogenic potential after the first 24 hours of treatment. Within the next 48 hours, all clonogenic potential in the continuous treatment group was lost (Fig. 7B).
Figure 7A. Effects of continuous and intermittent carboplatin exposure on SKOV3 cell proliferation, based on total dose of all treatments (total mass method). Points represent mean (n=3); bars represent SEM. Arrows indicate 12-hour carboplatin treatment for intermittent group. * indicates significant differences between groups of each day (P < 0.05).
Figure 7B. Effects of continuous and intermittent carboplatin exposure on SKOV3 cell proliferation, based on total exposure as integrated over time (AUC method). Points represent mean (n=3); bars represent SEM. Arrows indicate 12-hour carboplatin treatment for intermittent group. * indicates significant differences between each treatment group of each day (P < 0.05). Intermittent treatment results have been superimposed from the TDTM experiment for comparison, the experiment was not repeated.
4.4 Efficacy Comparison of Continuous and Intermittent Carboplatin

Suppression of tumor growth of the two dosing schedules was assessed in a murine model of human ovarian cancer. Female SCID mice inoculated IP with SKOV3 human ovarian adenocarcinoma cells were treated 7 days post-inoculation with continuous or intermittent carboplatin, or no treatment in the case of control animals (n=6/group). After 14 days of treatment, the average tumor weight was measured to be 0.869 ± 0.229 g, 1.354 ± 0.096 g and 2.013 ± 0.180 g for continuous, intermittent and control arms, respectively (Fig. 8). This translates into suppression of tumor growth by 57% and 32% due to continuous and intermittent carboplatin, respectively, relative to control.
Figure 8. IP tumor burden in SCID mice after 14 days of continuous carboplatin, intermittent carboplatin or no treatment (control). Total carboplatin dose was 880 µg in each treatment arm. Treatment was initiated 7 days after IP SKOV3 cell inoculation. Columns represent mean (n=5-6), bars represent SEM. * indicates significant differences between groups (P < 0.05).
4.5 Assessment of Proliferation, Apoptosis and Angiogenesis

To determine the effects of each treatment regimen on cell death, tumor sections extracted from mice in each treatment arm were immunostained for cleaved Caspase-3 and TUNEL. Caspase-3 activity in tumors treated continuously was significantly greater than controls and intermittently treated tumors (Fig. 9 A, Fig. 10). Caspase-3 activity showed an increase of 112% compared to non-treated controls $p < 0.05$, while intermittent treatment had a much less pronounced increase of 10% compared to non-treated controls. Similarly, TUNEL staining in tumors from the continuous treatment group showed an increase in TUNEL activity of 289% compared to non-treated controls $p < 0.05$, which was significantly greater than the increase in the intermittent treatment group of 54% compared to non-treated controls (Fig. 9 B, Fig. 11). Tumor proliferation was assessed via Ki-67 immunostaining. Continuous treatment with carboplatin resulted in the lowest index of Ki-67, measured at 48% of control. Intermittent treatment resulted in higher activity of Ki-67, when compared to the continuous group. Activity of Ki-67 in the intermittent treatment was measured to be 73% of control. (Fig. 9 C, Fig. 12). Both continuous and intermittently treated tumors showed significantly lower levels of Ki-67 than the control treatment arm. Finally, the degree of angiogenesis in tumors was measured via CD-31 immunostaining. Levels of CD-31 were lower as a consequence of continuous chemotherapy when compared to intermittently treated tumors, while both treatments showed significantly lower CD-31 immunostaining than in control tumors (Fig 9 D, Fig. 13).
Figure 9. Changes in tumor cell death, proliferation, and angiogenesis after 14 days of continuous or intermittent carboplatin therapy, illustrated by indices of caspase-3 (A), TUNEL (B), Ki-67 (C), and CD-31 (D). Columns represent mean (n = 3-6), bars represent SEM. * indicates significant differences between groups (P < 0.05)
Figure 10. Immunohistochemical analysis of tumors. Representative images consistent with apoptosis as reported by (CASP3) on day 21 post-inoculation (x20 magnification, scale bar 20 µm) in three separate mice from each treatment group. Each section was taken from the center of each tumor.
Figure 11. Immunohistochemical analysis of tumors. Representative images consistent with necrosis and apoptosis as reported by (TUNEL) on day 21 post-inoculation (x20 magnification, scale bar 20 µm) in three separate mice from each treatment group. Each section was taken from the center of each tumor.
Figure 12. Immunohistochemical analysis of tumors. Representative images consistent with tumor proliferation as reported by (Ki67) on day 21 post-inoculation (x20 magnification, scale bar 20 µm) in three separate mice from each treatment group. Each section was taken from the center of each tumor.
Figure 13. Immunohistochemical analysis of tumors. Representative images consistent with tumor angiogenesis as reported by (CD-31) on day 21 post-inoculation (x20 magnification, scale bar 20 µm) in three different mice from each treatment group. Each section was taken from the center of each tumor.
**Figure 14.** Linear correlation of markers of apoptosis illustrated between indices of Caspase-3 and TUNEL.
5 Discussion

5.1 Current Treatment Status and Alternatives

Past and current treatments of ovarian cancer have largely involved debulking surgery followed by intermittent chemotherapy with various cytotoxic agents (Omura, Blessing et al. 1986; Stewart, Aabo et al. 1991; Piccart, Bertelsen et al. 2000). Thus far, these treatments have mostly been unsuccessful, with patients eventually succumbing to the disease (Guarneri, Piacentini et al.). The introduction of IP chemotherapy may be viewed as a progressive step forward. Stage III clinical trials have shown IP administration of cisplatin to produce significant survival advantages over intravenous administration (Alberts, Liu et al. 1996; Armstrong, Bundy et al. 2006). However, these and other clinical studies have focused on the utilization of cisplatin as the platinum drug of choice. Cisplatin is an effective agent in the treatment of ovarian cancer, yet its dose limiting toxicities have proved problematic, and it is plausible that toxicities associated with IP chemotherapy have been in fact due to the choice of drug (Markman, Bundy et al. 2001; Armstrong, Bundy et al. 2006). An alternative platinum agent, carboplatin, yields similar efficacy with less toxicity. As a result carboplatin has replaced cisplatin in the IV chemotherapy setting (Ozols, Bundy et al. 2003). The observed differences between the two platinums are attributed to their chemical stability, or lack of. Carboplatin is reported to have greater degree of chemical stability. Wenclawiak et al. report 86% degradation of cisplatin in 8 hours, in aqueous solution at 37° Celsius. In similar conditions, carboplatin remained stable for days (Wenclawiak and Wollmann 1996). Based on the greater stability and the more favorable toxicity profile we have focused our studies on carboplatin.
5.2 Treatment Regimens

Another overlooked issue in current treatment efforts is that of treatment-free periods. These intervals that exist in between therapeutic cycles may be one of the main contributors to treatment failures, by way of promoting tumor growth acceleration and drug resistance development (De Souza, Zahedi et al. 2011). Metronomic treatment with cytotoxic agents has shown to produce better efficacy as a result of increased frequency of drug administration (Brade and Tannock 2006; De Souza, Zahedi et al. 2010). Previous animal studies have shown the benefits of a continuous chemotherapy approach using taxane drugs, which are cell-cycle specific agents (Vassileva, Moriyama et al. 2008; Zahedi, De Souza et al. 2009; De Souza, Zahedi et al. 2010; De Souza, Zahedi et al. 2011). Platinum agents, although not cell cycle specific (El-Kareh, Labes et al. 2008), can still potentially benefit from prolonged exposure. Dose dense therapy in combination of carboplatin and paclitaxel is reported to be well tolerated and beneficial in platinum resistant disease (Cadron, Leunen et al. 2007; Sharma, Graham et al. 2009; Baird, Tan et al. 2010). In the present study, we demonstrate that elimination of drug-free periods with carboplatin leads to a reduction of tumor proliferation and increase in efficacy when compared to intermittent therapy.

5.3 Continuous Drug Delivery and the Use of the Alzet Pump

Catheter use in intraperitoneal chemotherapy is seen as the main cause of failure of IP chemotherapy. Estimates show that only 50 – 60% of patients are able to complete the entire course of IP therapy (Walker, Armstrong et al. 2006). Some of the reported complications associated with catheter use are bowel complications, infection, leaking, blockage and general access problems (Fujiwara, Markman et al. 2005).
The use of injectable and implantable biomaterials is seen as a potential alternative for IP chemotherapy. One such system is the PoLi_{gel} (Zahedi, De Souza et al. 2009). Studies performed in murine animal models have shown this system to deliver continuous dose of docetaxel over a period of 2 weeks (Zahedi, De Souza et al. 2009). Additionally the PoLi_{gel} was shown to be both biodegradable and biocompatible (De Souza, Zahedi et al. 2009). However, the current formulation is not appropriate for the platinum agents due to their hydrophilicity. Therefore a modified formulation has yet to be developed for platinum agents.

In the present, there have been no reports of prolonged polymeric drug delivery systems for carboplatin. As a consequence, the Alzet pump delivery was a promising alternative. In vitro release of carboplatin-loaded Alzet pumps showed a sustained release profile of carboplatin over a period of 14 days. Further, all animals with implanted Alzet pumps did not show any signs of discomfort or distress. Some of the disadvantages of the Alzet pump include the invasive surgical procedure involved with peritoneal implantation, limited drug reservoir volume and its restriction of use in humans. Nevertheless the Alzet pumps served as a reliable continuous delivery system for carboplatin over the duration of the study.

5.4 Efficacy Comparison of Continuous and Intermittent Therapy

The suppression of tumor growth with continuous and intermittent carboplatin was compared using a murine model of human ovarian cancer. SCID mice bearing IP SKOV3 tumors were treated with continuous or intermittent IP carboplatin for 14 days, such that animals in both groups received the same cumulative carboplatin dose of 48.9 mg/kg over the study period. Reports in literature suggest 60-100 mg/kg to be a maximum tolerable IP dose for mice. Limitations with the Alzet pump and available carboplatin formulation restricted the dose to 48.9 mg/kg. The
administered dose resulted in differences in efficacy between continuous and intermittent carboplatin treatment. When compared to non-treated controls, continuous carboplatin treatment resulted in a total tumor mass inhibition of 57%, which is significantly greater than the 33% inhibition that seen after intermittent therapy (Fig. 8).

It is likely for drug free periods which occur with intermittent carboplatin chemotherapy to result in a decreased and subtherapeutic concentration of carboplatin within days of dosing. Intracellular concentrations of platinum agents depend on influx and efflux transporters. Copper-transporting P-type adenosine triphosphatases (ATP7A) and ATP7B are transporters that reduce cellular concentrations of platinum agents. Over expression of ATP7B has been associated with platinum resistance. Studies have shown an increase in platinum free periods to lead to higher degree of platinum resistance (Markman, Rothman et al. 1991), the possible outcome of over expression of efflux transporters.

Additionally, drug free periods may play a direct role in tumor repopulation. In vitro studies in multicell spheroids have shown a net increase in tumor growth in between treatment cycles of combination of cisplatin and etoposide (Durand and Vanderbyl 1990). The cell population within a tumor mass in heterogeneous. Intermittent therapy may target more sensitive cells, as an outcome the subsequent tumor repopulation during drug free periods becomes increasing dominated by chemo resistance cells (Goldie and Coldman 1984; Sobrero and Bertino 1986), increasing in number with subsequent drug free periods, leading to a net increase of cells within the tumor. Additional mechanisms of platinum resistance observed during drug free periods of intermittent therapy may play further roles contributing to poor efficacy of intermittent therapy seen in the presented experiments.
To further investigate the effects of carboplatin dosing schedule on drug efficacy, the degree of cell death in tumors extracted from these same mice were measured. Immunostaining for cleaved caspase-3, a marker of apoptosis, showed a 112% increase in activity in tumors from the continuous treatment group, and a mere 10% increase in intermittently treated tumors as compared to non-treated control tumors. TUNEL labeling, also indicative of apoptotic cell death, showed continuous exposure to lead to 289% greater labeling than seen in non-treated control tumors, whereas intermittent treatment lead to only a 54% increase. The TUNEL assay is thought to be highly sensitive for apoptosis. However reports have shown the TUNEL technique to lead to labeling of viable cells (Kockx, Muhring et al. 1998), necrotic cells and (Levin, Bucci et al. 1999) and cells undergoing autolysis. Caspase-3 and TUNEL assays are two independent markers of apoptosis. Staining of Caspase-3 reflects the presence of a cytosolic protein involved in a proteolytic cascade leading up to apoptosis (Cryns and Yuan 1998). Whereas the TUNEL assay involves the labeling of fragmented strands of DNA resulting from apoptosis (Duan, Gamer et al. 2003). Nonetheless the two methods for detection of cell death through apoptosis showed an excellent correlation r = 0.998 (Fig. 14) reinforcing the results obtained in the different treatment arms.

5.5 Cell and Tumor Proliferation with Continuous and Intermittent Therapy

It has been shown that accelerated tumor cell repopulation occurs during treatment-free periods (Durand and Vanderbyl 1990; Vassileva, Allen et al. 2008). During cycles of chemotherapy with cyclophosphamide and 5-fluorouracil, layers of cancer cells proximal to blood vessels or tumor periphery are killed; however, treatment-free periods that follow each cycle allow for the activation of dormant cells, which proliferate at increasingly faster rates (Kim and Tannock 2005). Elimination of drug-free breaks allows the drug to continuously affect cells, theoretically reducing
tumor size. Previous work with paclitaxel and docetaxel showed an increase in treatment efficacy with continuous therapy by halting acceleration of cell proliferation seen in treatment free periods. In addition, continuous therapy with paclitaxel and docetaxel lead to greater tumor burden reduction as compared to intermittent therapy (Vassileva, Moriyama et al. 2008; De Souza, Zahedi et al. 2010).

Using clonogenic assays we demonstrated that continuous therapy with carboplatin completely inhibits proliferation of SKOV3 cells in a way not achievable with intermittent carboplatin. Continuous exposure halted in vitro cell clonogenicity within 24 hours. In contrast, intermittent carboplatin led to a decrease in proliferative potential immediately following drug exposure. Over the whole duration of the study, the number of clonogenic cells fell to a greater extent after each treatment in the intermittent exposure treatment arm. Over 9 days, cells exposed to intermittent treatment have accumulated DNA damage produced by carboplatin exposure, as a result, the difference in the number of clonogenic cells increased over time. This was observed at each exposure cycle, evidencing that intermittent carboplatin led to recovery of cell proliferation after each treatment, while continuous carboplatin eliminated clonogenicity within 24 hours of the first treatment cycle. Figure 7A demonstrates the loss of clonogenic cells after the first 24 hours of treatment with carboplatin. The rapid loss of clonogenic potential may lead some to believe that the cells in the continuous treatment arm have been killed within the first 24 hours of treatment, resulting to the abrupt loss of clonogenic potential. This, however, is not the case. Figure 6 shows cell viability to be close to 100% after a 24 hour exposure with carboplatin at 50 µg/ml; a dose that is greater than the dose given at 24 hour intervals to the continuous treatment group of 27 µg/ml in the clonogenic studies. Further, reduction of carboplatin dose of the continuous treatment group to 13.5 µg/ml lead to an eventual demise of clonogenicity, however this result was seen 72 hours after
continuous exposure of carboplatin. The result of viable yet non-cycling cells in the continuous treatment arms maybe a result of cell cycle arrest induced by carboplatin. Reports have shown cytotoxic agents to cause cell cycle arrest (Barlogie, Drewinko et al. 1976; Gohde, Meistrich et al. 1979). *In vitro* studies in squamous cell carcinoma of the head and neck have shown carboplatin to inhibit the cell cycle during the synthesis phase (S-phase) (Coleman, Stewart et al. 2002). A major characteristic of S–phase is the synthesis and replication of DNA. Carboplatin covalently binds to DNA, thereby halting further synthesis. On a nuclear level, carboplatin induced damage is repaired primarily by the nucleotide excision repair system (Reed 1998). Failure or inefficiency of this mechanism may lead to cell death through apoptosis. Based on the presented arguments, it is plausible that cells in the continuous treatment arm were dosed with low levels of carboplatin, as a result some of the cell remained viable yet non-cycling, resulting in viable cells lacking clonogenic potential.

To investigate this phenomenon *in vivo*, levels of Ki-67 in tumors extracted from mice following continuous and intermittent treatments with carboplatin were measured. Ki-67 is exclusively present in cells during mitosis and is, therefore, a marker of proliferation (Scholzen and Gerdes 2000). After a 14-day treatment period, continuously treated tumors exhibited 33% less Ki-67 immunostaining than intermittently treated tumors. Thus, *in vivo* continuous therapy with carboplatin is more effective at inhibiting tumor cell proliferation than intermittent carboplatin.

### 5.6 Continuous Therapy and Angiogenesis

The formation of new vasculature via angiogenesis is a process that plays a key role in tumor survival and proliferation (Kerbel 2006; Khosravi Shahi 2006). Studies have shown low-
dose, high-frequency chemotherapy to produce greater inhibition of angiogenesis than conventional chemotherapy (Hanahan, Bergers et al. 2000; Klement, Baruchel et al. 2000). Intermittent dosing of chemotherapy produces a similar response on endothelial cells as it does on cancerous cells. Immediately after treatment, proliferation of endothelial cells is inhibited; however, following the treatment-free period, endothelial cells re-enter proliferation at a rapid rate (Klement, Baruchel et al. 2000). Experiments with low-dose platinum agents administered more frequently have reportedly produced antiangiogenic effects (Shen, Wang et al.). The present study shows continuous IP carboplatin to produce an antiangiogenic effect. CD-31 labeling of tumors showed that continuous carboplatin therapy produces a greater inhibitive effect on angiogenesis than intermittent therapy (Fig. 9).

6 Conclusions and Future Directions

6.1 Conclusion

Overall, we have shown that a continuous carboplatin dosing schedule results in greater suppression of tumor growth in the xenograft model of SKOV3 human ovarian cancer. Continuous treatment was shown to lead to greater tumor cell death while inhibiting proliferation and angiogenesis to a significantly greater extent than intermittent carboplatin at the same cumulative dose. Clinical implementation of more frequent dosing schedules, where treatment-free periods are reduced or eliminated as in the case of continuous chemotherapy, seems to be a promising strategy that can potentially lead to better treatment outcomes associated with platinum chemotherapy.
6.2 Future Experiments

Based on the presented experiments, continuous intraperitoneal therapy seems to be a promising treatment approach for ovarian cancer. However, studies discussed in this thesis were based on carboplatin monotherapy. Studies have shown combination therapy to yields higher efficacy compared to monotherapy (Matsuo, Lin et al.). Hence, combination therapy with other cytotoxic agents by way of continuous administration may lead to higher efficacy. Potential candidate drugs to explore for continuous combination therapy with carboplatin are paclitaxel, gemcitabine and bevacizumab.

The current chemotherapeutic treatment standard for ovarian cancer involves combination therapy using carboplatin and paclitaxel. As previously discussed, the two agents produce their cytotoxic effects by different mechanisms of action. Additionally toxicity and drug resistance involved with the individual drugs is different, therefore the combination of the two agents produce a synergistic effect without causing excess toxicity. The ICON-4/AGO-OVAR 2.2 clinical trial has shown combination of carboplatin and paclitaxel to result in longer overall survival of 29 months, compared to 24 months observed in patients treated with carboplatin alone in patients with ovarian cancer. Combination of carboplatin and paclitaxel in continuous and localized IP therapy may lead to higher efficacy for patients with ovarian cancer.

Reports suggest that 70% of ovarian cancer patients relapse following primary treatment with cytoreductive surgery and standard chemotherapy with carboplatin and paclitaxel (Matsuo, Lin et al.; Matsuo, Bond et al. 2009). Platinum resistance is a common occurrence among the relapsed patients (Gore, Fryatt et al. 1990). Gemcitabine is a cytotoxic agent that produces its cytotoxic effect by disrupting DNA polymerization. In addition, gemcitabine inhibits ribonucleotide reductase; disrupting DNA repair mechanisms, making this agents especially useful due to the fact that resistant platinum cancer cells are seen to have an enhanced DNA repair mechanisms (Peters,
Van Moorsel et al. 2006). Therefore continuous combination therapy with carboplatin and gemcitabine has the potential to increase treatment efficacy of ovarian cancer.

Bevacizumab is a human monoclonal antibody. Bevacizumab functions by inhibiting the growth of new blood vessels, contributing to regression of newly formed vasculature and altering of vascular function and tumor blood flow of cytotoxic agents (Bachelder, Crago et al. 2001; Hicklin and Ellis 2005). Monotherapy with bevacizumab has shown response rates of approximately 20%, however when used in combination with other cytotoxics, response rates as high as 80% have been reported (Markman).
References


Gore, M. E., I. Fryatt, et al. (1990). "Treatment of relapsed carcinoma of the ovary with cisplatin or carboplatin following initial treatment with these compounds." Gynecologic Oncology 36(2): 207-211.


Markman, M. "Addition of bevacizumab to weekly paclitaxel significantly improves progression-free survival in heavily pretreated recurrent epithelial ovarian cancer." Gynecologic Oncology.


