BIODEGRADABLE PACLITAXEL-LOADED PLGA MICROSPHERES FOR REGIONAL TREATMENT OF PERITONEAL CANCERS

DISSERTATION

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the Degree of Doctor of Philosophy in the Graduate School of
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By

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*****

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ABSTRACT

For the last several decades, there has been interest in applying intraperitoneal (IP) chemotherapy to treat malignant peritoneal-confined tumors with the premise that IP therapy was capable of providing higher drug exposure to the tumor, which would not be achievable by systemic drug delivery. Clinical trials have demonstrated IP chemotherapy is not only safe, but can provide a pharmacokinetic advantage, improved objective response, and prolonged survival. However, the efficacy of this treatment has been primarily limited to microscopic residual disease (i.e. tumor diameter < 2 cm) presumably due to poor drug penetration into the tumor. For this reason, in addition to the high costs and potential complications associated with the use of indwelling peritoneal catheters, IP treatment has not gained wide acceptance, in spite of its demonstrated survival advantage. The overall goal of this study was to develop a method to improve IP chemotherapy by overcoming some of its limitations.

Our laboratory has shown that at apoptosis-inducing concentrations, paclitaxel can enhance tumor porosity and consequently drug penetration in solid tumors. Paclitaxel has shown significant activity against many types of human solid tumors. The FDA-approved formulation (Taxol®) uses Cremophor EL to solubilize
paclitaxel. Cremophor causes potential life-threatening hypersensitivity reactions and Cremophor micelles can sequester and reduce the free fraction of paclitaxel, thereby limiting drug uptake into tissues. Thus, we have developed paclitaxel-loaded PLGA microspheres that release the drug at specified rates to take advantage of the unique pharmacodynamic properties of paclitaxel, as well as eliminate Cremophor-associated complications.

We have primarily used three paclitaxel microsphere formulations that release paclitaxel at vastly different rates (e.g., ranging from <15% to >50% release over 24 hr under in vitro conditions) so that the fast-release microspheres would induce apoptosis, thereby enhancing the tumor penetration of the slow-release microspheres. The physical properties of the microspheres were designed to prolong drug residence in the peritoneal cavity. Hence, pharmacokinetic studies of IP administered paclitaxel microspheres showed consistently low levels of paclitaxel in plasma while maintaining elevated concentrations in the peritoneal cavity up to 168 hr. The spatial distribution of paclitaxel microspheres after IP administration demonstrated a passive targeting advantage with localization of the microspheres at metastatic IP tumor sites. In addition, paclitaxel microspheres demonstrated greater antitumor activity and longer survival time in mice bearing advanced ovarian or pancreatic tumors, compared to the Cremophor formulation. Dose fractionation by using two types of microspheres with different release rates resulted in greater drug delivery into solid tumors compared to the Cremophor formulation or individual
microsphere treatment. Penetration of the microspheres in tumors enhanced drug exposure and retention within the tumor over time. In summary, the paclitaxel-loaded PLGA microspheres may be useful for IP therapy of peritoneal cancers.
DEDICATION

Dedicated to my parents,
my brother Tab,
and finally my fiancée, Chihjane
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I wish to express my sincere thanks and gratitude to my thesis advisor, Dr. Jessie Au. Her love and enthusiasm for science has been truly inspiring. The high personal and scientific expectations she demands of herself and others have largely contributed to my overall scientific training and growth. Working with her has been an enriching experience from which I have gained many intangible skills. Her financial support has allowed me to pursue numerous interesting opportunities during my graduate studies.

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Abstracts


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CHAPTER 1

BACKGROUND INFORMATION

1.1 Introduction

For the last several decades, there has been interest in the application of intraperitoneal (IP) chemotherapy for the treatment of malignant peritoneal-confined tumors with the premise that IP therapy delivers higher drug exposure to the tumor than that achievable by systemic intravenous (IV) drug delivery (1, 2). Clinical trials have demonstrated improved objective response and prolonged survival using IP therapy in primarily low-bulk ovarian cancer patients. Two completed phase III trials conducted by the Gynecologic Oncology Group (GOG) show up to an 11% increase in complete response rate and a 6-8 month improvement in overall survival in ovarian cancer patients who were treated with similar IP cisplatin regimens (3, 4). An ongoing phase III GOG trial has shown a longer progression-free duration (24 vs 19 months) using IP cisplatin and Taxol® (5). Despite its demonstrated survival benefits, the limitations of IP therapy such as high costs, possibility of infection, and limited drug penetration has made the treatment less widely accepted by the medical community.
Therefore, the work presented in this dissertation has focused on improving IP therapy to make it more appealing and effective. The goals of this dissertation are to (a) investigate the drug disposition after IP administration, (b) develop an alternative formulation to improve drug disposition, (c) characterize this new formulation in vitro and in vivo, (d) evaluate the antitumor efficacy of the new formulation against peritoneal tumors, and (e) compare the new with the current Cremophor treatment in terms of antitumor activity, tumor penetration, ease of use, and safety.

### 1.2 Anatomy and Physiology of the Peritoneal Cavity

The abdominal cavity, bounded above by the diaphragm and below by the upper plane of the pelvis, is the largest hollow space of the body. The abdominal cavity is lined by the peritoneum, which is composed of two layers: a loose connective tissue layer and a layer composed of mesothelial cells. The peritoneum assists in supporting the abdominal organs as well as resisting infection and storing fat. This extensive membrane covers not only the inside wall of the cavity (parietal peritoneum) but also every organ or structure contained in it including the greater part of the digestive tract, the liver and pancreas, the spleen, and the kidneys (visceral peritoneum). The peritoneal cavity, which is the space between the visceral and parietal peritoneum is only a potential space. Normally, it contains a small amount of serous fluid that permits free movement of the abdominal viscera inside the peritoneal cavity. However, obstruction of lymphatic drainage sites by tumor cells can lead to diminished efflux of peritoneal fluid, causing formation of ascites (6, 7).
Clearance of solutes and fluids from the peritoneal cavity can occur through diffusion into the blood circulation or convection into lymphatic vessels. The parietal peritoneum is supplied by a number of arteries located in the abdominal wall and eventually drains into the vena cava. The arterial blood supply to the visceral peritoneum arises from the celiac and mesenteric arteries, but the veins draining the visceral peritoneum empty into the portal vein. The consequence of this vascular arrangement is that drugs or other solutes, which are absorbed primarily across the visceral peritoneum are subject to the first pass effect.

The distributed model has been used to describe the drug absorption where the solute diffuses through the tissue and transfers between the extracellular fluid and blood in a spatially distributed capillary network (1, 8). The model states that the drug absorption is governed by $D\sqrt{pa}$ where the diffusion coefficient ($D$) and capillary permeability ($p$) are dependent on the properties of the drug, but not the capillary surface area per unit tissue volume ($a$) (9). Hydrophilic drugs have small ($pa$) products and are considered diffusion rate-limited. Hydrophobic drugs are more permeable and are flow rate-limited.

Lymphatic drainage from the peritoneal cavity occurs primarily through stomatas, which are openings located in the subdiaphragmatic surface. The omentum, mesentery, and other abdominal viscera also have well-established lymphatic networks. The lymphatic system transports 70% of the peritoneal fluid and can remove excess fluid or microparticulates from the peritoneal cavity (10). Absorption
of macromolecules with MW > 20,000 is governed by lymph flow rate, but lymphatic transport does not play an important role for low molecular weight (MW<1000) solutes (11, 12).

1.3 Peritoneal Cancers

1.3.1 Overview of Ovarian Cancer

The ovaries are essential reproductive organs that are located at the base of the peritoneal cavities in women. Each of these grayish-pink organs is 6.4 cm³ in size and weighs about 2 to 3.5 grams (13). The ovaries have two primary functions: storage of the eggs until ovulation and secretion of the hormones estrogen and progesterone.

The American Cancer Society has estimated that 25,400 women will be diagnosed with ovarian cancer and 14,300 women will die from this disease in 2003 (14). It is one of the leading causes of cancer death because 75% of all women with ovarian cancer are not diagnosed until the disease has metastasized beyond the ovary (15). This is due to the fact that symptoms of early-stage ovarian cancer are often vague and can mimic other common medical problems, so the disease often goes undetected until it has progressed to a more advanced stage. Symptoms can include: nausea, abdominal swelling, pelvic pressure, back or leg pain, loss of appetite, and general abdominal discomfort problems such as gas, bloating, indigestion, or cramps.

Similar to other cancers, the survival rate is heavily influenced by the stage of the disease at the time of diagnosis. For the small percentage of women who are fortunate enough to have their cancer diagnosed before it has spread beyond the ovary,
the 5-year survival rate is 80%-95%. However, the majority of women have advanced, metastatic ovarian cancer in which the 5-year survival rate is only 15%-25% (15).

1.3.2 Staging and Treatment for Ovarian Cancer

The Federation Internationale de Gynecologic et d’Obstetrique (FIGO) and the American Joint Committee on Cancer (AJCC) have classified the progression of ovarian cancer into 4 stages and Table 1.1 lists the criteria for each stage.

Treatment for ovarian cancer is primarily determined by the stage of the disease. Age and general health are also taken into consideration. The main treatment options for ovarian cancer are surgery, chemotherapy, and radiation therapy. The extent of surgery performed depends on the disease progression as well as the woman’s health. Surgery may be sufficient for women with early stages of ovarian cancer where the cancer is localized. The surgery can include a hysterectomy (removal of the uterus), bilateral salpingectomy (removal of the fallopian tubes), bilateral oophorectomy (removal of both ovaries) and an omentectomy (removal of the omentum). For these advanced cases of ovarian cancer, the surgeon removes as much tumor as possible known as debulking, even though all of it cannot be removed. In debulking, surgeons try to leave behind tumors no more than 2 cm in diameter. Studies have shown that tumor debulking improves a patient's outlook for survival by reducing the amount of cancer still needed to be treated by chemotherapy or radiation therapy (16).
Radiation therapy may be used in a small number of patients by using high energy X-rays to kill cancer cells that may remain in the pelvic area after surgery. Radiation therapy is not used frequently for ovarian cancer treatment. If used, radiation therapy is used to treat localized disease (17, 18).

Chemotherapy is more frequently used to treat postsurgical residual disease. Systemic chemotherapy of paclitaxel and a platinum-containing compound such as cisplatin or carboplatin is considered the standard front-line treatment program for women with advanced ovarian cancer (19). These anticancer drugs are normally administered as an intravenous (IV) infusion for 3, 6, or 24 hr. The overall response was 72%-73% (19, 20). The progression-free survival and overall survival time was 15.5-22 months and 31-52 months, respectively.

1.3.3 Overview of Pancreatic Cancer

The pancreas is located between the stomach and the duodenum. Its length varies from 12.5 to 15 cm and its weight from 60 to 100 grams. The pancreas has both exocrine and endocrine functions, whereby it secretes digestive enzymes and hormones, respectively. Over 95% of the cells in the pancreas form exocrine glands and ducts, which carry the digestive enzymes to the common bile duct and eventually to the small intestine. A small percentage of the cells in the pancreas are endocrine cells. These cells are arranged in small clusters called islets (of Langerhans). The islets release two hormones, insulin and glucagon, that are important in controlling the amount of glucose in the blood.
The American Cancer Society has estimated that 30,700 people will be diagnosed with pancreatic cancer and 30,000 people will die from this disease in 2003, resulting in the fifth leading cause of cancer death in the United States (14). Similar to ovarian cancer patients, pancreatic cancer patients have a poor prognosis because the disease is often advanced and metastastic at the time of diagnosis. Only 10% of pancreatic cancer patients have the disease localized within the pancreas at the time they are diagnosed. Early stages are often asymptomatic or symptoms are similar to many common ailments (e.g. abdominal or back pain, weakness, loss of appetite, and weight loss), resulting in missed diagnoses.

The survival rate is heavily influenced by the stage of the disease at the time of diagnosis. For the small percentage of patients who are fortunate enough to have their cancer diagnosed before it has spread beyond the pancreas, the 5-year survival rate is 20%. For patients with advanced disease, the 5-year survival rate of all stages is less than 1% with most patients dying within 1 year (21, 22).

1.3.4 Staging and Treatment for Pancreatic Cancer

The AJCC have adopted a TNM classification system for pancreatic cancer, which describes the extent of the primary tumor (T), the spread to nearby lymph nodes (N), and the presence of metastasis (M). Table 1.2 summarizes the criteria for using this system of tumor classification. However, many doctors particularly surgeons prefer to classify pancreatic tumors simply as either resectable, locally advanced, or metastatic. In patients with respectable tumors, the surgeon is able to remove all visually observed tumor nodules. Patients with locally advanced
disease have too much cancer spread to tissues around the pancreas or into blood vessels to permit complete removal, although the cancer has not yet spread to distant organs. These tumors are considered unresectable. In patients with metastatic pancreatic cancer, the spread to distant organs has been identified.

Treatment for pancreatic cancer patients is primarily determined by the extent of the disease. If the tumor is resectable and localized within the pancreas, a total pancreatectomy (removal of the pancreas) or a pancreaticoduodenal resection known as the Whipple procedure are conducted. Surgical resection is not an option for most patients with advanced disease. For unresectable, locally advanced and metastatic tumors, the standard chemotherapy is gemcitabine administration (23, 24). Radiation therapy has been used often to manage advanced pancreatic cancer since it has palliative clinical benefits in reducing visceral pain and stabilizing the size of the primary tumor mass (25). However, advanced pancreatic cancer responds unfavorably to surgery, chemotherapy, or radiation therapy. The median survival time is 5 to 9 months, regardless of treatment (23, 26).

1.4 Paclitaxel

Paclitaxel is an anticancer drug isolated from the stem bark of *Taxus brevifolia* (Pacific yew). It consists of a taxane ring with a side chain at position C-13 that is essential for its anti-tumor activity (27). Paclitaxel has shown widespread activity against many types of cancer cells including ovarian and pancreatic cancer. The IC$_{50}$ of paclitaxel was 4-5 nM and 1-7.5 nM for ovarian and pancreatic carcinoma cell lines, respectively (28, 29). In clinical trials, paclitaxel has demonstrated
significant activity against many types of human tumors including ovarian, breast, colon, non-small cell lung, head and neck cancers (30-32). Paclitaxel has multiple pharmacological effects in human cancer cells, including polymerization and stabilization of microtubules, blockade of cells at the G2/M phase of the cell cycle, inhibition of DNA synthesis, and induction of apoptosis (33).

Paclitaxel possesses low solubility in water. Paclitaxel is currently formulated in Cremophor EL (polyoxyethylated castor oil derivative) and ethanol (w/v 1:1). It is then diluted in normal saline or 5% dextrose solution for administration. The amount of Cremophor co-administered with paclitaxel is much higher per dose than other agents using this vehicle (34). This has significant importance because of the problems that have been associated with Cremophor. The clinical use of this vehicle has been linked with severe life-threatening hypersensitivity reactions as well as leaching of IV infusion sets (35). This vehicle has also been linked to the nonlinear pharmacokinetics seen in paclitaxel (36). Also, previous studies in our lab have shown that Cremophor EL, at concentrations above the critical micellar concentration of 0.01% w/v, forms micelles which sequestered paclitaxel, thereby reducing the free fraction of the drug (37). This has not been a problem for systemic paclitaxel therapy because under this setting, paclitaxel is released after Cremophor is metabolized. This is not the case for regional delivery where Cremophor is not eliminated. With the many problems associated with this vehicle, steps should be taken to develop an alternative formulation that does not require the use of Cremophor for IP therapy.
1.5 Regional Drug Delivery

The purpose of regional drug delivery such as IP chemotherapy is to achieve high drug exposure in the tumor while minimizing drug toxicity to normal tissue. For effective IP therapy, high drug concentrations must be maintained in the peritoneal cavity while limiting absorption into the systemic circulation in order to provide a tumor targeting benefit. Regional chemotherapy has already been successfully used in intravesical treatment of superficial bladder cancer, topical treatment of skin cancer, IP treatment of advanced ovarian cancer, and intrathecal therapy for brain cancer (38-41).

1.6 Biodegradable Delivery Systems

Biodegradable polymers have been commonly used in the biomedical field as resorbable sutures, orthopedic fixation devices, and drug delivery systems (42, 43). These polymers are utilized because of their biocompatibility (i.e. the polymers and their degradation products do not induce inflammatory responses in blood or tissues). Biodegradable delivery systems do not need to be surgically removed; they can be metabolized and excreted by natural pathways. Since drug loading and release characteristics are highly dependent on the properties of the polymer, the biodegradable delivery systems can be customized for drug release spanning from hours to months, thus possessing the potential for reduced toxicity, improved efficacy and patient compliance.
1.7 Poly(lactide-co-glycolide) (PLGA)

PLGA composed of polylactic acid and polyglycolic acid has been extensively used in microparticulate drug delivery systems (44-46). In aqueous solutions, PLGA undergoes homogenous, bulk degradation in which random chain cleavages of ester bonds occur throughout the polymer matrix. PLGA hydrolyzes to yield lactic acid and glycolic acid monomers, which are normal byproducts of cellular metabolism. Drug release in microparticulate drug delivery systems is governed by polymer degradation, which are determined by the properties of the PLGA polymer. A low molecular weight PLGA polymer degrades faster than PLGA composed of high molecular weight polymer chains. PLGA degradation rate is inversely correlated to the crystallinity of the polymer since water does not penetrate as easily into the crystalline regions (47). Thus, a copolymer such as PLGA, which is amorphous, will degrade faster than either of its semicrystalline homopolymers. Factors such as microsphere size, shape, and surface morphology can also influence PLGA degradation (48). A high surface area to volume ratio increases water penetration and uptake, which enhances polymer degradation.

1.8 Overview of the dissertation

The remainder of this document is divided into six chapters. Each chapter begins with a brief introduction to state the purpose of the study. Applied methodologies are detailed in the materials and methods section. The major findings and their significance are summarized in the results and discussion sections. Tables
and figures are included in each chapter. The references, supplemental tables and figures, and raw data are appended at the end of this dissertation.

Chapter 2 focuses on the pharmacokinetics and tissue distribution of paclitaxel solubilized in Cremophor after IP administration. Using autoradiography, the spatial distribution of the drug indicated only significant regional exposure within the peritoneal cavity. However, the drug was rapidly cleared from the peritoneal cavity after IP administration with no significant amount of drug detected in the peritoneal lavage at 24 hr. This reduces drug exposure and diminishes the potential pharmacokinetic advantage gained by using regional drug delivery.

Chapter 3 discusses the preparation and in vitro characterization of biodegradable PLGA paclitaxel-loaded microspheres. This formulation was developed to prolong peritoneal drug retention, which was not achieved with the Cremophor formulation in the previous chapter. In vitro release studies demonstrated that a wide range of release rates could be achieved by adjusting various microsphere parameters such as microsphere size or molecular weight of the PLGA polymer.

Chapter 4 describes the pharmacokinetics and tissue distribution after IP administration of the paclitaxel microspheres developed in the previous chapter compared to the Cremophor formulation. The microspheres were able to provide a pharmacokinetic advantage over the Cremophor formulation in terms of peritoneal-to-plasma concentrations. The microspheres were observed to localize at peritoneal tumor metastatic sites, which could enhance antitumor activity.
Chapter 5 evaluates the antitumor efficacy of the Cremophor formulation and the paclitaxel microspheres against peritoneal tumors. Using death as an endpoint, the survival studies demonstrated that the paclitaxel microspheres were more successful in prolonging median survival time using equal and equi-toxic doses in tumor-bearing mice while reducing treatment frequency, compared to the Cremophor formulation. Dose fractionation by using two types of microspheres with different release rates demonstrated the most antitumor activity in both tumor models. However, individual microsphere treatments with different release rates showed different antitumor effects in different tumor models.

Chapter 6 investigates the pharmacodynamic effects of drug treatments used in the preceding chapter. Drug penetration into solid peritoneal tumors after IP administration was evaluated using the Cremophor formulation and paclitaxel microsphere and the results showed that paclitaxel-induced apoptosis through alteration of the tumor structure can enhance the penetration into solid tumors, but only if the drug is retained in the peritoneal cavity after apoptosis has occurred. Penetration of the microspheres into the tumor was observed, which enhanced drug exposure and retention in the tumor over time. As a result, the microspheres demonstrated greater drug penetration into solid tumors after IP administration,
compared to the Cremophor formulation. Dose fractionation by using two types of microspheres with different release rates resulted in greater drug delivery into solid tumors compared to individual microsphere treatment.

Chapter 7 summarizes the perspectives and contributions of this dissertation research and addresses possible investigations in the future.
| Stage I (24%) | Limited to the ovaries | 1A | Limited to one ovary; no ascites; no tumor on the surface of the ovary |
|              |                      | 1B | Limited to both ovaries; no ascites; no tumor on the surface of the ovaries |
|              |                      | 1C | Present on the surface of one or both ovaries; ascites present in peritoneal washings; |
| Stage II (6%) | Limited to the pelvis | 2A | Invasion of fallopian tubes or uterus; no ascites |
|              |                      | 2B | Invasion of bladder, the sigmoid colon, or the rectum; no ascites |
|              |                      | 2C | Combination of 1C with 2A/2B |
| Stage III (55%) | Spread to the abdominal cavity or lymph nodes | 3A | No tumors can be seen with naked eye, but histologically confirmed microscopic seeding of abdominal peritoneal surfaces; no spread to lymph nodes; |
|              |                      | 3B | Tumors < 2 cm seen in abdomen; no spread to the lymph nodes |
|              |                      | 3C | Tumors >2 cm seen in abdomen; spread to the lymph nodes; |
| Stage IV (15%) | Distant metastasis | 4 | Spread to the inside of liver and other organs |

**Table 1.1. Clinical staging for ovarian cancer.** The FIGO system classifies ovarian cancer into stages based on extent of metastatic spread. For each stage, the percentage of patients who are classified within that stage at the time of diagnosis is also listed.
<table>
<thead>
<tr>
<th>Primary tumor (T)</th>
<th>TX</th>
<th>Primary tumor not assessable</th>
</tr>
</thead>
<tbody>
<tr>
<td>(&lt; 20%)</td>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>Tumor limited to pancreas (&lt; 2 cm)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>Tumor limited to pancreas (&gt; 2 cm)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>Tumor extends directly into duodenum, bile duct, or peripancreatic tissues</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>Tumor extends directly into stomach, spleen, colon, or adjacent large vessels</td>
</tr>
<tr>
<td>Regional lymph nodes (N)</td>
<td>NX</td>
<td>Regional lymph nodes not assessable</td>
</tr>
<tr>
<td></td>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
<tr>
<td>Distant Metastasis (M)</td>
<td>MX</td>
<td>Distant metastasis not assessable</td>
</tr>
<tr>
<td></td>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

**Table 1.2. Clinical staging of pancreatic cancer.** The TNM system classifies pancreatic cancer based on the involvement of the primary tumor, lymph nodes, and advanced metastases. The majority of pancreatic cancer patients have advanced, unresectable tumors at the time of diagnosis.
2.1 Introduction

For the last several decades, there has been interest in the application of intraperitoneal (IP) chemotherapy for the treatment of malignant peritoneal-confined tumors with the premise that IP therapy delivers higher drug exposure to the tumor than that achievable by systemic intravenous (IV) drug delivery (1, 2). Multiple studies have shown that IP therapy is safe and can provide a pharmacokinetic advantage. The ratio of the area of drug concentration-time curve (AUC) after IP and IV administrations is 20 for cisplatin, 300 for 5-fluorouracil, 470 for doxorubicin, and 1000 for Taxol® (49-51). Clinical trials have demonstrated improved objective response and prolonged survival using IP therapy in low-bulk ovarian cancer patients. Two completed phase III trials conducted by the Gynecologic Oncology Group (GOG) show up to an 11% increase in complete response rate and a 6-8 month improvement
in overall survival in ovarian cancer patients who were treated with similar IP cisplatin regimens (3, 4). An ongoing phase III GOG trial has shown a longer progression-free duration (24 vs 19 months) using IP cisplatin and Taxol® (5).

Paclitaxel has been one of the most important anticancer drugs developed in the past two decades and has demonstrated significant antitumor activity against many types of human solid tumors (52, 53). Paclitaxel has multiple pharmacological effects in human cancer cells, including polymerization and stabilization of microtubules, blockade of cells at the G₂/M phase of the cell cycle, inhibition of DNA synthesis, and induction of apoptosis (54). The significant activity of paclitaxel against ovarian cancer has led to the evaluation of IP Taxol®, where paclitaxel is formulated in 50:50 Cremophor:ethanol. A completed Phase II trial using IP Taxol® showed an overall 30% complete response, but only one of the cured patients initially had macroscopic disease (tumor size > 0.5 cm) (55).

Preclinical pharmacokinetics and tissue distribution of paclitaxel formulated in Cremophor have been studied in mice after IP administration (56-58). However, the limitations of the analytical method precluded the characterization of the plasma concentration-time profiles during the early absorption phase and the later terminal phase (56). Consequently, the kinetics of paclitaxel absorption after IP administration has not been established. Drug concentrations of various tissues over time after IP administration of paclitaxel formulated in Cremophor have been
examined, although the peritoneal fluid concentration has never been analyzed in mice (57). Clinical studies have observed an elimination half-life (e.g. 73-140 hr) from the peritoneal fluid (51, 59).

The purpose of the present study is to obtain a better understanding of the peritoneal clearance of paclitaxel formulated in Cremophor after IP administration to develop methods for improving IP therapy. To accomplish this goal, it is imperative to examine the rate and extent of clearance of paclitaxel in Cremophor from the peritoneal cavity, not simply the systemic appearance of the drug. The time course of drug distribution into various tissues is also important. Whole-body autoradiography enables visualization of most organs available on the same section, thus allowing the comparisons of drug concentrations in various organs without disrupting the blood flow to those tissues.
2.2 Materials and methods

2.2.1 Chemicals and reagents

Paclitaxel was a gift from Bristol-Myers Squibb Co. (Princeton, NJ) or purchased from Handetech (Houston, TX). Cephalomannine was obtained from the National Cancer Institute (Bethesda, MD). Both compounds were stored at 4°C. 3\(^{-}\)\(^{3}\)H-Paclitaxel (specific activity, 10.6 Ci/mmol), which was obtained either through the National Cancer Institute (Bethesda, MD) or Moravek Biochemicals, Inc. (Brea, CA), was stored at -70°C. High performance liquid chromatographic (HPLC) analysis showed that paclitaxel, 3\(^{-}\)\(^{3}\)H-paclitaxel, and cephalomannine were >99% pure. Cremophor EL\textsuperscript{®} was purchased from Sigma Chemical Company (St. Louis, MO). HPLC solvents were purchased from Fisher Scientific Company (Fair Lawn, NJ). All chemicals and reagents were used as received.

2.2.2 Animal protocol

Female nu/nu balb/C mice (6-8 weeks old) were obtained from Charles River/NCI Laboratories. Upon arrival, the animals were housed in cages (5 mice/cage) at the animal facility regulated at 72 ± 4°F with automatic 12-hr light/dark cycles. Mice were allowed at least 3-5 days to acclimate to the new surroundings and had free access to sterilized rodent diet and water ad libitum.

2.2.3 Drug formulation and administration

As our overall goal is to determine the fate of IP paclitaxel as it is administered to patients, the present study used the same solvent as the commercial formulation. Paclitaxel was dissolved in Cremophor EL-ethanol (1:1, v/v) according
to the specifications of the FDA-approved formulation, Taxol®. The mixture was then diluted with sterile 0.9% (w/v) sodium chloride to a final concentration of 1 mg/ml. Dosing solutions were administered either IP or IV, between 8 am and 12 pm. Angiocatheters (18-gauge, 1.3 mm; Becton-Dickinson; Sandy, UT) were used to instill the fluid. The abdominal region was massaged briefly before collecting the peritoneal lavage samples.

At predetermined times, mice were sacrificed using isofluorane (Abbott Laboratories, North Chicago, IL). Plasma samples were obtained by immediate centrifugation (2000 g for 30 sec) of blood samples collected through the retro-orbital venous plexus. Peritoneal samples were obtained by rinsing the peritoneal cavity with two volumes of 2 mL physiologic saline. All samples, dosing solutions, and the remaining animal carcasses were stored at -70°C until analysis.

2.2.4 HPLC analysis

Plasma and peritoneal lavage samples were analyzed by using our previously reported column-switching HPLC assay (60). Briefly, samples (100-400 µl) were spiked with an internal standard, cephalomannine (10 µg/ml) and vortexed for 30 sec. Ethyl acetate (2-3x sample volume) was added. The mixtures were vortexed for 1 min, centrifuged (4000 g for 5 min), and the supernatant was collected. This procedure was repeated twice. The organic extracts were pooled and evaporated under constant air stream at room temperature. The residue was reconstituted in 200 µL of 37.5% acetonitrile in water, vortexed for 1-2 min, sonicated for 1 min,
centrifuged at 4000 g for 5 min, and 50-100 µL of the supernatant was injected into the HPLC system. Standard curves were established based on the area-response ratio of the drug to the internal standard, using at least five data points.

2.2.5 Pharmacokinetic data analysis

The plasma concentration-time data of paclitaxel were analyzed by WinNonlin v3.0 (Pharsight Corp., Mountain View, CA) using both compartmental and noncompartmental methods. For compartmental analysis of the IV data, equations for two- or three-compartment models with elimination from the central compartment were used to fit the data using the Gauss-Newton least-squares regression analysis algorithm to determine the best-fit parameters. The goodness of fit of the models to the experimental data was analyzed using Akaike Information Criterion (AIC) and Schwartz Criterion (SC). When comparing several models for a given set of data, the model associated with the smallest value of AIC or SC is regarded as giving the best fit out of that set of models (61). In the noncompartmental analysis of the IP data, AUC and area under moment curve (AUMC) were calculated using the trapezoid rule. The mean residence time (MRT) was calculated as AUMC/AUC (62). The bioavailability (F) was calculated using Equation 1, assuming minimal variation among all of the mice used in the study.

\[
F = \frac{Dose_{i.v.} \times AUC_{i.p.}}{AUC_{i.v.} \times Dose_{i.p.}} \times 100\%
\]  

(1)
Absorption kinetics from the peritoneal cavity were analyzed by two methods: deconvolution and a modified Loo-Riegelman method, which analyzes the data based on a three compartment model (63). The average absorption rate was determined by the slope of the amount-remaining-to-be-absorbed (ARA) vs. time profile.

2.2.6 Whole-body tape autoradiography

The time course of paclitaxel distribution into peritoneal and systemic tissues was studied by administering an IV or IP dose of radiolabeled drug in tracer quantity and calculating the tissue concentrations using whole body autoradiography. Briefly, mice were given a dose of paclitaxel formulated in Cremophor (10 mg/kg non-radiolabeled paclitaxel and 1 mCi/kg \(^3\)H-paclitaxel). At predetermined time points, mice were euthanized by CO\(_2\)-induced hypoxia. After sacrifice, the carcasses were immediately placed in hexane cooled with dry ice as previously described with minor modifications (64). The frozen carcasses were embedded in a carboxymethyl cellulose gel (Bondex International Inc., St. Louis, MO). The gel molds were again immersed in dry ice/hexane mixture at -70°C for 20 min and stored at –20°C until sectioning. Multiple coronal sections of 30-40 µm were obtained using a PMV 450 cryomicrotome (Stockholm, Sweden) and maintained at -25°C. Whole-animal sections were mounted on Scotch tape 800 (3M Co., St. Paul, MN). Multiple sections were taken from various planes, allowing for complete examination of all major organs. Sections were freeze-dried at -25°C for up to 3 days and gradually warmed to room temperature.
Photographs of the dried sections were taken using a 35 mm camera (Nikon N50) and Kodak Ektachrome 100 color film (Kodak Inc., Rochester, NY). Commercially available, pre-calibrated tritium microscale autoradiography standards (Amersham Corporation, Arlington Heights, IL) with levels of activity ranging from 109.4 to 0.10 µCi/g and tissue sections for quantitative autoradiography were placed against tritium-sensitive film (Hyperfilm tritium autoradiography film; Amersham, Arlington Heights, IL) and exposed for 7 days at 4°C.

2.27 Photographic development and image processing

The applied sections were removed from the autoradiography films for film development. The film was moved through a number of processing tanks in succession at room temperature. The film was placed in a developer (Kodak formula D-19) for 5 min before being moved into the stop tank (Kodak Indicator Stop Solution; 16 ml per liter) for 30 sec. Next, the film was placed into acid-hardening fixing bath (Kodak formula F-5) for 5 min and then washed using deionized water for 5 min. Finally, the film was placed in a rinsing tank (Kodak Photo-Flo 200 Solution; 5.5 mL per 1.10 liter) for 30 sec before hanging it to air-dry.

The autoradiographs were analyzed using computer-assisted video densitometry. A digitized image of the developed x-ray film was captured using a scanner (MicroTek ScanMaker V310). The image was analyzed using the Image J software (http://rsb.info.nih.gov/nih-image/download.html). The densitometry measurements of the pre-calibrated microscale tritium standards were used to generate
a standard curve using a least-squares regression of a second-order polynomial function. Tissue concentrations were obtained for each image and the average values were used.
2.3 Results

2.3.1 Plasma pharmacokinetics of intravenous and intraperitoneal paclitaxel formulated in Cremophor

Figure 2.1 shows the plasma concentration-time profiles after IV and IP administration of paclitaxel dissolved in Cremophor/ethanol and the pharmacokinetic parameters are shown in Table 2.1 and 2.2, respectively. The plasma concentrations of IV paclitaxel declined tri-exponentially with a terminal $t_{1/2}$ of 11 hr. Analysis using AIC and SC showed that the IV data was best described by a three compartment model rather than a two compartment model. The initial volume of distribution in the plasma compartment ($V_1$) of 176 ml/kg, presumably due to extensive binding of paclitaxel to plasma proteins (65). The volume of distribution at steady state exceeded the total body water volume of 0.6 ml/g by more than 3-fold, indicating extensive drug uptake and binding in the peripheral tissues (66). After IP administration, the plasma concentration rose rapidly and reached a peak concentration at 1.75 hr. Using Equation 1, the bioavailability of the IP dose was calculated to be 34%. These pharmacokinetic parameters of IV and IP of paclitaxel are consistent with the literature (56, 59).

2.3.2 Pharmacokinetics of intravenous and intraperitoneal paclitaxel formulated in Cremophor in the peritoneal cavity

Figure 2.2 displays the pharmacokinetic profile of paclitaxel recovered from the peritoneal lavages. After IV administration, no significant amount of paclitaxel was recovered at any time. After IP administration, paclitaxel
concentrations in the peritoneal cavity showed a rapid mono-exponential decline over time. By 8 hr, less than 4% of the administered dose remained in the peritoneal cavity and reached negligible levels within 12 hr. The disappearance rate was 0.378 hr\(^{-1}\) and the peritoneal MRT was calculated to be 2.48 hr.

### 2.3.3 Kinetics of the Absorption of the Intraperitoneal Dose

Figure 2.3 shows the absorption rate-time plots, as analyzed by the Loo-Riegelman and deconvolution methods, respectively. Both analyses show rapid absorption of paclitaxel from the peritoneal cavities in mice; 50% of the absorption was completed in 2 hr and greater than 80% in 6 hr. Linear regression analysis indicated an average absorption rate of 0.304 hr\(^{-1}\). The maximum cumulative dose fraction absorbed into the systemic circulation from the peritoneal cavity reached a plateau of 33%, in agreement with the bioavailability calculated using the plasma AUC data (see above).

### 2.3.4 Tissue Distribution of Paclitaxel Formulated in Cremophor

Figures 2.4 and 2.5 show representative whole body sections of animals obtained at early and late time points after IV and IP administration of \(^{3}\text{H}\)-paclitaxel, respectively. It is noted that the autoradiographs detected total radioactivity and did not distinguish the unchanged paclitaxel from its metabolites. Figure 6 compares changes in paclitaxel concentrations in tissues with time after IV and IP administration.
At 15 min after IV administration, most of the radioactivity was found in the liver and in parts of the gastrointestinal tract, whereas low levels of radioactivity were detected throughout the body. The kidney, spleen, heart and lung showed the highest intensity at 15 min, followed by a decline over time. Within 2 h, radioactivity in most organs and tissues had decreased to levels close to or equal to the background signal (2 µCi/g). Since the small intestine and large intestine are located on multiple planes throughout the abdominal cavity, several segments of the gastrointestinal tract can be seen in one section. The small intestine and the large intestine were differentiated by the color of the intestinal contents and location within the abdominal cavity. The small intestine showed peak activity at 1h whereas the large intestine showed peak activity at 2 hr. A surge in radioactivity in the large intestine coincided with a decrease of similar magnitude seen in the small intestine, indicating movement from the small intestine to the large intestine.

After IP administration, all of the radioactivity was confined to the peritoneal cavity at all times. Little to no radioactivity distinguishable from the background level was detected in the brain, kidneys, heart, lungs, or other systemic tissues. At 15 min, the radioactivity resided primarily in the space surrounding the visceral tissues. Most of the radioactivity was primarily found in the liver and small intestine primarily at early time points and concentrated in the large intestine at later time points. Peak concentrations in the liver and small intestine were obtained at 2 hr while the large intestine showed peak activity at 8 hr. The GI transit is much slower after IP administration compared to IV administration. This may indicate some local
toxicity observed in the gastrointestinal tract. Abdominal pain and other gastrointestinal toxicities such as constipation and bowel obstruction are often associated with IP therapy (51, 67, 68).

Table 2.3 compares the tissue paclitaxel concentrations between IP and IV administration. The ratios of $AUC_{0\rightarrow8\ hr}$ after IP and IV administrations were $>1$ for the tissues that are localized in the peritoneal cavity (i.e., stomach, intestines, kidney), and were $<1$ for systemic tissues (i.e., heart, lung, liver). The pharmacokinetic advantage observed in the peritoneal tissues primarily occurred at later time points (i.e. 2 hr, 8 hr), thereby contributing largely to the drug exposure (AUC) after IP administration. However, the overall AUC is only slightly enhanced after IP administration.
2.4 Discussion

Comparison of the tissue distribution data established that IP administration of paclitaxel formulated in Cremophor resulted in localization of the drug in the peritoneal cavity whereas IV administration resulted in much broader distribution into all tissues. After IV administration, systemic tissues that are highly perfused (e.g. lung, kidney) experienced high concentrations at early time points, but tissue concentrations rapidly declined over time (i.e. undetectable within 8 hr). The spatial distribution of the drug over time as observed in the autoradiographs support the reported elimination pathway of paclitaxel, in which the drug undergoes hepatic metabolism and biliary excretion into the gastrointestinal tract prior to fecal excretion (69). After IP administration, high drug concentrations were observed exclusively in the peritoneal cavity. However, most of the drug remained concentrated in the dosing solution, leading to low peritoneal tissue concentrations initially observed in the autoradiographs. Therefore, IP therapy can provide direct exposure to peritoneal tumors and limit systemic toxicity (1).

One of the main drawbacks to IP therapy is the limited drug penetration in tumors mathematically characterized by Dedrick and Flessner (70). Various preclinical studies utilizing different anticancer agents have shown a maximum drug penetration of only 1-2 mm from the tumor surface after ip administration (71-73). Consequently, in clinical trials, patients with the smallest volume of residual disease achieved the highest objective response rate (i.e. 80% complete response) (3). Therefore, IP therapy has been primarily used currently to treat low-bulk disease.
Our laboratory has demonstrated that high concentrations of paclitaxel can induce apoptosis and reduce tumor cell density, allowing subsequent drug exposure to penetrate further into tumors (74). The cytotoxic effect of paclitaxel is dependent on both drug concentration and exposure duration (75-77). Thus, it is necessary to sustain elevated concentrations of paclitaxel in the peritoneal cavity to have significant anti-tumor activity. However, the pharmacokinetic data from this study showed that the drug was rapidly absorbed from the peritoneal cavity with a peritoneal MRT of 2.48 hr. This was consistent with the estimated lag time observed in the liver and gastrointestinal tissue concentrations. Since most of the drug was absorbed within 12 hr (i.e. 5 t₁/₂), the 34% bioavailability suggests that much of the dose was absorbed directly into the portal (visceral) circulation and excreted either as parent drug or metabolites into the bile. The rapid absorption resulted in little to no difference in drug exposure (i.e AUC) to various peritoneal tissues after IP administration compared to that after IV administration.

In humans, Taxol® has a considerably longer peritoneal MRT of 40.7 hr, but it has been shown that peritoneal transport and clearance can be linearly scaled between species (9). However, even this prolonged exposure was still unable to yield significant antitumor activity in clinical settings (55). The Cremophor vehicle used to solubilize paclitaxel has been shown to increase peritoneal MRT by more than 5-fold (59). However, it is also known that Cremophor can form micelles, which can sequester the drug and reduces the free fraction of paclitaxel (37, 78). Therefore, it is
possible that though the Cremophor could enhance peritoneal drug retention, it may also be responsible for limiting tumor penetration and reducing the efficacy of the drug.

The results from this study show that IP administration of paclitaxel does confer a pharmacokinetic advantage over the standard IV route of administration. Unfortunately, the rapid clearance from the peritoneal cavity negates much of the potential benefit of this route of regional drug delivery. However since paclitaxel is limited to only the peritoneal cavity after intraperitoneal administration, the pharmacokinetic advantage could be enhanced if the drug could be retained longer in the peritoneal cavity. Increasing the drug retention could also enhance tumor penetration by drug diffusing into paclitaxel-induced apoptotic areas of the tumor. Since Cremophor micelles entrap paclitaxel, it would be desirable to develop a Cremophor-free formulation that would retain the drug in the peritoneal cavity for prolonged periods of time.
### Table 2.1. Pharmacokinetic parameters after iv bolus of paclitaxel formulated in Cremophor.

The administered dose was 10 mg/kg. At least 3 mice were used for each timepoint.

<table>
<thead>
<tr>
<th>AUC ((ug/ml)*hr)</th>
<th>AUMC ((ug/ml)*hr²)</th>
<th>MRT (hr)</th>
<th>Cl (ml/hr/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.9</td>
<td>52.1</td>
<td>3.1</td>
<td>590.8</td>
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</table>

<table>
<thead>
<tr>
<th>t_{1/2α} (hr)</th>
<th>t_{1/2β} (hr)</th>
<th>t_{1/2γ} (hr)</th>
<th>Vdss (ml/kg)</th>
<th>V₁ (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.6</td>
<td>11.0</td>
<td>1817.8</td>
<td>176.0</td>
</tr>
</tbody>
</table>
Table 2.2. Pharmacokinetic parameters after ip bolus of paclitaxel formulated in Cremophor. The administered dose was 10 mg/kg. At least 3 mice were used for each timepoint.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>IP/IV Concentration Ratio</th>
<th>IP/IV AUC Ratio</th>
<th>IP/IV C&lt;sub&gt;max&lt;/sub&gt; Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>1 hr</td>
<td>2 hr</td>
</tr>
<tr>
<td>Liver</td>
<td>0.08</td>
<td>0.42</td>
<td>1.16</td>
</tr>
<tr>
<td>Stomach</td>
<td>-</td>
<td>0.96</td>
<td>1.49</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>0.28</td>
<td>0.30</td>
<td>2.41</td>
</tr>
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<td>0.41</td>
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<td>0.89</td>
</tr>
<tr>
<td>Lung</td>
<td>0.26</td>
<td>0.42</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Table 2.3. Ratio of ip concentrations to iv concentrations were determined in various tissues. AUC was calculated by the trapezoid rule. Ratio of AUC and C<sub>max</sub> (ip/iv) was also determined.
Figure 2.1. Plasma concentration-time profiles after IP (circles) and IV (squares) administration of paclitaxel formulated in Cremophor (10 mg/kg). At least 3 mice were used for each timepoint. Mean ± S.D.
Figure 2.2. Concentration-time profiles of paclitaxel recovered from the peritoneal cavity after IP administration of dose of 10 mg/kg. No significant amount of paclitaxel was detected in the peritoneal cavity after IV administration. At least 3 mice were used for each timepoint. Mean ± S.D.
Figure 2.3. Loo-Riegelman plot: amount absorbed (circles) and amount remaining to be absorbed (ARA) (squares) over time. Loo-Riegelman method was modified to account for a three compartment model. Solid line represents regression analysis to fit data.

\[ y = 20.171e^{-0.3038x} \]

\[ R^2 = 0.9927 \]
Figure 2.4. Cumulative dose fraction absorbed over time determined by the deconvolution method.
Figure 2.5. Whole body autoradiographs of mice after IV administration of $^3$H paclitaxel. Representative sections were used to illustrate timecourse of Taxol absorption. Upper row are pictures of actual sections taken. Bottom row are the same sections overlaid with the autoradiogram to show the spatial relationship of the signal. Autoradiograms have been digitally tinted with red for easier visualization.
Figure 2.6. Whole body autoradiographs of mice after IP administration of $^3$H paclitaxel. Representative sections were used to illustrate timecourse of Taxol absorption. Upper row are pictures of actual sections taken. Bottom row are the same sections overlaid with the autoradiogram to show the spatial relationship of the signal. Autoradiograms have been digitally tinted with red for easier visualization.
Figure 2.7. Tissue concentration-time profiles determined by digital videodensitometry. IP and IV adminstration of a tracer dose of $^3$H-paclitaxel in conjunction with a cold 10 mg/kg paclitaxel dose. No radioactivity was detected in the brain following either adminstration route (limit of detection = 1-2 µg/g). IP: closed symbols; IV: open symbols. At least 3 mice were used for each timepoint. Mean ± S.D.
CHAPTER 3

PREPARATION AND CHARACTERIZATION OF PACLITAXEL-LOADED POLY-LACTIC-CO-GLYCOLIC ACID (PLGA) MICROSPHERES FOR INTRAPERITONEAL DELIVERY

3.1 Introduction

Paclitaxel has been one of the most important anticancer drugs developed in the past two decades and has demonstrated significant antitumor activity in clinical trials against many types of human solid tumors (52, 53). Paclitaxel has multiple pharmacological effects in human cancer cells, including polymerization and stabilization of microtubules, blockade of cells at the G2/M phase of the cell cycle, inhibition of DNA synthesis, and induction of apoptosis (27, 54).

Due to its significant activity against ovarian cancer, paclitaxel has been used for intraperitoneal (IP) chemotherapeutic treatment with the advantage that regional drug administration can deliver higher drug concentrations to the peritoneal tumors (51, 55). As discussed in the previous chapter, IP therapy has provided improved objective response and prolonged survival in ovarian cancer patients, although it has been less effective against large, bulky tumors (3, 4).
Due to its low aqueous solubility, paclitaxel is solubilized in Cremophor EL-ethanol (1:1 v/v, Taxol®) and diluted with sterile 0.9% (w/v) sodium chloride just prior to administration. However, there have been many problems associated with the use of the Cremophor vehicle. The clinical use of this vehicle has been linked with severe life-threatening hypersensitivity reactions as well as leaching of iv infusion sets (35, 59, 79). In addition, paclitaxel can be entrapped within Cremophor micelles at Cremophor concentrations above the critical micellar concentration of 0.01% w/v (37). During systemic administration, Cremophor can be metabolized by the liver, which releases paclitaxel from the micelles. However, during IP therapy, Cremophor is not metabolized and paclitaxel remains trapped in the Cremophor micelles. This results in a reduced free fraction of paclitaxel and limits the extent of drug penetration into peritoneal tumors. Hence, a Cremophor-free formulation is needed for IP administration.

Developing new formulations for paclitaxel has been historically difficult due to the low aqueous solubility of the drug. Alternative paclitaxel formulations have included cosolvents, emulsions, cyclodextrin complexes, liposomes, and microspheres (46, 80-84). Precipitation of paclitaxel may occur in cosolvent and emulsion systems upon dilution with infusion liquids. The cyclodextrins used to increase aqueous solubility of paclitaxel are toxic and ineffective. Liposomes may not be physically stable for long periods of time (e.g. drug recrystallization may occur during reconstitution after storage). Biodegradable polymeric microspheres are physically stable and are not known to produce toxicity. As a result, controlled release polymeric
microsphere formulations have gained wide acceptance and have even been successful in treating patients with advanced cases of cancer (85-87). The microspheres can improve treatment efficacy while reducing toxicity (88-90). The release rate profiles of drugs from microspheres are a function of the physicochemical properties of the polymer. Therefore, microspheres can be tailored to obtain the appropriate release kinetics by regulating variables such as the polymer molecular weight and composition (91). In addition, the size of the microspheres can be adjusted to retard the removal from the peritoneal cavity (92, 93). Results from Chapter 2 showed that IP Taxol® was rapidly cleared from the peritoneal cavity, thereby limiting the exposure and effectiveness of paclitaxel. The microspheres can be designed to maintain drug concentrations and prolong drug retention in the peritoneal cavity.

Poly(lactic-co-glycolic) acid (PLGA) is a biodegradable, biocompatible FDA-approved polymer. Because of its broad range of performance characteristics, it has commonly been used in the biomedical field as resorbable sutures, orthopedic fixation devices, and drug delivery systems (42, 43). The purpose of the present study was to develop paclitaxel microsphere formulations using PLGA as the carrier, characterize the formulations, and determine the suitability of the formulation for intraperitoneal drug delivery.
3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Paclitaxel was obtained from Hande Tech Co. (Houston, TX), and cephalomannine was obtained from the National Cancer Institute (Bethesda, MD). Both compounds were stored at 4°C. HPLC analysis showed that paclitaxel and cephalomannine were >99% pure. Polyvinyl alcohol (PVA) was purchased from Sigma Chemical Company (St. Louis, MO). HPLC solvents were purchased from Fisher Scientific Company (Fair Lawn, NJ). Poly(DL-lactide-co-glycolide) (PLGA) with intrinsic viscosities ranging from 0.18-0.92 dL/g and a copolymer ratio of 50:50 or 75:25 (lactic acid:glycolic acid, LA:GA) was obtained from Birmingham Polymer Inc. (Birmingham, AL). All chemicals and reagents were used as received.

3.2.2 HPLC Instrumentation

The HPLC system consisted of a SpectroFlow 400 solvent delivery system (Applied Biosystems, Foster City, CA), Waters 510 pump (Water Associates, Milford, MA), Waters 714 Autosampler, Hewlett-Packard 1040A HPLC detection system, an electrically actuated multifunction ten-pore HPLC valve (Valco Instruments Company, Houston, TX), and a model 680 automated gradient controller (Waters Associated, Milford, MA). The Hewlett-Packard ChemStation 3D integrating software was used to analyze chromatograms. The HPLC stationary phase consisted of a cleanup column (Nova-Pak C₈, 4 µm particle, 3.9 mm × 75 mm) from Waters (Ireland) and an analytical column (Bakerfield C₁₈, 5 µm particle, 4.6 mm × 250 mm) obtained from Mallinkrodt Baker (Phillipsburg, NJ). Samples were injected into the
cleanup column and eluted with a mobile phase consisting of 37.5% acetonitrile at a rate of 1.0 mL/min. The analytical mobile phase consisting of 49% acetonitrile was directed through the analytical column at a flow rate of 1.2 mL/min. The UV detector was set at 229 nm and its limit of sensitivity for paclitaxel was 10 ng per injection.

3.2.3 Preparation of Paclitaxel-Loaded PLGA Microspheres

The microspheres were fabricated with different stirring rates, different drug loadings, different lactide:glycolide ratios, and a wide range of molecular weights of PLGA. The drug loading was calculated as the (mass of the drug) divided by the (mass of the drug and polymer).

The microspheres were prepared by the solvent evaporation process as previously described with minor modifications (94). Briefly, paclitaxel was dissolved in a solution of PLGA in 5 mL of methylene chloride. The organic solution was emulsified in 20 mL of 1% PVA solution used to stabilize the emulsion and prevent microsphere fusion during the fabrication process. The resulting emulsion was rapidly mixed using either a homogenizer for 30 sec or a magnetic stir bar for 3 min. The mixture was then diluted with 0.1% PVA with a final volume of 500 ml, and stirred at 1000 rpm at room temperature and ambient pressure until the solvent evaporation was completed. The microspheres were collected by centrifugation, washed with cold, deionized water, and resuspended in 250 ml water. This procedure was repeated 3 times to remove the residual PVA surfactant. The resulting microspheres were then
frozen using liquid nitrogen and dried in a Freezone 4.5 lyophilizer (Labconco Corporation; Kansas City, MO). The dried microspheres were stored in the refrigerator at 4°C.

3.2.4 Entrapment Efficiency Determination

Paclitaxel-loaded microspheres (3 mg) were dissolved in 1 ml methylene chloride. The solution was mixed with the internal standard, cephalomannine (10 µg/100 µl), and vortexed vigorously for 1 min. A 100 µl aliquot was removed and evaporated under a nitrogen stream. The residue was reconstituted with 200 µl of mobile phase, vortexed, and centrifuged at 4000 g for 5 min. One hundred µl of the supernatant was used for HPLC analysis using our previously reported column-switching HPLC assay (60). The encapsulation efficiency is calculated as the (mass of encapsulated drug) divided by the (mass of the drug used in preparation).

3.2.5 Microsphere Size Determination

Paclitaxel microspheres were suspended in distilled water. The solution containing the microspheres were placed dropwise onto metal foil and allowed to air-dry. The dried microspheres were coated with gold-palladium for a total time of 80 sec under an argon atmosphere to ensure a uniform coating. The size and surface morphology of the microspheres were examined using a Phillips XL 30 scanning electron microscope. Digital images were recorded and analyzed using Optimas imaging software v. 6.5 (Media Cybernetics). At least 100 microspheres for each sample were studied.
3.2.6 Glass Transition Temperature (Tg) Determination

Thermograms of the polymer and the microspheres were obtained by using a computer-interfaced Perkin-Elmer differential scanning calorimeter (DSC), Model 7. Cold running water was connected to the DSC cell for controlled cooling to subambient temperatures. The temperature calibration was accomplished with the melting transition of indium standard. The samples (5-15 mg), which were stored in the refrigerator at 4°C prior to analysis, were sealed in aluminum pans. Thermal scanning was conducted at a heating rate of 5°C/min and under a nitrogen atmosphere.

3.2.7 In Vitro Release Study

In screw-capped tubes, paclitaxel microspheres (3 mg) were suspended in 3 ml of release media (PBS containing 0.1% w/v Tween 80), which facilitated microsphere suspension and drug solubility. The tubes were placed into an orbital shaker maintained at 37°C and shaken at 200 rpm. At specified time points, the tubes were centrifuged at 2000 rpm for 5 min and 2.5 ml of the supernatant (referred to as release study samples) were carefully removed of which 2.0 ml was stored at -25°C for later HPLC analysis. Then, the microspheres were redispersed in 2.5 ml of fresh, drug-free release media to maintain sink conditions during the release study and the tubes were placed back into the orbital shaker until the next sampling time.

Release study samples were analyzed for drug content using HPLC. The sample was mixed with the internal standard, cephalomannine (1 µg/100 µl), and extracted with 8 ml of ethyl acetate. The mixture was vortexed vigorously, centrifuged at 4000 g for 5 min, and the supernatant was collected. This procedure
was repeated twice. The organic extracts were pooled and evaporated under constant air stream at room temperature. The residue was reconstituted in 200 µL of 37.5% acetonitrile in water, vortexed for 1-2 min, sonicated for 1 min, centrifuged at 4000 g for 5 min, and 50-100 µl of the supernatant was injected into the HPLC system.

Mass balance, calculated as the sum of \(((\text{cumulative amount of drug released}) + (\text{amount of drug recovered from the microspheres})) \div (\text{initial amount of drug loaded into the microspheres})\) was determined at the end of the release study. After the final sampling timepoint, the remaining microspheres were dissolved using 2 ml of methylene chloride. The HPLC protocol for determining microsphere entrapment efficiency described in section 3.2.4 was used to determine the remaining drug content in the microspheres used in the release study.

### 3.2.8 Statistical Analysis

Pearson correlation coefficients were used to evaluate the extent of a relationship between two data sets. Coefficients of determination were calculated. Statistical differences among groups were analyzed using ANOVA. A p-value of 0.05 was considered statistically significant.
3.3 Results

3.3.1 Characterization of Paclitaxel-Loaded PLGA Microspheres

Table 3.1 summarizes the properties for various batches of microspheres. The average yield was 81.5 ± 6.3% (mean ± SD), and was inversely correlated with microsphere size, ranging from 89% yield for 3 µm microspheres to 79% yield for 38 µm microspheres ($r^2 = 0.38$, $p < 0.05$). The encapsulation efficiency was 82.4 ± 6.7% (mean ± SD), and was positively correlated with microsphere size, ranging from 69% efficiency for 3 µm microspheres to 94% efficiency for 38 µm microspheres ($r^2 = 0.57$, $p < 0.01$). There was no significant correlation between drug loading or PLGA molecular weight and yield or encapsulation efficiency.

There was a positive correlation between the glass transition temperature ($T_g$) of PLGA and the polymer molecular weight ($r^2 = 0.74$, $p < 0.01$). There were no significant differences between the $T_g$ of the unprocessed PLGA polymer and that of the PLGA microspheres.

SEM photographs of paclitaxel-loaded PLGA microspheres are shown in Figure 3.1. The microspheres were generally spherical in shape and smooth in surface morphology. No discrete drug particles (i.e. needle-like paclitaxel crystals) were observed on the microsphere surface or within microsphere cross-sections. No appreciable drug degradation ($< 0.1\%$) was detected under storage at 4°C for 3 months.
3.3.2 **In Vitro Release Profile**

Figures 3.2 - 3.5 show the cumulative release-time profiles of paclitaxel-loaded PLGA microspheres in release media. The release was characterized by an initial burst release (average of 17.1%/day), followed by a slower sustained release (average of 0.8%/day). A secondary burst release (average of 1.1%/day) was observed after 3 weeks, but it was not significantly greater than the drug release rate in the preceding 3 weeks.

The average mass balance was 87.3% for all of the microspheres. However, the mass balance was greater for the low molecular weight PLGA microspheres (average of 93.7% for 13.0 kDa vs. 81.8% for 45.1, 92.3, 139.3 kDa, p < 0.07) and was significantly greater for larger size microspheres, irrespective of PLGA molecular weight (average of 94.6% for 10 and 30 µm vs. 82.6% for 3 µm, p < 0.04).

**3.3.2.1 Effect of PLGA molecular weight on paclitaxel release from PLGA microspheres**

Release profiles of paclitaxel from PLGA microspheres prepared using PLGA of different molecular weights are shown in Figure 3.2. The extent of drug release evaluated over 28 days was greatest for microspheres prepared with the lowest molecular weight PLGA (70.5% for 13.0 kDa vs. 34.7%-44.4% for 45.1, 92.3, and 139.3 kDa PLGA, p < 0.001); these microspheres also showed the highest initial burst release compared to higher molecular weight PLGA microspheres (48.1% for 13.0 kDa vs. 7.6-10.2% for 45.1, 92.3, and 139.3 kDa PLGA, p < 0.001).
3.3.2.2 Effect of LA:GA molar ratio on paclitaxel release from PLGA microspheres

Release profiles of paclitaxel from PLGA microspheres prepared using different molar ratios of lactic acid (LA) and glycolic acid (GA) are shown in Figure 3.3. Given similar polymer molecular weight and microsphere size, microspheres prepared with 50:50 PLGA released significantly more paclitaxel than microspheres prepared with 75:25 PLGA; the extent of drug release over 28 days was 34.6 and 12.1% for LA:GA molar ratio of 50:50 and 75:25, respectively (p < 0.01).

3.3.2.3 Effect of microsphere size on paclitaxel release from PLGA microspheres

Release profiles of paclitaxel from PLGA microspheres with different microsphere sizes are shown in Figure 3.4. The extent of release over 28 days was evaluated in microspheres composed of 13.0 and 45.1 kDa PLGA. At the average size of 3, 10, and 30 µm, the cumulative release over 28 days was 70.5, 55.5, and 44.0% for 13.0 kDa PLGA microspheres and 36.4, 36.4, and 24.3% for 45.1 kDa PLGA microspheres, respectively. The data suggests a trend of an inverse relationship between microsphere size and cumulative release, but the correlation was not significant (r²=0.80, p = 0.29 for 13.0 kDa; r²=0.94, p = 0.16 for 45.1 kDa).

3.3.2.4 Effect of drug loading on paclitaxel release from PLGA microspheres

Release profiles of paclitaxel from PLGA microspheres prepared using different drug loadings are shown in Figure 3.5. The extent of release over 28 days was evaluated in microspheres composed of 13.0 and 45.1 kDa PLGA. At the
average loading of 2, 5, and 10%, the cumulative fraction released after 28 days was 56.9, 70.5, and 61.6% for 13.0 kDa PLGA microspheres and 50.3, 36.0, and 43.3% for 45.1 kDa PLGA microspheres, respectively, indicating no relationship between drug loading and cumulative release ($r^2=0.13$, $p = 0.77$ for 13.0 kDa; $r^2=0.04$, $p = 0.87$ for 45.1 kDa).
3.4 Discussion

This chapter describes the preparation of paclitaxel-loaded PLGA microspheres. The encapsulation efficiency was high, averaging about 82%. This may be a result of the retention of paclitaxel in the organic phase, due to its high oil/water partition coefficient of > 99, and thereby became encapsulated as the microspheres solidify (95). The encapsulation efficiency represents (fraction of the total dose) minus (dose amount that is not entrapped or free drug). The free drug can leak into the aqueous media at the microsphere surface during preparation. Hence, the untrapped fraction is a function of the surface area normalized to the microsphere volume. Consequently, the larger microspheres, which have smaller surface area/volume ratio, showed higher encapsulation efficiencies.

The \( T_g \) of the microspheres increased with increasing PLGA molecular weight. This is consistent with findings in the literature (44, 91, 96). \( T_g \) is dependent on intra-polymer chain interactions, which increase with increasing polymer molecular weight. Also, the microsphere preparation was not found to affect the \( T_g \) of the polymer, thereby suggesting no significant drug-polymer interactions. The absence of discrete drug particles or crystals in the microsphere further suggests that the drug was incorporated into the polymer matrix as both paclitaxel and PLGA were readily soluble in methylene chloride.

An overall mass balance of 87.3% was observed. Drug degradation and formation of epi-taxol can account for an additional 5-10% of the mass balance (97). The mass balance was highest for the larger microspheres (i.e. 10 and 30 \( \mu \)m) with a
mass balance of 94.6%. In addition, microspheres (i.e. 3 µm, 13.0 kDa PLGA) that were observed to form aggregates in the release media, resulting in larger effective diameters, also showed a high mass balance of 90.5%. In contrast, the average mass balance for the smallest size microspheres prepared with high molecular weight PLGA (i.e. 3 µm and 45.1, 92.3, 139.3 kDa) showed a lower mass balance of 78.7, suggesting that mass balance could be affected by microsphere size. The reason of the effect of the microsphere size on mass balance is unclear.

Drug release from biodegradable microspheres, composed of a hydrophobic polymer matrix such as PLGA, occurs in three phases (98-101). The initial burst release from the microspheres occurs through dissolution of the drug present at or near the microsphere surface. The second slower, zero-order release is due to hydrolytic degradation of the polymer thereby extending aqueous diffusion further into the microsphere to solubilize deeply entrapped drug molecules (42). The final phase may show an additional burst in drug release due to breakdowns in the microsphere structural integrity. The observance of the final phase depends on the rate of drug depletion, which is governed by drug solubility, from the microspheres, relative to degradation rate of the polymer. The present study showed that the release of paclitaxel from PLGA microspheres followed this classic model of drug release. Furthermore, it was observed that the rate of drug release depended on polymer molecular weight, polymer composition, and microsphere size.

The microspheres with the lowest molecular weight PLGA showed the highest initial burst release compared to higher molecular weight PLGA microspheres.
Lower molecular weight polymer chains have more polymer chain flexibility and consequently, lower glass transition temperatures, which are dependent on the free volume of the PLGA polymer chains (102). Fewer polymer chain entanglements improve drug mobility. During microsphere preparation, drug can be deposited at the oil/water interface as the solvent evaporates. Enhanced drug mobility can increase surface drug deposition, resulting in a greater burst release (103). Also, more polymer chain mobility of low molecular weight PLGA enhances aqueous diffusion into the microsphere, thereby improving polymer permeability and degradation, which in turn increases drug release (98).

A greater cumulative drug release was observed for the microspheres prepared with the 50:50 PLGA copolymer as compared to the 75:25 copolymer. PLGA is a copolymer of two homopolymers: polylactic acid (LA) and polyglycolic acid (GA). Both polymers have crystalline structures. However, the crystallinity is decreased in the copolymer because the two polymer chains can not be packed as tightly as each individual polymer (104). Accordingly, the 75:25 PLGA has greater crystallinity compared to the 50:50 copolymer. Higher crystalline polymers have more tightly packed polymer chains, which reduces interchain volume, thereby restricting aqueous diffusion and limiting polymer degradation and drug release. In addition, PLGA polymers with higher LA compositions (e.g. 75:25 PLGA) are more hydrophobic because of the additional methyl group on the LA chemical structure. The higher hydrophobicity reduces the penetration by the aqueous media and degradation of the polymer (42).
The size of the microspheres also affected the initial burst release of paclitaxel from the microspheres. Smaller microspheres possess a higher surface area-to-volume ratio than larger microspheres. Increased surface area enhances polymer and paclitaxel exposure to the aqueous media, resulting in a larger initial burst and enhanced polymer degradation. Moreover, the smaller microspheres have shorter diffusion path lengths, thereby increasing the penetration by the aqueous media.

The release rate was found to be independent of drug loading. It has been reported that drug loading can affect microsphere release rate, but this applies to water-soluble drugs, where the release is rate-limited by the diffusion of the drug from the microspheres to the aqueous media (105-107). The void volume resulting from dissolution of drug molecules creates pathways for further aqueous penetration (108). Increasing the drug loading minimizes the distance between adjacent drug molecules, thereby reducing the aqueous pathway tortuosity. For drugs with low aqueous solubility like paclitaxel, the drug release is rate-limited by the solubility. Hence, increasing the drug loading did not enhance the drug release from the microspheres.

Other paclitaxel microsphere formulations using PLGA blended with other polymers and chemicals have been developed, but most have not been evaluated in vivo, even fewer against solid tumors (109-112). However, there are a few notable paclitaxel microsphere formulations that have been evaluated in clinical trials.

An albumin-stabilized paclitaxel nanoparticle formulation (ABI-007) has been developed for systemic use and has shown considerable activity in metastatic breast, head and neck, and rectal cancer in several Phase II clinical trials (113, 114).
Given its small size (< 200 nm), this formulation would not be suitable for IP therapy since it would likely be rapidly cleared from the peritoneal cavity based on observations from Chapter 2.

Another paclitaxel-loaded microsphere formulation (Paclimer®) uses a polyphosphoester polymer matrix for drug delivery. In preclinical studies, Paclimer® (50 µm microsphere diameter) retarded the growth of subcutaneous non-small cell lung tumors relative to Taxol®, but did not produce tumor regression (115). Further studies showed no significant survival advantage as compared to Taxol® in intraperitoneal H29 colon xenograft tumor model (53 days vs 40 days, p = 0.3) (116). Because of the slow release rate of Paclimer® (i.e. only 25% over two weeks), the formulation may not have provided significant concentrations for antitumor activity. Also, tumor penetration may have been very limited due to its large size. The clinical trial evaluation of Paclimer® was recently aborted.

IP delivery of microspheres should enhance peritoneal distribution and tumor penetration while reducing peritoneal clearance via capillary perfusion and lymphatic drainage. The present study describes the preparation and characterization of several microspheres with different properties and a wide range of release profiles. Additional studies to evaluate the pharmacokinetics and antitumor activity of the microspheres are discussed in the later chapters.
<table>
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<th>Batch</th>
<th>Int Visc (dl/g)</th>
<th>Mol Wt (kDa)</th>
<th>LA:GA</th>
<th>Drug loading (%)</th>
<th>Encap. Eff. (%)</th>
<th>Yield (%)</th>
<th>Initial Burst (%/d)</th>
<th>Actual Size (μm)</th>
<th>Tg (°C)</th>
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Table 3.1. Characterization parameters of paclitaxel-loaded PLGA microspheres.

a The molecular weight represents the weight-average molecular weight.
b The drug loading was calculated as the (mass of the drug) divided by the (mass of the drug and polymer).
c The encapsulation efficiency is calculated as the (mass of drug encapsulated) divided by the (mass of the drug used in preparation).
d The yield is calculated as the (mass of microspheres produced) divided by the (mass of the drug and polymer used in preparation).
e The initial burst was calculated as the release rate during the initial 24 hr.
Figure 3.1. SEM images of paclitaxel-loaded PLGA microspheres.
Panel A: Surface images illustrating microsphere shape and surface morphology (3, 10, and 30 µm microspheres at various magnifications)
Panel B: Cross-section image illustrating internal structure of microspheres
Figure 3.2. In Vitro Release Study of Paclitaxel Microspheres: Effect of PLGA Molecular Weight. Mean ± SD (n = 3)
Figure 3.3. In Vitro Release Study of Paclitaxel Microspheres: Effect of PLGA composition. Mean ± SD (n = 3)
Figure 3.4. In Vitro Release Study of Paclitaxel Microspheres: Effect of microsphere size. Top panel: 13.0 kDa PLGA microspheres. Bottom panel: 45.1 kDa PLGA microspheres. Mean ± SD (n = 3)
Figure 3.5. In Vitro Release Study of Paclitaxel Microspheres: Effect of drug loading. Top panel: 13.0 kDa PLGA microspheres. Bottom panel: 45.1 kDa PLGA microspheres. Mean ± SD (n = 3)
CHAPTER 4

PHARMACOKINETICS AND TISSUE DISTRIBUTION FOLLOWING INTRAPERITONEAL ADMINISTRATION OF PACLITAXEL-LOADED MICROSPHERES

4.1 Introduction

For the last several decades, there has been interest in the application of intraperitoneal (IP) chemotherapy for the treatment of malignant peritoneal-confined tumors under the premise that IP therapy delivers higher drug exposure to the tumor than that achievable by systemic intravenous (IV) drug delivery (1, 2). Clinical trials have demonstrated improved objective response and prolonged survival using IP therapy in low-bulk ovarian cancer patients (3, 4).

Paclitaxel with its demonstrated significant antitumor activity in clinical trials against many types of human solid tumors has been one of the most important anticancer drugs developed in the past two decades (52, 53). The significant activity of paclitaxel against ovarian cancer has led to the evaluation of IP Taxol®, where
paclitaxel is formulated in 50:50 Cremophor:ethanol. As discussed in Chapter 3, the use of Cremophor presents several difficulties including severe life-threatening hypersensitivity reactions (34, 37).

To circumvent the Cremophor-associated side effects, a number of paclitaxel microsphere formulations have been developed in the last 10 years and have shown some antitumor activity against solid tumors in animals and humans (46, 84, 102, 111-113). However, most of these formulations have not been evaluated as regional drug delivery devices for intraperitoneal (IP) therapy. One recently developed paclitaxel-loaded microsphere formulation (Paclimer®) had entered Phase I clinical trial for patients with advanced ovarian cancer. Paclimer® (50 µm microsphere diameter) used a polyphosphoester polymer matrix for drug delivery (115). Survival studies using IP H29 colon xenograft tumor model demonstrated a longer median survival time was achieved in the Paclimer® group (53 days), relative to the Taxol® group (40 days) (116). However, the survival advantage was not significant (p < 0.3). Because of slow release rate of Paclimer® (i.e. only 25% over two weeks), the formulation may not have provided sufficient concentrations for antitumor activity. Also, tumor penetration may have been very limited due to its large size. The clinical trial evaluation of Paclimer® was recently aborted.

Our laboratory has demonstrated that drug penetration into tumors can be enhanced by reducing high tumor cell density through apoptosis induction (76, 77). Based on our studies, it was concluded that drug-induced apoptosis resulted in more rapid drug diffusion in tumors by creating a larger fraction of interstitial space and/or a
decrease in tortuosity. Apoptosis and consequently, tumor penetration is dependent on both paclitaxel concentration and exposure duration (74, 97). Inability to provide concentrations sufficient to induce apoptosis or allow sufficient time (i.e. 16-24 hr) for apoptosis to occur resulted in diminished drug penetration in tumors. Therefore, it is necessary to utilize a formulation that can release high concentrations of paclitaxel at the outset yet continue to release drug for tumor penetration after apoptosis has occurred.

Results from Chapter 2 showed that paclitaxel formulated in Cremophor was rapidly cleared from the peritoneal cavity after IP administration so that negligible concentrations could be detected in the peritoneal fluid after 24 hr, thereby limiting the exposure and effectiveness of the Cremophor formulation. As discussed in Chapter 3, several different paclitaxel-loaded PLGA microsphere formulations with different properties and a wide range of release profiles were developed to extend the drug retention in the peritoneal cavity after regional drug delivery, thereby allowing subsequent drug penetration after apoptosis occurs. The purpose of this study was to characterize the pharmacokinetics and tissue distribution after intraperitoneal administration of the paclitaxel-loaded PLGA microspheres in comparison to the Cremophor formulation.
4.2 Materials and Methods

4.2.1 Chemicals and Reagents

All compounds and solvents used for preparation of paclitaxel-loaded PLGA microspheres are listed in Chapter 3. Rhodamine B and acridine orange were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals and reagents were used as received.

4.2.2 Preparation and Characterization of Paclitaxel-Loaded PLGA Microspheres

Paclitaxel microspheres were prepared by the solvent evaporation method as previously described in Chapter 3. The 3 µm microspheres prepared with 13.0 kDa 50:50 PLGA were selected for this study because the smaller microspheres resulted in a homogenous suspension in solution and are likely to provide a more uniform distribution in the peritoneal cavity as compared to larger microspheres, which tend to settle as a result of gravity. In addition, the greater initial burst release for the smaller microspheres would provide the high drug concentration required to induce apoptosis. Also, the tendency of these microspheres to aggregate because of the low molecular weight of the PLGA polymer and its associated low Tg (29°C), would reduce the microsphere clearance from the peritoneal cavity.

4.2.3 Animal Care

Female nu/nu balb/C mice 6-7 weeks old were obtained from Charles River/NCI Laboratories. Animals were treated in accordance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Upon
arrival, the animals were housed in cages (5 mice/cage) at the animal facility regulated at 72 ± 4°F with automatic 12-hr light/dark cycles. Mice were allowed at least 3-5 days to acclimate to the new surroundings prior to experimentation. Mice had free access to sterilized rodent diet (Teklad #7912; Harlan, Madison, WI) and water ad libitum.

4.2.4 Drug Formulation and Administration

Mice were given an IP injection of paclitaxel, dissolved either in Cremophor/ethanol as described for the FDA approved formulation, Taxol®, or formulated in PLGA microspheres. The amount of paclitaxel was the same for both formulations (10 mg/kg). For the Cremophor formulation, paclitaxel was dissolved in Cremophor EL-ethanol (1:1, v/v) and then diluted with sterile 0.9% (w/v) sodium chloride to a final concentration of 1 mg/ml. The paclitaxel microspheres were dispersed in PBS containing 0.01% (w/v) of Tween 80. Drug administration occurred between 8 am and 12 noon. The abdominal region was massaged briefly following dose administration to facilitate fluid distribution in the peritoneal cavity.

At predetermined times, mice were anesthetized using isofluorane (Abbott Laboratories, North Chicago, IL). Blood samples collected through the retro-orbital venous plexus. Plasma samples were obtained by immediate centrifugation (2000 g for 30 sec). Afterwards, two volumes of 2 mL of physiologic saline were instilled into the peritoneal cavity using an angiocatheter (18-gauge, 1.3 mm; Becton-Dickinson; Sandy, UT). The peritoneal lavage samples were collected and centrifuged at 750 g for 5 min and the resulting supernatant and the microsphere pellet were stored
separately. Abdominal tissues, including liver, small intestine, large intestine, omentum, and mesentery were surgically removed, quickly rinsed with water, and placed in screw-capped tubes. Fecal contents of the intestines were flushed out with 2 mL distilled water and stored in separate tubes. The duration was less than 5 min for recovering the peritoneal lavage and less than 10 min for tissue removal. All samples, dosing solutions, and the remaining animal carcasses were stored at -70°C until analysis.

4.2.5 HPLC Analysis

Plasma, peritoneal lavage, and tissue samples were analyzed by HPLC. Using at least 5 data points, standard curves were established based on the area-response ratio of the drug to the internal standard, cephalomannine. Plasma and peritoneal lavage (supernatant) samples were prepared for HPLC analysis as previously described in Chapter 2. Peritoneal lavage (pellet) samples were prepared for HPLC analysis as previously described in Chapter 3 for microsphere encapsulation efficiency determination.

Abdominal tissue samples were spiked with an internal standard, cephalomannine (10 µg/100 µl) and homogenized in 2 mL distilled water. The homogenizer was rinsed with an additional 2 mL of distilled water to collect residual tissue remaining on the homogenizer and pooled with the tissue homogenate. To eliminate sample cross-contamination, the homogenizer was further rinsed with a 50 mL of distilled water followed by 50 mL of acetonitrile between samples. Eight mL of ethyl acetate was added to the tissue homogenate for drug extraction. The mixture
was vortexed for 1 min, centrifuged (4000 g for 5 min), and the supernatant was collected. This procedure was repeated twice. The organic extracts were pooled and evaporated under constant air stream at room temperature. The residue was reconstituted with mobile phase (37.5% acetonitrile), vortexed and sonicated each for 1 min, centrifuged at 4000 g for 5 min, and 50-100 µL of the supernatant was injected into the HPLC system. The detection limit of paclitaxel was 10 ng/ml for plasma and peritoneal lavage, and 20-30 ng/g for tissue.

4.2.6 Pharmacokinetic Analysis

The pharmacokinetic data was analyzed with WinNonlin v3.0 (Pharsight Corp., Mountain View, CA) using noncompartmental methods; area-under-the-curve (AUC) was determined using the trapezoid rule. The significance of the differences was analyzed using Student’s t test. Differences were considered significant at p < 0.05.

4.2.7 Microsphere Distribution in the Peritoneal Cavity

Mice were administered IP with 10 mg/ml of fluorophore-loaded PLGA microspheres (3 µm diameter) dispersed in PBS containing 0.01% (w/v) of Tween 80. As controls, mice were administered blank vehicle (PBS with 0.01% Tween 80) and or a combination of rhodamine dissolved in vehicle and blank microspheres (3 µm diameter). The PLGA microspheres containing either rhodamine (red fluorescence) or acridine orange (yellow fluorescence) were prepared in the same manner as the paclitaxel microspheres as described in Chapter 3 with the exception that a fluorophore instead of paclitaxel was loaded in the microspheres. For comparison, the
distribution of commercially available fluorescently labeled latex beads (2.0 µm diameter; Sigma-Aldrich, St. Louis, MO) was also studied since the fluorescently-labeled latex beads do not degrade over time and consequently maintain constant fluorescent intensity.

At pre-determined times, mice were sacrificed. A sagittal midline incision was made to expose the tissues within the peritoneal cavity. An ultraviolet lamp (Ultra-Violet Products, Inc., San Gabriel, CA) operating at a wavelength of 254 nm was used to visualize the distribution of the fluorescent signal. Areas exhibiting strong fluorescence were noted. Photographs were taken using a Contax camera using high-speed film or using a digital camera at high exposure durations under various lighting conditions.
4.3 Results

4.3.1 Characterization of Paclitaxel-Loaded PLGA Microspheres

Paclitaxel-loaded PLGA microspheres prepared using the solvent evaporation method were characterized in vitro to obtain basic formulation parameters. The drug content, calculated as (mass of the drug) divided by (mass of the drug and polymer), was 4.0 ± 0.1% (mean ± SD, n = 3). The encapsulation efficiency, calculated as (drug mass in the produced microspheres) divided by (total drug mass used for the preparation), was 80.2 ± 2.2% (mean ± SD, n = 3).

The average size of the paclitaxel microspheres used in this pharmacokinetic study was 3.63 ± 1.69 µm (mean ± SD, n > 300). Over 95% of the microspheres fall within the range of 2 – 7 µm. SEM analysis showed a smooth microsphere surface, as described in Chapter 3.

4.3.2 Plasma and Peritoneal Cavity Pharmacokinetics

Table 4.1 summarizes the pharmacokinetic parameters after IP administration of paclitaxel formulated in Cremophor or PLGA microspheres. Figure 4.1 shows the plasma concentration-time profile after IP administration of both paclitaxel formulations. The maximum plasma concentration (C\text{max}) after administration of the microspheres was 80% lower compared to the Cremophor formulation (0.85 vs 0.17 µg/ml, p < 0.05). The cumulative plasma concentration over time (AUC) of the microspheres was 16% lower compared to the Cremophor formulation, indicating a lower extent of absorption. Plasma concentrations were undetectable after 168 hr for both formulations.
Figure 4.2 shows the concentration-time profiles of paclitaxel recovered from the peritoneal lavage. For the Cremophor formulation, peritoneal lavage concentrations declined rapidly at an initial rate of 0.53 hr\(^{-1}\), resulting in less than 0.1% of the dose recovered at 24 hr and undetectable concentrations at 168 hr. For the microspheres, the decline in drug concentration of the peritoneal lavage samples was slower (i.e. 0.17 hr\(^{-1}\)), resulting in significantly higher peritoneal concentrations after 6 hr (p < 0.05), and 3-4 fold higher peritoneal AUC, compared to the Cremophor formulation.

4.3.3 Tissue Distribution of Paclitaxel

Previous autoradiographic results described in Chapter 2 showed that after IP administration, paclitaxel was primarily confined to tissues in the peritoneal cavity with little to no drug detected in systemic tissues. Hence, the present study focused on the tissues in the peritoneal cavity. Figure 4.3 shows the paclitaxel concentration-time profiles in tissues after IP administration of the Cremophor and PLGA microsphere formulations. For the Cremophor formulation, peak concentrations were observed at 1 hr for all tissues except the large intestine, which showed a maximal concentration at 6 hr. After reaching maximum concentration, tissue concentrations rapidly declined at an average rate of 0.15 hr\(^{-1}\) and were not detectable by 168 hr.

For the microspheres, peak tissue concentrations were also observed at 1 hr for liver and small intestine and at 6 hr for the large intestine. The concentrations of these tissues subsequently declined at an average rate of 0.05 hr\(^{-1}\), which was 3-fold lower than the Cremophor formulation. The cumulative AUC in these tissues was 2-4
fold higher for the microspheres, compared to the Cremophor formulation. The tissue that showed the most differences was the connective tissue, comprised of the omentum and the mesentery. For the microspheres, the paclitaxel concentration increased over time with $C_{\text{max}}$ occurring at 24 hr, whereas for the Cremophor formulation, the peak concentration of 27.9 $\mu$g/g was observed at 1 hr and declined subsequently.

Microsphere aggregates were observed in 2 of 20 mice and found in the lower portions of the abdominal cavity near the large intestine and bladder after 12 hr post-administration; HPLC analysis indicates 5-10% of the total dose was contained in these aggregates.

4.3.4 In Vivo Distribution of Fluorescent Microspheres

The high drug concentrations in the omentum and mesentery observed for the microspheres, but not for the Cremophor formulation, suggested localization of the drug-entrapped microspheres. This was tested using fluorescent microspheres. The results are shown in Figure 4.4 - 4.7.

The control group treated with physiological saline showed a low level of auto-fluorescence in peritoneal tissues. In contrast, mice treated with fluorophores showed much higher fluorescence. For the mice given rhodamine dissolved in PBS (i.e. not entrapped in microspheres), a homogeneous signal of high fluorescent intensity was observed throughout the abdominal cavity 15 min after administration, which subsequently declined to a level not distinguishable from the background auto-fluorescence at 24 hr.
In contrast, for the mice treated with rhodamine-entrapped microspheres, high intensity of fluorescence was localized in the folds of the gastrointestinal tract and other tissues at 15 min and remained on the surface of the diaphragm, omentum and the mesentery at 24 and 96 hr. The fluorescent intensity was lower at 96 hr, compared to earlier timepoints. Fluorescence was occasionally detected in the lower abdominal region near the bladder and large intestine in 4 out of 24 mice.

Similar distribution patterns were observed with acridine orange-loaded microspheres and fluorescent latex beads with fluorescent aggregates found on the omentum, mesentery, and diaphragm at 24 hr (Figure 4.7). There was no visible change in the fluorescence intensity of the latex beads over time.
4.4 Discussion

Results from the present study demonstrate the advantages of the microsphere formulation. The plasma AUC after microsphere administration was lower compared to the Cremophor formulation, which suggests less systemic absorption from the microsphere formulation, and hence, a lower risk of systemic toxicity (117, 118).

After IP administration of both formulations, paclitaxel concentration in the peritoneal lavage declined in a biphasic manner. The fluid from the carrier vehicle was observed to be rapidly absorbed over the first 12 hr, which resulted in the initial decline in peritoneal lavage concentrations. This was consistent with findings in the literature (119, 120). However, the rate of decline in peritoneal lavage concentrations was >3-fold slower for the microspheres, resulting in >3-fold increase in AUC, compared to the Cremophor formulation. This suggests a lower clearance of the microspheres from the peritoneal cavity, compared to the Cremophor formulation.

Despite attempts to maintain a homogenous distribution through physical manipulation of the abdomen immediately after administration, there was some settling of the microspheres in 10%-15% of the mice as a result of gravity. Localization of the microsphere aggregates near the large intestine could contribute to the higher overall exposure to this tissue. The formation of these microsphere aggregates after the absorption of fluid from the carrier vehicle could contribute to higher tissue concentrations in the large intestine at 6 hr. Since the fecal contents of
the large intestine showed peak amounts of paclitaxel at 6 hr, the higher large intestinal concentrations at 6 hr could also be due to residual fecal matter after intestinal flushing.

The omentum and mesentery (connective) tissues showed an increase in paclitaxel concentrations over time after microsphere administration, indicating drug accumulation in these tissues. The measured paclitaxel concentration included both the entrapped and released drug. The localization of the fluorophore-loaded PLGA microspheres in the omentum, mesentery, and diaphragm suggests that the increase in connective tissue concentrations was due to microsphere (i.e. entrapped drug) accumulation. Since the fluorescently labeled latex beads, which are not degradable and maintain constant fluorescent intensity, also showed a similar distribution pattern, this confirms that the high tissue concentrations in the omentum and mesentery were due to microsphere accumulation. This distribution pattern is consistent with other findings reported in the literature (121, 122).

The distribution of the microspheres in the peritoneal cavity are influenced by gravity, diaphragmatic movements from respiration, and the peristaltic movements of the gastrointestinal tract (123). Movements of the microspheres diminish as the fluid from the carrier vehicle is gradually absorbed by the lymphatics. The omentum, diaphragm, and mesentery are fused double-layered membraed structures that span over various organs (124). These thin membranes are rich in lymphatic vessels and
represent major drainage sites of excess fluid and particulate matter in the peritoneal cavity. Therefore, the microspheres are likely to be more concentrated in these tissues.

Microsphere aggregates may result in regional toxicity since high amounts of paclitaxel are concentrated in a localized area, though it is unlikely since the majority of the drug is encapsulated, this spatial distribution may provide a possible passive targeting advantage. In IP animal tumor models, tumor nodules have been reported to develop with high (>80%) success rates at the omentum, mesentery, and intestinal surface (122, 125, 126). The microspheres were observed to aggregate in these locations where tumor nodules frequently develop.

The animal tumor models were established to represent advanced, metastatic disease observed in clinical cancer patients with peritoneal tumors. Abdominal peritoneum, mesenteric/pericolonic fat, lymph nodes, small and large intestine serosa, and the liver are common (>70%) metastatic sites for peritoneal tumors such as ovarian and pancreatic cancer in clinical patients (127). Ovarian and pancreatic tumors are both known to metastasize via peritoneal dissemination of cancer cells to other peritoneal surfaces (126, 128). It is possible that similar to the fate of the microspheres, peritoneal tumor cells circulate within the peritoneal cavity and accumulate in the lymphatic drainage sites, leading to tumor development and ascites formation.
In conclusion, the paclitaxel-loaded PLGA microspheres are a viable regional drug delivery alternative to Cremophor formulation for treatment of peritoneal tumors. The microspheres were observed to aggregate at common metastatic sites for peritoneal cancers. Though the released drug only represents a small fraction of the total dose, the drug would be constantly released within the vicinity of the tumor. The microspheres possessed a much longer mean residence time in the peritoneal cavity compared to the Cremophor formulation, allowing for the possibility of enhanced tumor penetration. The addition of a slow-releasing microsphere formulation to the current fast-releasing microsphere formulation could further enhance antitumor activity, where the fast-releasing microspheres would be responsible for inducing apoptosis and increasing tumor porosity while the slow-releasing microspheres would be responsible for furthering drug penetration into the tumor by sustaining drug exposure. Further investigation should be performed to optimize the release profile of the microsphere formulation and study the anti-tumor effect of the microspheres in the animal tumor models.
Table 4.1. Pharmacokinetic Parameters for Peritoneal Tissues Following Intraperitoneal Administration of the Cremophor formulation or Paclitaxel Microspheres.

AUC was calculated by the trapezoid rule.
Dose = 10 mg/kg paclitaxel equivalent (n = 3 or 4).

<table>
<thead>
<tr>
<th>Plasma</th>
<th>$C_{\text{max}}$ (ug/ml)</th>
<th>$t_{\text{max}}$ (hr)</th>
<th>AUC (ug/ml*hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>Tx</td>
<td>MS</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.9</td>
<td>1</td>
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<table>
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<tr>
<th>Peritoneal Lavage</th>
<th>$C_{\text{max}}$ (% of dose)</th>
<th>$t_{\text{max}}$ (hr)</th>
<th>AUC (% of dose*hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>Tx</td>
<td>MS</td>
</tr>
<tr>
<td></td>
<td>75.0</td>
<td>64.8</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peritoneal Tissue</th>
<th>$C_{\text{max}}$ (ug/g)</th>
<th>$t_{\text{max}}$ (hr)</th>
<th>AUC (ug/g*hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>MS</td>
<td>Tx</td>
<td>MS</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>21.4</td>
<td>1</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>3.0</td>
<td>6.3</td>
<td>1</td>
</tr>
<tr>
<td>Large Intestine</td>
<td>4.2</td>
<td>2.8</td>
<td>6</td>
</tr>
<tr>
<td>Connective Tissue</td>
<td>19.8</td>
<td>27.9</td>
<td>24</td>
</tr>
</tbody>
</table>
Figure 1. Plasma concentration-time profile following IP administration of Cremophor formulation (circles) or paclitaxel microspheres (triangles) at equivalent paclitaxel doses of 10 mg/kg. Plasma concentrations were not detectable at 96 hr with Cremophor formulation and 168 hr with microspheres. Mean ± S.D. (n = 3 or 4). * denotes p < 0.05.
Figure 2. Peritoneal lavage concentration-time profile following intraperitoneal administration of Cremophor formulation or paclitaxel microspheres at equivalent paclitaxel dose of 10 mg/kg. Peritoneal concentrations were not detectable at 168 hr with Cremophor formulation.
Mean ± S.D. (n = 3 or 4). * denotes p < 0.05.
Figure 3. Tissue concentration-time profiles following IP administration of Cremophor formulation (triangle) or paclitaxel microspheres (square) at equivalent paclitaxel dose (10 mg/kg). Tissue concentrations could not be detected at 168 hr with Cremophor formulation. Liver concentrations could not be detected at 168 hr after either formulation. Mean ± S.D. (n = 3 or 4). * denotes p < 0.05.
Figure 4.4. Spatial distribution after intraperitoneal administration of blank PBS containing 0.01% Tween 80. Images taken under normal and ultraviolet light.
Figure 4.5. Spatial distribution after intraperitoneal administration of rhodamine solution containing blank PLGA microspheres. Upper panel: 15 min; Lower panel: 24 hr. Images taken under normal and ultraviolet light.
Figure 4.6. Spatial distribution of rhodamine-entrapped PLGA microspheres after intraperitoneal administration. Left panel: 15 min; Middle panel: 24 hr; Right panel: 96 hr. Images taken under normal and ultraviolet light.
Figure 4.7. Localization of fluorescent microspheres 24 hr after intraperitoneal administration. Left panel: Acridine orange-loaded PLGA microspheres: omentum, diaphragm. Right panel: Fluorescent latex (polystyrene) beads: lower abdomen, diaphragm. Images taken under normal and ultraviolet light.
5.1 Introduction

In 2003, 25,400 and 37,000 people will be diagnosed with new cases of ovarian and pancreatic cancer, and 14,300 and 30,000 people will die from these respective diseases (1). Patients with early-stage diseases are often asymptomatic or present with vague symptoms (e.g. abdominal or back pain, weakness, loss of appetite, weight loss), resulting in missed diagnoses. At the time of diagnosis, < 25% of ovarian cancer patients and < 10% of pancreatic cancer patients have organ-confined diseases, resulting in poor prognosis. Overall 5-year survival rates of 15%-25% and < 1% for ovarian and pancreatic cancer, respectively (2-4).

As previously discussed, intraperitoneal (IP) chemotherapy for the treatment of malignant peritoneal-confined tumors such as ovarian and pancreatic cancer have been utilized in the past under the premise that IP therapy is capable of delivering higher drug concentrations to the tumor than that achievable by systemic intravenous (IV) drug delivery (5, 6).
Previous studies have shown that regional drug delivery can be safely administered and can provide a pharmacokinetic advantage (7-9). Furthermore, using IP therapy with drugs such as cisplatin, mitoxantrone, 5-fluorouracil, and paclitaxel, clinical trials have demonstrated improved objective response and prolonged survival, compared to conventional systemic delivery (10-12).

However, limitations of IP chemotherapy have prevented it from gaining widespread acceptance among the medical community. The antitumor efficacy of IP therapy has been primarily limited to microscopic residual disease (i.e. tumor diameter < 2 cm), most likely due to the inability to elevate drug concentrations in the tumor core, which is necessary to prevent tumor progression and regrowth (13). Higher doses can not be administered due to regional toxicity, primarily abdominal pain (7, 10, 14). Therefore, frequent IP treatments are necessary. Unfortunately, IP therapy is associated with considerable costs and requires patient hospitalization and indwelling catheter implantation. Peritoneal catheters have several complications including catheter malfunction or occlusion, bowel perforation or obstruction, and tissue infection (15). Therefore, it is necessary to improve upon several aspects of IP therapy to exploit its demonstrated survival advantages.

Because of its demonstrated significant antitumor activity against many types of human solid tumors, particularly ovarian cancer, IP delivery of paclitaxel has been evaluated for cancer treatment (16-18). Paclitaxel has multiple pharmacological effects in human cancer cells, including polymerization and stabilization of microtubules, blockade of cells at the G2/M phase of the cell cycle, inhibition of DNA
synthesis, and induction of apoptosis (19). The IC$_{50}$ of paclitaxel after 96 hr treatment was 4-5 nM and 4-7.5 nM for several ovarian and pancreatic carcinoma cell lines, respectively (20, 21).

Results from Chapter 2 showed that IP delivery of paclitaxel dissolved in the Cremophor vehicle, as specified under the current FDA-approved formulation (Taxol®), resulted in rapid clearance from the peritoneal cavity, which limited drug penetration into tumors and antitumor activity because the antitumor effects of paclitaxel are dependent on both paclitaxel concentration and exposure duration (22-24). Chapter 3 describes the preparation of paclitaxel-loaded PLGA microspheres with the purpose of increasing drug retention in the peritoneal cavity and eliminating the complications associated with Cremophor. Results in Chapter 4 showed that the paclitaxel microspheres provided a targeting advantage over the Cremophor formulation. Localization of the microspheres at metastatic sites of peritoneal tumors may enhance antitumor activity.

Several microsphere formulations have been developed and successful in treating patients with advanced cases of cancer. Lupron® is a 6-month poly(D,L-lactic acid) microsphere delivery system for a luteinizing hormone-releasing hormone analogue, leuprolide used for the treatment of prostate cancer, endometriosis, and central precocious puberty (25-27). Biodegradable 5-fluorouracil microspheres composed of 50:50 PLGA polymer has been used for the radiosensitization of glioblastoma. Significant concentrations of 5-fluorouracil were still present in the cerebrospinal fluid of patients at 1 month after implantation (28, 29). Cisplatin
microspheres composed of biodegradable poly(D,L-lactic acid) matrix have been used for IP therapy (30). The microspheres were developed to release cisplatin at a constant rate for 3 weeks. Studies have shown improved survival in tumor-bearing rats compared to cisplatin and has been safely administered to patients in clinical studies (31, 32).

The purpose of the present study was to determine whether IP delivery of paclitaxel microspheres could be more effective in treating peritoneal tumors than the Cremophor formulation. Efficacy was measured in terms of antitumor activity, safety, and convenience by evaluating the effects of the following parameters of drug delivery: (a) systemic vs. local delivery, (b) Cremophor vs. microsphere formulation given at equi-toxic doses, (c) Cremophor vs. microsphere formulation given at equal doses, (d) Cremophor vs. microsphere formulation given as a single dose, (e) Cremophor vs. microsphere formulation given as multiple doses, (f) microsphere size, and (g) drug release from microspheres. Two different tumor models (i.e. SKOV3 ovarian and Hs766T pancreatic tumors) were used to evaluate the effects of different tumor growth rates.
5.2 Materials and Methods

5.2.1 Chemicals and Reagents

HPLC-grade paclitaxel was obtained from Hande Tech Co. (Houston, Tx) and was stored at 4°C. Acetonitrile, ethanol, ethyl acetate, methylene chloride were of HPLC-grade and purchased from Fisher Scientific Company (Fair Lawn, NJ). Tween 80 and Cremophor EL® were purchased from Sigma Chemical Company (St. Louis, MO). Poly(DL-lactide-co-glycolide) (PLGA) was obtained from Birmingham Polymer Inc. (Birmingham, AL). All chemicals and reagents were used as received.

5.2.2 Paclitaxel Formulation Preparation

Paclitaxel was formulated in Cremophor EL-ethanol (1:1, v/v) and then diluted with sterile 0.9% (w/v) sodium chloride prior to administration. Paclitaxel microspheres were prepared using the solvent evaporation method and characterized as described in Chapter 3.

5.2.3 Animal Care

Female, athymic or nu/nu balb/C mice 5-7 weeks old were obtained from Charles River/NCI Laboratories (Wilmington, MD). The pancreatic tumor model used the athymic mice whereas the ovarian tumor model used the balb/C mice. Upon arrival, animals were housed in cages (5 mice/cage) at the animal facility regulated at 72 ± 4°F with automatic 12-hr light/dark cycles. Mice had free access to sterilized rodent diet (Teklad #7912; Harlan, Madison, WI) and water ad libitum. Animals were treated in accordance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.
5.2.4 Peritoneal Tumor Models in Mice

SKOV3 ovarian carcinoma cell line was obtained from ATCC (Manassas, VA) and maintained in McCoy’s culture media. Hs766T pancreatic cancer cell line was a gift from Dr. Byoungwoo Ryu (John Hopkins Medical Institute, Baltimore, MD) and maintained in Dulbecco's modified Eagle's media. Using these cell lines, only 30%-40% of the mice developed tumors following IP injections. Therefore, subclones were cultivated to increase the tumor establishment rate. This was accomplished by injecting parent SKOV3 and Hs766T tumor cells IP into immunodeficient mice (20 x 10^6 cells in a volume of 0.5 ml). After 3 weeks, the ascites fluid was collected and centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and discarded. The tumor cell pellet was redispersed in cell culture media and plated in a culture flask. As the cells reached confluency, the cells were injected IP into mice to begin another cycle of tumor development and subclone culture. This procedure was performed 2-3 times to develop metastatic subclones, such that IP injection of the subclones resulted in tumors in 100% of the mice. Mice were euthanized when they showed (a) > 30% initial body weight gain for the pancreatic tumor model or > 20% initial body weight loss for the ovarian tumor model and (b) palpable tumors, visible abdominal swelling, or loss of righting reflex.

5.2.5 Survival Study

Pilot studies were conducted to determine the time course of tumor establishment. Briefly, tumor cells were implanted in mice. At predetermined time points, mice were sacrificed and autopsied; the results showed establishment of
multiple tumor nodules in 100% of the mice after 4 weeks for the SKOV3 model and after 10 days for the Hs766T model. In the present study, drug treatment was initiated at 50% of the median survival time of the untreated, control mice; the median survival time was 8 weeks for SKOV3 ovarian tumors and 3 weeks for Hs766T pancreatic tumors. Multiple treatment regimens (described in Results) were evaluated. Control mice were given IP blank microspheres (i.e. no drug) dispersed in saline.

Toxicity was determined by post-treatment body weight loss. A body weight loss of 10% was considered to be acceptable and well-tolerated, a well-established standard (33, 34). Post-mortem autopsies were performed on mice to determine whether the cause of death was disease-related. Mice, which died within 10 days post-treatment and showed a body weight loss of > 15%, were considered toxicity-related deaths. Veterinary consultation confirmed that a constricted colon observed in these mice, reducing lumen diameter and thereby restricting gastrointestinal transit, was likely a result of drug toxicity. Mice, which died within 10 days post-treatment with internal hemorrhaging within the abdominal cavity, were considered deaths as a result of faulty injections. These mice, which did not die as a result of tumor burden, were censored. Mice, which died after 10 days post-treatment and presented with tumor nodules and/or tumor infiltration into organs, were considered deaths as a result of tumor burden.
Survival was used as the pharmacodynamic endpoint. Survival times and body weight measurements were recorded for at least 100 days after treatment. Three weeks or more after the last recorded disease-related death, the remaining mice were sacrificed and autopsies were conducted. Mice with no visible tumors were considered cured.

5.2.6 Statistical Analysis

Median survival time and increase in lifespan (ILS), calculated as the ratio of the median survival time of the experimental group to that of the control group, were determined for each treatment group. The use of ILS enabled the comparison of drug effects from different treatment groups. Control groups from different studies were pooled for statistical analysis unless there were significant differences between groups, in which case, individual control groups were used. The levels of significance in the differences in survival times were analyzed using the log-rank and Wilcoxon test using SAS (Cary, NC). Differences were considered significant at p < 0.05. Pearson correlation coefficients were used to evaluate the relationship between two data sets.
5.3 Results

5.3.1 Survival Curve of Treatment Groups for SKOV3 and Hs766T Tumor Model

Tables 5.1 and 5.2 show the treatment dose and schedule for the SKOV3 and Hs766T tumor model, respectively. The results from the survival studies are summarized in Table 5.3 and 5.4 for the SKOV3 and Hs766T tumor model, respectively. The control group showed insignificant weight loss (2.3% ± 1.7%, mean ± SD), indicating that the PLGA polymer used in microsphere preparation produced little to no toxicity. The body weight loss for the Cremophor treatment groups was 5.6% ± 1.9% (mean ± SD). The body weight loss for the microsphere treatment groups ranged from 0.8% for slow-release microsphere group to 16.6% for the combination microsphere group. For each drug-loaded microsphere treatment group, the relationship between body weight loss and \textit{in vitro} paclitaxel release from the microspheres in PBS was evaluated. Results showed a significant positive correlation between body weight loss and amount of paclitaxel released over the first 72 hr ($r^2 = 0.90$, $p < 0.0001$). A release of more than 40 mg/kg paclitaxel in 72 hr resulted in > 15% body weight loss and lethality.

5.3.2 Effect of Administration Route on Antitumor Activity

The first study compared the antitumor activity of IP and systemic (IV) therapies in the SKOV3 tumor model using a single formulation. Paclitaxel was dissolved in Cremophor and administered IV or IP (i.e. 15 mg/kg biweekly for 4 weeks). Figure 5.1 shows the Kaplan-Meier plot depicting the surviving fraction as a
function of time. The survival times of the IV and IP treatment groups were significantly longer, compared to the untreated control group (23% and 132% longer, respectively, \( p < 0.05 \) for both treatments). The ILS of the IP group (TX15x8ip) was 88% longer, compared to the IV group, but the improvement was not significant due to the limited sample size (\( p = 0.5 \)).

### 5.3.3 Effect of Paclitaxel Formulation on Antitumor Activity

The following study compared the antitumor activity of the microspheres and the Cremophor formulation at both equi-toxic doses and equal doses. The study was conducted in both SKOV3 and Hs766T tumor models.

The results for the study with equi-toxic doses are shown in Figure 5.2. Mice were given a single IP, maximum tolerated dose (i.e. 40 mg/kg for the Cremophor formulation and 120 mg/kg for the microspheres). For both tumor models, the survival times of the Cremophor (TX40x1) and microsphere (MS40sm80sm2) groups were significantly longer, compared the control group (69% and 117% longer, respectively, for SKOV3 model, \( p < 0.0001 \) for both treatment groups; 54% and 113% longer, respectively, for Hs766T model; \( p < 0.001 \) for both treatment groups). Compared to the Cremophor group, the microsphere group showed a significantly longer ILS (29% longer in SKOV3 model, \( p < 0.001 \) and 38% longer in Hs766T model, \( p < 0.05 \)) and a higher cure rate (33% vs. 0% for SKOV3 model, 25% vs. 6% for Hs766T model).

The results for the study with equal doses are shown in Figure 5.3. Mice were given IP doses of equivalent paclitaxel amounts (120 mg/kg). For both tumor
models, the survival times of the Cremophor (TX15x8ip for the SKOV3 model; TX40x3 for the Hs766T model) and microsphere (MS40sm80sm2) treatment groups were significantly longer, compared to the control group (132% and 105% longer, respectively, for SKOV3 model; 72% and 104% longer, respectively, for Hs766T model; p < 0.01 for both treatment groups in both tumor models). Compared to the Cremophor group, the microsphere group (MS40sm80sm2) showed a higher cure rate (33% vs. 25% for SKOV3 model; 25% vs. 14% for Hs766T model) and a trend of a longer survival time, but the difference was not significant (7% longer in SKOV3 model, p = 0.4; 19% longer in Hs766T model, p = 0.5).

5.3.4 Effect of Treatment Frequency of Paclitaxel Formulation on Antitumor Activity

The next study evaluated the effect of treatment frequency (i.e. single dose vs. multiple dose) on antitumor activity using the Cremophor formulation and the microspheres. Figure 5.4 shows the results in the Hs766T tumor model. The single dose groups were given a single IP, maximum tolerated dose (MTD) of either 40 mg/kg of the Cremophor formulation (TX40x1) or 120 mg/kg of the microspheres (MS40sm80sm2). For the multi-dose study, the MTD dose (total of 3 doses) of the Cremophor formulation (TX40x3) was administered weekly for 3 weeks and the MTD dose (total of 2 doses) of the microsphere formulation (MS40sm80sm2x2) was repeated 3 weeks after the initial dose. Subsequent doses of the multi-dose treatments were administered after the animals had fully recovered from initial doses (i.e., 100% of initial body weight).
For both paclitaxel formulations, the survival times for the single dose and multiple dose groups were significantly longer, compared to the control group (54% and 79% longer, respectively, for Cremophor formulation; 104% and 160% longer, respectively, for microspheres; p < 0.001 for both formulations). Multiple dosing showed an additional 46%-54% improvement in ILS over the single dose for both formulations, but it was not significant due to the limited sample size (p = 0.1 for Cremophor formulation and p = 0.3 for microspheres). The lack of statistical significance was due to the equal cure rate between the single and multiple dose groups. If these tumor-free cures were excluded, the improvement was significant (p < 0.05 for both tumor models).

5.3.5 Effect of Microsphere Size on Antitumor Activity

The subsequent study evaluated the antitumor activity of the paclitaxel microspheres of two different sizes (3 and 30 µm). Studies were conducted in both SKOV3 and Hs766T tumor models. Paclitaxel formulated in PLGA microspheres was administered IP at equal doses (i.e., 40 mg/kg in the SKOV3 model and 120 mg/kg in the Hs766T model). The microsphere formulations in both tumor models contained equal amounts of small microspheres because a secondary aim of the survival study was to evaluate maximal antitumor activity. The survival curves for both studies are shown in Figure 5.5.

For both tumor models, the survival times of the groups treated with small microspheres (MS40sm80sm2 for SKOV3 model; MS60sm60sm2 for Hs766T model) and large microspheres (MS40lg80lg for SKOV3 model; MS60sm60lg for Hs766T
model) were significantly longer compared to the control group (85% and 105% longer, respectively, for SKOV3 model, \( p < 0.01 \) for both microsphere groups; 138% and 188% longer, respectively, for Hs766T model; \( p < 0.001 \) for both microsphere groups). The ILS of the small microsphere group was 11% longer in SKOV3 model (\( p = 0.2 \)) and 17% shorter in Hs766T model (\( p = 1.0 \)), compared to the large microsphere group. However for both tumor models, the small microsphere group delayed the initial tumor-related death (62% for SKOV3 model; 9% for Hs766T model) and exhibited 3-4 fold lower variability in antitumor activity, defined as the range between the first and last death (24 vs. 106 days for SKOV3 model; 16 vs. 47 days for Hs766T model), compared to the large microsphere group.

5.3.6 Effect of Microsphere Release Rate on Antitumor Activity

The final study compared the antitumor activity of the paclitaxel microspheres with two different release rates. The \textit{in vitro} release profiles for the fast-release and slow-release microspheres (i.e. MS40sm and MS80sm2, respectively) are shown in Figure 5.6. Studies were conducted in both SKOV3 and Hs766T tumor models. As shown in Figure 5.7, different effects were observed in the two tumor models.

In the SKOV3 tumor model, the survival times of the microsphere treatment groups were significantly longer, compared to the control group (42% longer for MS40sm and 81% longer for MS80sm2, \( p < 0.001 \) for both microsphere groups). The slow-release microsphere group showed a trend of a longer ILS, compared to the fast-release microsphere group (27% longer, \( p = 0.3 \)). The lack of
statistical significance was due to the equal cure rate between the slow-release and fast-release microsphere groups. If these tumor-free cures were excluded, the improvement was significant ($p < 0.001$).

In the Hs 766T tumor model, the survival times of the fast-release and slow-release microsphere treatment groups were longer, compared to the control group (84% longer for MS40sm, $p < 0.01$ and 24% longer for MS80sm2, $p = 0.11$). Contrary to the SKOV3 model, the fast-release microsphere group showed a significantly longer ILS, compared to the slow-release microsphere group (48% longer, $p < 0.05$). However, neither microsphere group produced any cured mice.

Relationships between median survival time and drug release from microspheres were evaluated for the SKOV3 and Hs766T tumor models as shown in Figure 5.8. Drug release profiles are characterized by the initial burst release rate and the cumulative drug release. For the Hs766T tumor model, a significant positive correlation between median survival time and initial burst release rate was observed ($r^2 = 0.7$, $p < 0.05$). Furthermore, there was a significant positive correlation between median survival time and cumulative drug release ($r^2 = 0.8$, $p < 0.01$). In contrast, no relationship was observed between median survival time and initial burst release ($r^2 = 0.04$, $p = 0.7$) or cumulative drug release ($r^2 = 0.08$, $p = 0.6$) for the SKOV3 tumor model.
5.4 Discussion

The purpose of the present study was to determine whether IP delivery of paclitaxel microspheres was more advantageous in treating peritoneal tumors compared to the Cremophor formulation. At equi-toxic doses, a significant (i.e. 29%-38%) increase in ILS was observed using the microspheres, compared to the Cremophor formulation for both tumor models. Because the microspheres were designed to release drug over a period of time, higher doses of the microspheres can be administered safely to the peritoneal cavity. The maximum tolerated dose (MTD) of the microspheres was 120 mg/kg with a 1:2 ratio of fast:slow release microspheres whereas the MTD for the Cremophor formulation was only 40-50 mg/kg, necessitating the use of multiple treatments (34, 35).

Compared to a single microsphere dose, multiple doses of the Cremophor formulation were necessary to administer equal doses of 120 mg/kg. At equal doses, the microspheres still prolonged ILS by 7%-19%, compared to the Cremophor formulation. Furthermore, increasing the frequency of IP treatments can increase costs and potential complications as previously discussed. In addition to higher antitumor efficacy and greater safety, paclitaxel microspheres could allow for fewer treatments, resulting in better cost-effectiveness, greater patient compliance and convenience, and fewer complications due to reduced dependence on indwelling peritoneal catheters. Therefore, paclitaxel microspheres demonstrated survival advantages and practical benefits.
Despite the drawbacks to frequent IP treatment, multiple doses are frequently required to control tumor growth or even eradicate the tumor in which case, multiple doses of the microsphere group (MS40sm80sm2) were observed to be more effective in prolonging lifespan, compared to multiple doses of the Cremophor treatment group (Tx40x3). The microsphere treatment delayed initial tumor burden-related death by 13 days and maintained this time delay interval during the study, resulting in the parallel survival curves for the microsphere treatment groups shown in Figure 5.4. In contrast, the Cremophor formulation delayed initial tumor burden-related death by 10 days, but this time delay interval decreased over time, resulting in converging survival curves for the Cremophor treatment groups. Tumor growth occurs when drug concentrations in the peritoneal cavity fall below therapeutic levels. Therefore, to delay tumor growth and extend survival time, it is necessary to maintain elevated therapeutic drug concentrations in the peritoneal cavity where the microspheres have demonstrated to be more successful as previously discussed in Chapter 4.

Compared to the small, 3 µm microsphere groups, the large, 30 µm microsphere groups were observed to improve survival times in the Hs766T tumor model, but not in the SKOV3 tumor model. Furthermore, the range or variability of antitumor activity for the treatment groups containing the large microspheres in both tumor models was 3-4 fold higher compared to the microsphere groups comprised exclusively of small microspheres. As discussed previously in Chapter 4, the large size of the microspheres may result in a heterogeneous peritoneal distribution and
diminished capacity for tumor penetration, leading to variable antitumor activity and survival times. Therefore, since the difference in ILS between the two microsphere groups was not statistically significant, the small microspheres represents the more useful microsphere formulation for treatment.

For both tumor models, the combination microsphere (MS40sm80sm2) group demonstrated the most antitumor activity without any toxicity-induced deaths. The combination microsphere (MS40sm80sm2) treatment was comprised of two parts: 1 part of small, fast-releasing microspheres (MS40sm) and 2 parts of small, slow-releasing microspheres (MS80sm2). We speculate the greater antitumor activity may be a result of enhanced tumor penetration. Our laboratory has shown that fractionation of the dose into two doses such that the first dose is sufficient to induce apoptosis, followed by the remaining dose with a time delay of 24 hr to allow for apoptosis can enhance the total drug delivery to solid tumors (36). Therefore, the combination microsphere treatment may provide the initial dose required to induce apoptosis with the fast-release microspheres. Reduction of the tumor cell density could enhance drug penetration of the slow-release microspheres into the tumor. This hypothesis will be tested in Chapter 6.

While the overall antitumor activity of the combination microsphere (MS40sm80sm2) treatment was most effective in prolonging survival time compared to the control group in both tumor models, the individual components (i.e. slow and fast-release microspheres) of the combination microsphere treatment showed different antitumor activities in the ovarian and the pancreatic tumor model. The slow-release
microsphere group was more successful in prolonging survival time in the ovarian tumor model while the fast-release microsphere group was more effective in the pancreatic tumor model. This suggests that microsphere release rates alone do not dictate antitumor activity.

Antitumor activity may be a function of microsphere release rate and tumor growth kinetics. Preliminary studies in our lab have shown a tumor doubling time of 3-5 days for the pancreatic tumor, which was consistent with the literature, whereas the reported tumor volume doubling time was 8 days for the ovarian tumor (37, 38). For the pancreatic tumor model, the release rate of the fast-release and slow-release microspheres were likely slower than the tumor growth rate, resulting in the observed antitumor effects where survival times were dependent on the drug release rates. However, for the ovarian tumor model, the release rate of the slow-release microspheres was likely slower than the tumor growth rate whereas the fast-release microspheres was likely faster than the tumor growth rate, resulting in the drug being cleared before exerting its antitumor effect. As a result, no relationship was observed between survival time and microsphere release rate. Therefore, it is necessary to tailor the release kinetics of the microspheres with the tumor growth rate to maximize antitumor effect.

This study has demonstrated that paclitaxel microspheres have several advantages in safety, antitumor efficacy, and convenience over the Cremophor formulation for IP therapy. However, treatment could be even more effective if the microsphere release rate can be synchronized with growth kinetics of the treated
tumors. This will become even more important in the management of malignant tumors in humans, where the tumor growth rate is typically slower than that in mice. Therefore, understanding the time course of the antitumor activity of the microsphere treatment on peritoneal tumors, which will be studied in Chapter 6, will be crucial to optimization of drug treatment.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Dose</th>
<th>Treatment Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.01% Tween 80 in saline</td>
<td>Single dose</td>
</tr>
<tr>
<td>TX10x4</td>
<td>40 mg/kg (4 x 10 mg/kg)</td>
<td>Biweekly for 2 weeks</td>
</tr>
<tr>
<td>TX40x1</td>
<td>40 mg/kg</td>
<td>Single dose</td>
</tr>
<tr>
<td>TX15x8ip</td>
<td>120 mg/kg (8 x 15 mg/kg) ip</td>
<td>Biweekly for 4 weeks</td>
</tr>
<tr>
<td>TX15x8iv</td>
<td>120 mg/kg (8 x 15 mg/kg) iv</td>
<td>Biweekly for 4 weeks</td>
</tr>
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<td>MS40sm</td>
<td>40 mg/kg (sm)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS40lg</td>
<td>40 mg/kg (lg)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS80sm2</td>
<td>80 mg/kg (sm2)</td>
<td>Single dose</td>
</tr>
<tr>
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<td>120 mg/kg (40sm+80sm2)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS40sm80lg</td>
<td>120 mg/kg (40sm+80lg)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS40sm40lg40sm2</td>
<td>120 mg/kg (40sm+40lg+40sm2)</td>
<td>Single dose</td>
</tr>
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</table>

TX - Cremophor formulation  
MS - Microsphere formulation  
sm - 3 μm, 50:50 PLGA microspheres  
sm2 - 3 μm, 75:25 PLGA microspheres  
lg - 30 μm, 50:50 PLGA microspheres

Table 5.1. Treatment regimen for SKOV3 ovarian survival study
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Dose</th>
<th>Treatment Frequency</th>
</tr>
</thead>
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<td>Control</td>
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<td>Single dose</td>
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<tr>
<td>TX40x1</td>
<td>40 mg/kg</td>
<td>Single dose</td>
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<td>40 mg/kg (sm)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS80sm2</td>
<td>80 mg/kg (sm2)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS40sm80sm2</td>
<td>120 mg/kg (40sm+80sm2)</td>
<td>Single dose</td>
</tr>
<tr>
<td>MS60sm60sm2</td>
<td>120 mg/kg (60sm+60sm2)</td>
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<td>MS60sm60lg</td>
<td>120 mg/kg (60sm+60lg)</td>
<td>Single dose</td>
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</tr>
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<td>240 mg/kg (40sm+80sm2) x 2</td>
<td>Every 3 weeks</td>
</tr>
</tbody>
</table>

TX - Cremophor formulation
MS - Microsphere formulation
sm - 3 μm, 50:50 PLGA microspheres
sm2 - 3 μm, 75:25 PLGA microspheres
lg - 30 μm, 50:50 PLGA microspheres

Table 5.2. Treatment regimen for Hs766T pancreatic survival study.
<table>
<thead>
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<th>Group</th>
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<th>Cure (%)</th>
<th>Median Survival Time (days)</th>
<th>Improved Lifespan (fold, vs. control)</th>
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TX - Cremophor formulation  
MS - Microsphere formulation  
sm - 3 μm, 50:50 PLGA microspheres  
sm2 - 3 μm, 75:25 PLGA microspheres  
lg - 30 μm, 50:50 PLGA microspheres

Table 5.3. Summary of SKOV3 ovarian survival study. Treatment groups were arranged by study. Cured mice were surgically documented complete responses. Median survival time was calculated as the time at which 50% of the animals had died. Improved lifespan (ILS) is calculated as the ratio of the median survival time of the experimental group to that of the control group. All treatment groups were significantly better than the control group.
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cure (%)</th>
<th>Median Survival Time (days)</th>
<th>Improved Lifespan (fold, vs. control)</th>
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</thead>
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TX - Cremophor formulation  
MS - Microsphere formulation  
sm - 3 µm, 50:50 PLGA microspheres  
sm2 - 3 µm, 75:25 PLGA microspheres  
lg - 30 µm, 50:50 PLGA microspheres

Table 5.4. Summary of Hs766T pancreatic survival study. Treatment groups were arranged by study. Cured mice were surgically documented complete responses. Median survival time was calculated as the time at which 50% of the animals had died. Improved lifespan (ILS) is calculated as the ratio of the median survival time of the experimental group to that of the control group. All treatment groups were significantly better than the control group.
Figure 5.1. Kaplan-Meier plot on the effect of administration route on survival using SKOV3 ovarian tumor model.
Figure 5.2. Kaplan-Meier plot on the effect of paclitaxel formulation at MTD dose on survival. Left panel: SKOV3 ovarian tumor model; Right panel: Hs766T pancreatic tumor model. The corresponding MTD was used for each paclitaxel formulation (i.e. 40 mg/kg for Cremophor formulation; 120 mg/kg for microspheres).
Figure 5.3. Kaplan-Meier plot on the effect of paclitaxel formulation at equal doses on survival. Left panel: SKOV3 ovarian tumor model; Right panel: Hs766T pancreatic tumor model. Equivalent doses of paclitaxel formulated in Cremophor or PLGA microspheres were administered at 120 mg/kg.
Figure 5.4. Kaplan-Meier plot on the effect of treatment frequency on survival using Hs766T pancreatic tumor model. Left panel: Cremophor formulation (single vs. multiple dose). Right panel: Microspheres (single vs. multiple dose).
Figure 5.5. Kaplan-Meier plot on the effect of microsphere size on survival.
Left panel: SKOV3 ovarian tumor model (40mg/kg)
Right panel: Hs766T pancreatic tumor model (120 mg/kg)
It was noted that both treatment groups in the Hs766T model contain small microspheres.
Figure 5.6. *In vitro* cumulative drug release from slow-release (MS80sm2) and fast-release (MS40sm) microsphere in PBS.
Figure 5.7. Kaplan-Meier plot on the effect of microsphere release rate on survival. Left panel: SKOV3 ovarian tumor model; Right panel: Hs766T pancreatic tumor model. Fast-release microspheres: MS40sm; Slow-release microspheres: MS80sm2
Figure 5.8. Median survival time vs. initial microsphere burst release rate and cumulative drug release. Left panel: Hs766T pancreatic tumor model. Right panel: SKOV3 ovarian tumor model.
6.1 Introduction

Paclitaxel, one of the most important anticancer drugs developed in the past two decades, has demonstrated significant antitumor activity against many types of human solid tumors (1, 2). In many human cancer cell lines, paclitaxel has been shown to produce immediate pharmacological effects as well as delayed cytotoxicity due to slow manifestation of apoptosis and significant intracellular concentrations (3). The significant activity of paclitaxel against ovarian cancer has led to the evaluation of intraperitoneal (IP) delivery of Taxol®, the commercial formulation of paclitaxel whereby the drug is solubilized in Cremophor:ethanol.

For the last several decades, there has been interest in the application of IP chemotherapy for the treatment of malignant peritoneal-confined tumors with the premise that IP therapy delivers higher drug exposure to the tumor than could be achievable by systemic drug delivery (4, 5). As discussed in Chapter 2, IP therapy has demonstrated improved objective response and prolonged survival in clinical trials.
using ovarian cancer patients. However, the efficacy of this treatment has been primarily limited to microscopic residual disease (i.e. tumor diameter < 2 cm) (6-8). The diminished effectiveness of IP treatment in treating bulky tumors is presumably due to poor drug penetration into the tumor. Various preclinical studies utilizing different antineoplastic agents have shown a maximum drug penetration of less than 1 mm from the tumor surface after IP administration (9-11).

In contrast to systemic therapy where drug delivery to tumor cells occurs through the blood circulation, drug delivery to cells in solid tumors following IP administration is determined by drug transport through the interstitial space (12, 13). The efficacy of IP treatment depends on the ability of the drug to penetrate the solid tumor. Drug penetration for paclitaxel has been difficult for the following reasons: (a) tumors may have limited access to the free drug because of paclitaxel entrapment in Cremophor micelles (14, 15); and (b) highly protein-bound drugs like paclitaxel and cisplatin behave as macromolecules, and are primarily transported by convection (16, 17). These drugs remain on the periphery of tumors or spheroids while low binding drugs such as 5-fluorouracil can easily cross cell membranes and distribute uniformly (18).

Our laboratory has previously reported that tumor cell density is a barrier to paclitaxel penetration into tumors (19). Drug treatment can induce apoptosis, which is dependent on both paclitaxel concentration and exposure duration (20, 21). Our laboratory further showed that removal of these apoptotic cells lowers cell density and increases the fraction of interstitial space, thereby promoting drug penetration into
tumors (22). Furthermore, our laboratory has shown that fractionation of the dose into two doses such that the first dose is sufficient to induce apoptosis, followed by the remaining dose with a time delay of 24 hr to allow apoptosis to occur, can enhance the total drug delivery to solid tumors (23).

Chapter 3 described the development of paclitaxel-loaded PLGA microspheres with different release rates. Results in Chapter 5 showed that several of the paclitaxel microspheres provided a significant survival advantage over any single paclitaxel formulation treatment. The best antitumor response was observed for the treatment group containing a combination of a fast-release and a slow-release microsphere formulation, whereby the fast-release formulation could provide drug concentrations to induce apoptosis while the slow-release formulation could sustain drug concentrations in the tumor.

The purpose of the present study was to test the hypothesis that a combination of a fast release and a slow release formulation can enhance drug delivery into solid tumors. This was studied using autoradiographical techniques to compare the penetration of paclitaxel into peritoneal tumors following IP administration of the Cremophor formulation, fast and slow-release microspheres given separately and in combination. Autoradiography has been used to provide quantitative data and high resolution spatial information related to the exposure of tissues from radio-labeled drugs (24, 25). Over the last 10 years, autoradioluminography has been the new
technology used to characterize autoradiographic tissue distribution by exposure of radio-treated sections to phosphor imaging plates, which are scanned by a specialized scanner, resulting in digital images (26-28).
6.2 Materials and Methods

6.2.1 Chemicals and Reagents

3"-\(^{3}\)H-Paclitaxel (specific activity, 14.7 Ci/mmol), obtained either through the National Cancer Institute (Bethesda, MD) or Moravek Biochemicals, Inc. (Brea, CA), was stored at -70°C. All chemicals and reagents used in the preparation and characterization of paclitaxel-loaded PLGA microspheres are as described in Chapter 3 and were used as received.

6.2.2 Animal Tumor Model

Female athymic mice obtained from Charles River/NCI Laboratories (Wilmington, MD) were used. Development of the ovarian animal tumor model using the SKOV3 cell line obtained from ATCC (Manassas, VA) was as described in Chapter 5. Drug treatment was initiated at 6 weeks after tumor implantation; our earlier studies showed establishment of multiple tumors in 100% of the mice after 4 weeks. The ovarian tumor model (SKOV3) was selected over the pancreatic tumor model (Hs766T), which was also used in Chapter 5 because the SKOV3 tumors contain more densely packed cells and fewer necrotic areas, compared to the Hs766T tumors. Necrotic areas can alter drug penetration into the tumor. Therefore, the effect of apoptosis-inducing treatment on tumor penetration would be more evident and not confounded by necrosis in the SKOV3 tumor model.
6.2.3 Tumor Penetration Study

For dosing solutions, preparation of paclitaxel formulations (Cremophor or PLGA microspheres) were prepared as described in Chapter 4. In both formulations, a mixture of $^3$H-labeled and nonradiolabeled paclitaxel at a total dose of 20 mg/kg (1.6 mCi/kg) were administered IP. At 6, 24, 72, and 168 hr, mice were euthanized using isofluorane (Abbott Laboratories, North Chicago, IL). Tumors (3-6 mm diameter) were removed from the mice. Tumor nodules were rinsed with distilled water, blotted-dried with gauze, and flash-frozen in liquid nitrogen. This procedure was completed in < 5 min. Tumors were cut into two halves. One half was stored at -70°C for future use. The second half was mounted with the cross-section face up onto cryostat chucks using O.C.T. embedding matrix (Miles Inc., Elkhart, IN). Using a Microm microtome cryostat (Waldorf, Germany), the tumors were cut into 20 µm sections at -25°C. Every 200 µm, the sections were thaw-mounted onto glass slides (3 section per slide) as depicted in Figure 1. Tumor sections of equal depth were used to study drug penetration for different treatment groups.

6.2.4 Autoradioluminographic Image Development

The autoradioluminography image capture was done by Quest Pharmaceutical Services, L.L.C. (Newark, DE). Commercially available, pre-calibrated microscale tritium autoradiography standards, which contain step-wise increments of radioactivity ranging from 0.1 to 109.4 nCi/mg (Amersham Biosciences Corp., Piscataway, NJ) were placed onto glass slides. Slides with the standards and sample samples were placed against Fuji BAS-TR phosphor imaging plates (Stamford,
Following a 1-week exposure at room temperature, the plates were scanned using Typhoon imaging system (Amersham Biosciences Corp., Piscataway, NJ). The limit of detection was 2 µg/g tissue.

6.2.5 Computer-Assisted Videodensitometric Analysis of Tumor Penetration

Pixel gray-scale analysis was performed using Optimas Image Analysis software (MediaCybernetics, Silver Spring, MD). To quantitatively describe the decline in drug concentration as a function of distance from the tumor surface, gray-scale intensities were sampled in four inward directions as shown in Figure 1. The average of the four gray-scale intensity measurements was used to minimize variability within the tumor. Background gray-scale values, which were typically <10% of the intensity in the experimental group, were determined by measuring gray-scale intensities of blank tumor sections (i.e. tumor removed from animals that were not treated with radiolabeled paclitaxel). After background subtraction, tumor concentrations were determined from the gray-scale intensities using a standard curve, which was generated by measuring the gray-scale intensities of known quantities of radioactivity from the microscale standards described above. AUC was calculated using the trapezoid rule. \( W_{1/2} \) was calculated as \(-\ln(2)/k\), where \( k \) is the initial slope of the log-linear, tumor concentration-depth profile, determined using data points from peak concentrations to the concentration at the detection limit.
6.2.6 **Histological Staining and Analysis of Drug Effect of Tumor Cells**

Tumor sections were stained with hematoxylin and eosin. The histological image showing the tissue structure and distribution of tumor cells, were captured using a Zeiss 35M Axiovert microscope (Thornwood, NY) and a Yamaguchi 3-color camera. Three microscope fields of non-necrotic areas in the tumor periphery were randomly selected for each tumor. Necrotic areas were determined based on morphological changes such as loss of membrane integrity, disintegration of cell structure, and complete cell lysis (29). Microscopic fields were also selected from the center fields of the tumor core, which were not necrotic.

Paclitaxel induces apoptosis and reduces tumor cell density (30-32). Hence, both parameters were evaluated. Tumor cell density was determined by counting the total number of cells in each field at 400x magnification. The apoptotic index was calculated as (number of apoptotic cells) divided by (number of total tumor cells) in each field at 400x magnification. Apoptotic cells were determined based on morphological changes in tumor cells such as chromatin condensation and margination, disappearance of nucleoli, formation of membrane blebs, formation of apoptotic bodies, and/or cell shrinkage. This method has been shown to yield the same results as the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (32, 33).
6.2.7 Statistical Analysis

The statistical significance of differences between treatment groups was analyzed using ANOVA with Student’s t-test. Differences were considered significant at p < 0.05.
6.3 Results

Table 1 describes the treatment dose for the Cremophor and microsphere treatment groups used in this tumor penetration study. The effect of drug treatment over time was studied using the Cremophor and fast-release microsphere groups. The effect of different drug formulations on tumor penetration was studied at later time points (i.e., > 24 hr) since previous studies have shown that significant levels of apoptosis were not observed until 16-24 hr after drug treatment (3, 19). The combination microsphere group was composed of equal parts of slow-release and fast-release microspheres.

6.3.1 Effect of drug treatment: apoptosis and necrosis

Figure 2 shows morphological changes in the tumor. For the saline-treated control tumors, apoptotic cells comprised an average of 2% of all tumor cells in the control tumors, consistent with other studies (34, 35). For these tumors, the apoptotic index in the tumor periphery was not significantly different than the apoptotic index in the tumor core (1.8% vs. 1.7%, p = 0.6). Also, little to no necrotic areas were observed in the control tumors.

The drug-treated groups showed different tumor morphology, compared to the control group. Compared to the control group, the drug-treated groups showed significantly higher apoptotic indices in the tumor periphery (10.0% vs. 2.1%, p < 0.0001), but similar apoptotic indices in the tumor core (1.6% vs. 1.7%, p = 0.8). Furthermore, the extent of apoptosis varied with the drug release rate, ranging from
5% for slow-release microsphere to 20% for combination microsphere treatment at 72 hr. Areas of necrosis were observed primarily in the periphery of drug-treated tumors.

### 6.3.2 Effect of drug formulation on apoptosis and tumor cell density in peritoneal tumors

The apoptotic indices and tumor cell densities at 6, 24, 72, and 168 hr for the control, Cremophor formulation, single and combination microsphere treatment groups are summarized in Table 2. The apoptotic index and tumor cell density did not change significantly over time in the control group (p < 0.05). Representative histological images of drug-treated tumors are shown in Figure 3. A significant inverse correlation between apoptotic index and tumor cell density was observed ($r^2 = 0.46$, p < 0.02).

A comparison of the tumor morphologies in these four groups showed the following findings. There was a time delay in the drug-induced apoptotic cells. The Cremophor formulation and fast-release microsphere treatment groups did not show appreciable apoptosis in 6 hr and 24 hr and the apoptotic indices were not significantly different, compared to the control group (p > 0.05 for in all cases). Significant (i.e., 7-15 fold) increase in the apoptotic indices were observed at 72 hr such that the apoptotic indices of the drug-treated groups were much higher than the corresponding apoptotic index for the control group. At 168 hr, the apoptotic indices for the drug-treated groups declined, but remained 2-7 fold higher compared to the control group (p > 0.05 for in all cases). Between the two drug treatment groups, the fast-release microsphere group showed higher levels of apoptosis, compared to the Cremophor
formulation (20% vs. 10% at 72 hr, 15% vs. 6% at 168 hr, \( p < 0.05 \) for both time points).

Since the tumor cell density was inversely correlated with apoptosis, this suggests that a similar time delay would be observed for this parameter in drug-treated tumors. However, it was observed that the tumor cell density at 6 hr for the Cremophor and the fast-release microsphere treatment groups was 10-15% lower, compared to the control group (175.5 and 171.4 vs. 199.8 cells/field, \( p < 0.05 \) for both formulations). Despite the inconsistencies observed at 6 hr, the remainder of the data supports the presence of a time delay. At 24 hr, the tumor cell densities of the Cremophor and the fast-release microsphere treatment groups were not significantly different, compared to the control group (202.0 and 197.8 vs. 199.8 cells/field, \( p > 0.05 \)). The tumor cell densities then decreased with time for the Cremophor and fast-release microsphere groups at 72 hr and 168 hr and were significantly lower, compared to the control group (182.7 and 168.8 vs. 199.8 cells/field at 72 hr, 178.6 and 160.6 vs. 199.8 cells/field at 168 hr, \( p < 0.05 \) in all cases). Finally, the fast-release microsphere group showed significantly lower tumor cell densities at 72 hr and 168 hr, compared to the Cremophor group (182.7 vs. 168.8 cells/field at 72 hr, 178.6 vs. 160.6 cells/field at 168 hr, \( p < 0.05 \) in all cases).
6.3.3 Effect of amount of released drug from microspheres on apoptosis and tumor cell density in peritoneal tumors

We evaluated the relationship between extent of drug release and drug effect. The extents of drug release were obtained from the data described in Chapter 5; the cumulative amount of paclitaxel released in PBS was 1.63 mg/kg for slow-release microspheres, 14.04 and 14.62 mg/kg for fast-release microspheres, and 15.67 and 17.35 mg/kg for the slow-fast combination microspheres at 72 hr and 168 hr, respectively. As shown in Figure 4, there was a significant positive correlation between paclitaxel release and apoptotic index ($r^2 = 0.82$, $p < 0.05$) and an inverse correlation between paclitaxel release and tumor cell density ($r^2 = 0.91$, $p < 0.05$). The fast-release and combination microsphere groups both showed significantly higher apoptotic indices (19.9% and 21.5% vs. 5.3%, $p < 0.01$) and lower tumor cell densities (168.8 and 164.9 vs. 185.2 cells/field, $p < 0.05$), compared to the slow-release microsphere group.

6.3.4 Effect of drug formulation on paclitaxel penetration in peritoneal tumors

Tumor concentration and drug penetration were determined using videodensitometric readings and image analysis of the autoradioluminographic images. Figure 5 shows the spatial drug distribution in tumors for mice treated with the Cremophor formulation, single, and combination microspheres.
The highest concentrations ($C_{\text{max}}$) for the drug treatment groups were located near the surface of the tumor and declined exponentially as a function of distance from the tumor surface except for the combination microsphere group. The $w_{1/2}$, $C_{\text{max}}$, and AUC values used to characterize drug penetration and tumor exposure are summarized in Table 3.

The $C_{\text{max}}$ and AUC of the Cremophor group were significantly lower, compared to all of the microsphere groups (6.2 $\mu$g/g vs. microsphere average of 69.0 $\mu$g/g, $p < 0.05$; 3.7 $\mu$g/g*mm vs. microsphere average of 19.4 $\mu$g/g*mm, $p < 0.05$). The fast-release and slow-release microsphere groups showed similar AUCs (9.7 and 12.3 $\mu$g/g*mm, $p = 0.1$), but a significantly longer $w_{1/2}$, indicating a slower concentration decline for the fast-release microsphere group (207.6 vs. 64.8 $\mu$m, $p < 0.01$).

The drug concentrations of the combination microsphere group did not follow a log-linear decline (i.e., local maximum at 500 $\mu$m), so the $w_{1/2}$ value could not be accurately determined. However, the combination microsphere group did show the highest peak tumor concentrations (129.9 $\mu$g/g vs. average of 27.7 $\mu$g/g, $p < 0.05$) and AUC (36.4 $\mu$g/g*mm vs. average of 8.6 $\mu$g/g*mm, $p < 0.05$), compared to all of the treatment groups. Because the total dose of the combination group was twice of the dose in the single microsphere groups, we also compared the dose-adjusted drug effects. The $C_{\text{max}}$ of the combination microsphere treatment was 69% greater than the sum of the peak concentrations for the slow-release and fast-release microspheres (129.9 $\mu$g/g vs. 77.0 $\mu$g/g ($21.3 + 55.7 \mu$g/g)). Likewise, the AUC of the combination
microsphere treatment was 65% greater than the sum of the AUCs for the slow-release and fast-release microspheres (36.4 µg/g*mm vs. 22.0 µg/g*mm (9.7 + 12.3 µg/g*mm)). Hence, the combination microsphere treatment groups showed greater dose-adjusted C_{max} and AUC, compared to the single microsphere treatment group.

Figure 6 shows the changes in w_{1/2} values over time for the Cremophor formulation and the fast-release microsphere groups. The w_{1/2} values for the Cremophor group was 154.2 ± 19.1 µm (mean ± SD) and did not change significantly over time. In contrast, the w_{1/2} values for the fast-release microsphere group increased over time from 129.8 µm to 291.4 µm. Similar to the apoptotic index and tumor cell density, there was a time delay in the w_{1/2}, C_{max}, and AUC values observed for the fast-release microsphere group. Compared to the Cremophor group, the w_{1/2}, C_{max}, and AUC values for the fast-release microsphere groups at 6 hr, 24 hr, and 72 hr were not significantly different (p > 0.05 in all cases), but were significantly greater at 168 hr (57% for w_{1/2}, 241% for C_{max}, 164% for AUC, p < 0.05 in all cases).

Figure 7 compares the localization of microspheres in tumors. The fast-release microspheres were primarily localized on tumor periphery (i.e. 10-20 cell layer depth from tumor surface) at 6 and 24 hr, followed by penetration to the inner parts of the tumor (i.e. 40-80 cell layer depth from tumor surface) at 72 and 168 hr. A similar distribution was observed for the combination microspheres at 72 and 168 hr, whereas the slow-release microspheres remained localized on the periphery of the tumor (i.e. 15-25 cell layer depth from tumor surface) at 72 hr. The presence of lymphocytes was also observed, suggesting some inflammation occurring in the tumor.
The drug retention in the tumor, expressed as % change in AUC, was greater for the combination microsphere group, compared to the Cremophor and the fast-release microsphere treatment groups. The AUC in the Cremophor formulation and the fast-releasing microspheres treatment groups both declined 32% from 72 hr to 168 hr, whereas the AUC of the combination microsphere treatment group increased by 14% over that time period.
6.4 Discussion

The time-dependent increase in apoptosis in the Cremophor and microsphere drug-treated groups is consistent with the reported delayed appearance of paclitaxel-induced apoptosis (3, 36). The 25%-50% decrease in the apoptotic indices from 72 hr to 168 hr was accompanied by a decrease in the tumor cell density, indicating continuing removal of apoptotic cells over this time period. The apoptotic index was significantly higher after treatment with the fast-release microspheres, compared to the slow-release microspheres. This data shows that apoptosis is not only a time-dependent pharmacodynamic effect, but also dependent on drug concentration, which is affected by the microsphere release rate.

Tumor cell density was inversely related to apoptotic index, although the tumor cell densities at 6 hr for the drug-treated groups were lower, compared to the control group whereas the apoptotic indices at 6 hr for the drug-treated groups were not significantly different, compared to the control group. Although the pathway mechanism for immune response activation by paclitaxel has not been fully elucidated, paclitaxel has been shown to induce cytokine expression and trigger other cellular immune responses (37, 38). The presence of lymphocytes, which are commonly involved in the late stages of cellular inflammatory response, indicated that there might have been an acute response (i.e. inflammation) to drug treatment since no significant changes in tumor cell density were observed in the control group. Furthermore, PLGA itself does not elicit an immune response (39, 40). Initial
vasodilation of tumor vessels and increased capillary permeability may cause temporary edema or increase in interstitial fluid, resulting in the apparent lower tumor cell density observed at 6 hr (41).

The tumor concentrations declined as a function of distance from the tumor surface. The drug penetration depth \( (w_{1/2}) \) of the Cremophor group did not change significantly over time and the drug remained primarily on the periphery. Previous studies have shown an increase in paclitaxel penetration over time with complete tumor penetration at 48-72 hr after treatment (19). However, this work was conducted in tumor histocultures, which maintained constant drug exposure and did not consider drug removal by blood flow or lymphatic vessels that occur in living systems. The results from Chapter 2 showed that the peritoneal half-life of the Cremophor formulation was 2-3 hr and drug concentrations in the peritoneal lavage could not be detected after 12 hr. Although the Cremophor formulation induced apoptosis and reduced tumor cell density, limited drug penetration was observed as a result of rapid peritoneal clearance of the Cremophor formulation. The pharmacokinetic data suggests that no significant amount of drug would be available for tumor penetration after apoptosis occurred.

In contrast, the drug penetration depth \( (w_{1/2}) \) of the fast-releasing microsphere treatment group increased over time. Significant increases in tumor penetration occurred only after significant reductions in tumor cell density were observed. The higher drug penetration could be due to penetration of intact microspheres, which could increase the drug exposure and the mean residence time of
the drug within the tumor. Infiltration of the microspheres into the tumor may have coincided with significant increases in apoptosis since paclitaxel-induced apoptosis has been known to increase tumor porosity and/or reduce interstitial fluid pressure in solid tumors, which could lessen the resistance to movement of microspheres or other large macromolecules (19, 42, 43). The lower apoptotic index and higher tumor cell density as a result of lower drug release likely resulted in the limited microsphere penetration of the individual slow-release microsphere treatment group.

The combination microsphere treatment group showed the highest fraction of apoptotic cells and the lowest tumor cell density, compared to all of the other treatment groups. Furthermore, the additive total dose used in the combination microsphere group produced a synergistic increase in drug penetration (i.e. $C_{\text{max}}$ and AUC), as follows. Based on the *in vitro* microsphere release profiles, the effective dose (i.e. amount of released drug) for the combination microspheres was only 15%-20% greater, compared to the fast-release microspheres. In comparison, the drug penetration (i.e., $C_{\text{max}}$ and AUC) of the combination microspheres was considerably greater (i.e. 3-10 fold in all cases) than that of the single microsphere groups. This suggests that dose fractionation using microspheres with two different release rates can enhance drug delivery to tumors, whereby the fast-release microspheres induced apoptosis to promote the penetration of the slow-release microspheres, which continued to release drug over longer periods of time. In this study, the combination microsphere treatment group showed the greatest pharmacodynamic effects at 72 and
168 hr, compared to all of the other treatment groups. This is consistent with the significant survival advantage for the combination microsphere treatment group as observed in the survival studies presented in Chapter 5.

In summary, the results in this chapter showed that paclitaxel-induced apoptosis, through alteration of the tumor structure, could enhance the penetration of microspheres into solid tumors. However, this can only occur if the drug is retained in the peritoneal cavity after apoptosis has occurred. Penetration of the microspheres can prolong drug exposure and retention within the tumor over time. Dose fractionation by using two types of microspheres with different release rates resulted in greater drug delivery into solid tumors compared to the Cremophor formulation or individual microsphere treatment.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Formulation</th>
<th>Total Dose</th>
<th>Evaluated Time Points (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>saline</td>
<td>0</td>
<td>6, 24, 72, 168</td>
</tr>
<tr>
<td>Cremophor formulation</td>
<td>Cremophor: ethanol (1:1, w:w)</td>
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<td>6, 24, 72, 168</td>
</tr>
<tr>
<td>Fast release microspheres</td>
<td>3 µm, 50:50 PLGA microspheres</td>
<td>20</td>
<td>6, 24, 72, 168</td>
</tr>
<tr>
<td>Slow release microspheres</td>
<td>3 µm, 75:25 PLGA microspheres</td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>Combination microspheres</td>
<td>3 µm, 50:50 PLGA microspheres &amp; 3 µm 75:25 PLGA microspheres</td>
<td>40</td>
<td>72, 168</td>
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Table 6.1. Treatment dose for Cremophor and microsphere groups.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Effect</th>
<th>6 hr</th>
<th>24 hr</th>
<th>72 hr</th>
<th>168 hr</th>
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<tr>
<td>Control</td>
<td>Apoptotic index (%)</td>
<td>2.1±0.4</td>
<td>1.6±1.1</td>
<td>1.4±0.6</td>
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<td></td>
<td>Tumor cell density (cells/field)</td>
<td>199.8±8.6</td>
<td>195.7±6.7</td>
<td>209.2±8.2</td>
<td>202.4±5.4</td>
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<td>Cremophor formulation</td>
<td>Apoptotic index (%)</td>
<td>3.2±1.1</td>
<td>4.4±1.7</td>
<td>10.2±2.3*</td>
<td>5.4±1.3*</td>
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<td></td>
<td>Tumor cell density (cells/field)</td>
<td>175.5±11.0</td>
<td>202.0±10.1</td>
<td>182.7±5.9*</td>
<td>178.6±7.7*</td>
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<td>Fast release microspheres</td>
<td>Apoptotic index (%)</td>
<td>2.5±0.8</td>
<td>4.7±1.0</td>
<td>19.9±5.6**</td>
<td>14.5±1.3**</td>
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<td></td>
<td>Tumor cell density (cells/field)</td>
<td>171.4±5.0</td>
<td>197.7±10.1</td>
<td>168.8±4.4**</td>
<td>160.6±7.1**</td>
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<td>Slow release microspheres</td>
<td>Apoptotic index (%)</td>
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<td>N/A</td>
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<td>Tumor cell density (cells/field)</td>
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<td>N/A</td>
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<td>Apoptotic index (%)</td>
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<td>N/A</td>
<td>21.5±4.5**</td>
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<td>Tumor cell density (cells/field)</td>
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<td>N/A</td>
<td>164.9±8.0**</td>
<td>155.3±6.0**</td>
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Table 6.2. Pharmacodynamic effects of treatment: apoptotic index and tumor cell density. The apoptotic index was calculated as (number of apoptotic cells) divided by (number of total tumor cells) in each field. (3 fields per tumor). (n = 3 or 4). * denotes $p < 0.05$ compared to Cremophor group. N/A denotes time points not studied.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Parameter</th>
<th>6 hr</th>
<th>24 hr</th>
<th>72 hr</th>
<th>168 hr</th>
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<tr>
<td>Cremophor formulation</td>
<td>$C_{\text{max}}$ (µg/g)</td>
<td>42.1±17.9</td>
<td>24.5±13.6</td>
<td>6.2±1.9</td>
<td>3.0±1.4</td>
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<td>$AUC_{0\rightarrow2\text{ mm}}$ (µg/g·mm)</td>
<td>14.9±9.0</td>
<td>8.7±1.5</td>
<td>3.7±1.5</td>
<td>2.5±1.1</td>
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<td>$w_{1/2}$ (µm)</td>
<td>131.2±66.6</td>
<td>158.5±77.4</td>
<td>150.1±50.0</td>
<td>177.3±37.6</td>
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<td>Fast release microspheres</td>
<td>$C_{\text{max}}$ (µg/g)</td>
<td>49.6±19.0</td>
<td>40.5±3.6</td>
<td>21.3±13.8</td>
<td>10.0±4.6    *</td>
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<td>$AUC_{0\rightarrow2\text{ mm}}$ (µg/g·mm)</td>
<td>18.8±9.1</td>
<td>12.5±2.5</td>
<td>9.7±4.7</td>
<td>6.8±3.9     *</td>
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<td></td>
<td>$w_{1/2}$ (µm)</td>
<td>129.8±53.1</td>
<td>154.7±63.3</td>
<td>207.6±53.8</td>
<td>291.4±46.0  *</td>
</tr>
<tr>
<td>Slow release microspheres</td>
<td>$C_{\text{max}}$ (µg/g)</td>
<td>N/A</td>
<td>N/A</td>
<td>55.7±14.8   *</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0\rightarrow2\text{ mm}}$ (µg/g·mm)</td>
<td>N/A</td>
<td>N/A</td>
<td>12.3±3.4    *</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>$w_{1/2}$ (µm)</td>
<td>N/A</td>
<td>N/A</td>
<td>64.8±33.5</td>
<td>N/A</td>
</tr>
<tr>
<td>Combination microspheres</td>
<td>$C_{\text{max}}$ (µg/g)</td>
<td>N/A</td>
<td>N/A</td>
<td>129.9±43.1  **</td>
<td>107.4±13.9 **</td>
</tr>
<tr>
<td></td>
<td>$AUC_{0\rightarrow2\text{ mm}}$ (µg/g·mm)</td>
<td>N/A</td>
<td>N/A</td>
<td>36.4±22.1   **</td>
<td>41.4±7.8    **</td>
</tr>
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<td></td>
<td>$w_{1/2}$ (µm)</td>
<td>N/A</td>
<td>N/A</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 6.3. Drug penetration into peritoneal tumors following drug treatment: $C_{\text{max}}$ and AUC. AUC was calculated using the trapezoid rule. (n = 3 or 4).
* denotes p < 0.05 compared to Cremophor group.
** denotes p < 0.05 compared to all groups.
N/A denotes time points not studied; ND denotes not determinable.
Figure 6.1. Drug penetration study procedure. Tumors were harvested from animals and flash-frozen in liquid nitrogen (n = 3 or 4). Frozen tumors were sectioned serially with a cryotome and thaw-mounted on glass slides (3 sections per slide). Autoradioluminography images were captured by exposure of slides with sections to phosphor imaging plates. Computer-assisted densitometry analysis was used to measure drug concentration as a function of distance.
Figure 6.2. Morphological changes after drug treatment: apoptosis and necrosis. Upper panel: Apoptotic cells (indicated with arrows) were determined based on morphological changes (i.e. formation of apoptotic bodies, chromatin condensation and margination). 400x magnification.
Lower panel: Necrotic patches of cells identified by morphological changes such as loss of membrane integrity, disintegration of cell structure, and complete cell lysis, were typically found on the tumor periphery. 400x magnification.
Figure 6.3. Pharmacodynamic effects of treatment: apoptosis induction. Histological images of peripheral region of the tumor image at 400x magnification. Inset: Autoradioluminography images overlaid on histological images showing drug penetration.
Figure 6.4. Apoptotic index and tumor cell density as a function of cumulative drug release from microspheres: comparison of slow-release, fast-release, and combination microspheres.
Figure 6.5. Spatial drug distribution in tumors over time for the Cremophor and microsphere treatment groups. Inset: Concentration-depth profile (log scale). Concentration-depth profiles obtained from autoradioluminography images (n = 3 or 4).
Figure 6.6. Drug penetration of the Cremophor formulation vs. fast-release microspheres over time. Upper panel: Log-linear drug distribution as a function of distance. Lower panel: Comparison of $W_{1/2}$ over time for the drug treatment groups. $W_{1/2}$ was calculated as $-\ln(2)/k$, where $k$ is the initial slope of the log-linear, drug concentration-depth profile.
Figure 6.7. Penetration of drug-loaded microspheres in peritoneal tumors over time. Fast-release microspheres restricted to tumor periphery at 6 hr and 24 hr with subsequent penetration at 72 hr and 168 hr. Similar distribution pattern was observed with the combination microspheres. In contrast, slow-release microspheres remained on the periphery of the tumor at 72 hr. 100x magnification. Arrows indicate lymphocyte involvement in tumors.
CHAPTER 7

PERSPECTIVES AND CONCLUSIONS

The work in this dissertation has made several contributions in the field of regional drug delivery, particularly intraperitoneal chemotherapy. The extensive nature of this work encompassed many areas of pharmaceutical drug development demonstrating the importance of a multidisciplinary approach to regional cancer chemotherapy.

The initial study evaluated the pharmacokinetics and tissue distribution following IP administration of paclitaxel formulated in Cremophor (Chapter 2). Results from this study showed that paclitaxel formulated in Cremophor was rapidly cleared from the peritoneal cavity after intraperitoneal administration, diminishing the pharmacokinetic advantages of regional drug delivery.

Biodegradable paclitaxel-loaded PLGA microspheres were developed to enhance drug retention in the peritoneal cavity. This alternative paclitaxel formulation was characterized in vitro (Chapter 3). By adjusting the microsphere parameters (e.g. microsphere size, molecular weight of the PLGA polymer, etc.), different drug release profiles could be achieved, resulting in a flexible drug delivery system.
The remaining part of this dissertation focused on the evaluation of the paclitaxel microspheres in vivo. A subsequent pharmacokinetic study was conducted to compare the pharmacokinetics and tissue distribution after intraperitoneal administration of the Cremophor formulation and the microspheres (Chapter 4). The microsphere formulation showed low drug concentrations in plasma while maintaining elevated concentrations in the peritoneal cavity up to 168 hr. The sheer size of the microspheres limited absorption across the peritoneum and reduced drainage by the lymphatic system. This was evident by the observed aggregation of microspheres at the main lymphatic drainage sites of the peritoneal cavity. This heterogeneous peritoneal distribution illustrates a potential ability for passive tumor targeting since peritoneal tumors in both mice and humans frequently metastasize to these lymphatic sites, resulting in a co-localization of drug and tumor.

The overall antitumor efficacy of the paclitaxel microspheres was evaluated through small-scale survival studies (Chapter 5). The paclitaxel microspheres demonstrated greater antitumor activity and longer survival time in mice bearing advanced ovarian or pancreatic tumors compared to the Cremophor formulation. Furthermore, the microspheres demonstrated this antitumor efficacy while reducing treatment frequency, which could result in additional benefits such as higher patient compliance, greater convenience, and fewer complications due to reduced dependence on indwelling peritoneal catheters. Since the two tumor models responded differently to microspheres of two different release rates, it demonstrates the need to tailor the microsphere release profile to the tumor growth kinetics to
optimize antitumor activity. Furthermore, a better understanding of interspecies differences between mice and humans such as natural posture positioning and peritoneal tumor size and growth rate is necessary for clinical evaluations.

The study showed that paclitaxel-induced apoptosis through alteration of the tumor structure can enhance the penetration into solid tumors, but only if the drug is retained in the peritoneal cavity after apoptosis has occurred (Chapter 6). Penetration of the microspheres can prolong drug release directly from within the tumor. Dose fractionation by using two types of microspheres with different release rates resulted in greater drug delivery into solid tumors compared to the Cremophor formulation or individual microsphere treatment. This research could be applicable to other areas such as gene delivery. Low transfection efficiency has been one of the obstacles to successful gene therapy. This may be overcome by enhanced delivery of the oligonucleotides to the tumor through microsphere encapsulation of the oligonucleotide in conjunction with an apoptosis-inducing drug such as paclitaxel.

In conclusion, the research in this dissertation has demonstrated a step-by-step approach in problem solving and translation from a conceptual idea to a tangible application. Using a novel way to enhance regional drug delivery to tumors, the developed microspheres represent an approach to enhance IP therapy for treatment of peritoneal tumors.
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