Development and characterization of a lipid-based nanoparticulate formulation of topotecan and its use in combination with Doxil™ for treatment of relapsed ovarian cancer models

by

NILESH ARUN PATANKAR
B.Pharm., Mumbai University, India, 1999
M.Pharm., Mumbai University, India, 2002
M.Sc., University of British Columbia, Canada, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
in
The Faculty of Graduate Studies
(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA
(VANCOUVER)

December, 2010

ABSTRACT

Platinum-refractory ovarian cancer is considered an incurable disease as current treatments are only palliative. Improvements in treatment will be realized as our understanding of the unique signaling pathways driving disease development increases and new therapeutics targeting these pathways are developed. It's important to recognize, however, that at this time new molecularly targeted agents are not replacing the drugs being used to treat cancer; they are being used in combination with existing standards of care. In light of this, it is important to explore novel approaches using existing agents that are designed to achieve maximum therapeutic benefits in dosage forms that are well tolerated. Like most cancers, ovarian cancer is treated with a combination of drugs selected on the basis of complementary mechanisms of action and non-overlapping toxicities. Synergistic drug combinations can achieve therapeutic effects equal to that achieved with single agents, but at significantly lower and better tolerated doses. The factors that govern synergistic drug:drug interaction are, however, poorly understood and it is argued in this thesis that drug interactions favoring synergy will be influenced by drug exposure time. An effective method to enhance drug exposure time involves the use of drug carriers and a goal of this thesis was to develop an effective combination regimen against recurrent ovarian cancer using a novel lipid nanoparticle (LNP) formulation of topotecan and Doxil®; a LNP formulation of doxorubicin that has already been approved for the treatment of relapsed ovarian cancer. The LNP formulation of topotecan developed through this thesis research, referred to as TopophoreC™, was 2- to-3-fold more toxic than free topotecan, however this product candidate showed significantly better anti-tumor activity when compared to free topotecan administered at equivalent doses. Combinations of this LNP topotecan formulation with Doxil® were therapeutically superior to combinations of free topotecan and Doxil® as

judged in two models of ovarian cancer. On the basis of these studies, it can be concluded that interaction between TopophoreC™ and Doxil® affect the pharmacokinetic behavior of Doxil® however the results provide proof of concept data to support the use of TopophoreC™ and Doxil® combination for treatment of recurrent ovarian cancer.

PREFACE

A work presented in this thesis consists of three manuscripts presented in chapters 2 to 4. Authors include Nilesh Patankar, Malathi Anantha, Euan Ramsay, Dawn Waterhouse, Dita Strutt, Julia Pritchard, Mariska van Grinsven, Maryam Osooly and Marcel Bally. Dr. Marcel Bally is my principle research supervisor at University of British Columbia. Ms. Malathi Anantha, Ms. Dita Strutt and Ms. Maryam Osooly are research assistants, and Ms. Julia Pritchard and Ms. Mariska van Grinsven were co-op students in Dr. Bally's lab who provided technical assistance in some parts of this project. Dr. Euan Ramsay and Dr. Dawn Waterhouse are follow researchers in this lab who provided meaningful comments on the respective manuscripts.

An identification of research question, literature review, experiment design, performance of experiments and data analysis were exclusively done by Mr. Nilesh Patankar under the supervision of Dr. Marcel Bally. All three manuscripts were written and finalized by Mr. Nilesh Patankar after careful review and approval from Dr. Marcel Bally.

All animal experimentation was carried out according to the guidelines of University of British Columbia Animal Care Committee. Approval of these studies was granted under Animal Care Certificate number: A05-1582. Cell culture experiments were approved by University of British Columbia under Biohazard Approval Certificate number: B06-0214.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AA : Atomic absorption spectrometry

ANOVA : Analysis of variance

ATCC : American type culture collection

AUC : Area under curve

Balb/C : Inbred strain of mouse

BWL : Body weight loss

CI : Combination index

CH : Cholesterol

CHE : Cholesteryl hexadecyl ether

Cu : Copper

DMPC : 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

DNA : Deoxyribobucleic acid

Doxil : Liposomal doxorubicin

DSPC : 1,2-distearoyl-*sn*-glycero-3-phosphocholine

EDTA : Ethylenediamine-tetra acetic acid

FBS : Fetal bovine serum

FDA : Food drug administration

^{3}[H] : Tritium radiolabel

H_2SO_4 : Sulfuric acid

HBS : HEPES buffered saline

HBSS : Hanks balanced salt solution

HEPES : N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

HPLC : High performance liquid chromatography

IC : Inhibitory concentration

i.p. : Intraperitoneal

i.v. : Intravenous

LNP : Lipid nanoparticle

LUV : Large unilamellar vesicle

MLV : Multilamellar vesicle

MEP : Median effect principle

MLV : Multilamellar vesicle

Mn : manganese

MTD : Maximum tolerated dose

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaOH : Sodium hydroxide

PALS : Phase Analysis Light Scattering

PARP : Poly-ADP ribose polymerase

PBS : Phosphate buffer saline

PEG : Polyethylene glycol

PK : Pharmacokinetic

S.C. : Subcutaneous

S.D : Standard deviation

S.E.M. : Standard error of mean

SCID : Severely compromised immunodeficient

SHE : Sucrose-HEPES-EDTA

SUV : Small unilamellar vesicle

$t_{1/2}$: Half-life

Tc : Phase transition temperature

TGI : Tumor growth increase

UV : Ultraviolet

Wt. : Weight

ACKNOWLEDGEMENTS

First and foremost I would like to thank my research supervisor Dr. Marcel Bally for showing faith in me and for accepting me as a graduate student in his lab. I am extremely grateful for his support and guidance in developing scientific creativity in me. I have learned a lot during my graduate studies under his supervision.

I would like to thank my co-supervisor Dr. Helen Burt and members of my research committee Dr. Pieter Cullis, Dr. Karen Gelmon, Dr. Dawn Waterhouse and Dr. Mary Ensom for their continuous and timely support and insights in developing my research project.

To my colleagues in the lab: A big thank you to Malathi for the help in HPLC work and for teaching me how to make liposomes. Special thanks to Hong for training me in tissue culture techniques. A very big thank you to the entire staff of animal care facility specially, Dana, Maryam, Dita, Young Joo Yang and Christina for their kind help in conducting endless animal studies. I would like to thank my office mates Emmanuel, Mihaela and Anitha for all those "thoughtful" discussions and funny moments we shared that kept the atmosphere always cheerful.

I would like to thank summer research students and co-op students specially Julia and Mariska for their assistance in the parts of this project. A big thank you to fellow graduate students Catherine, Maite, Jessica, Jesse and all the members of Experimental Therapeutics who have helped me at some or the other stage and for keeping the work atmosphere always positive.

Thank you Corrinna and Margaret for giving me the opportunity to assist your group in helping with the clinical trials. It was a very valuable experience.

I would like to thank my family and friends. It has always been through their love and support that I have accomplished all that I have.

I express my appreciation and gratitude to the Faculty of Pharmaceutical Sciences, UBC for providing me an opportunity to avail quality training in terms of research.

Thank you to BC Innovation Council and University of British Columbia for providing the financial support during the course of my training.

Finally, my biggest thanks go to my wife Vinaya for her throughout support and patience in countless things during this time and for always being there on my side.

This thesis is dedicated to
my parents, my sister and
my wife

1. INTRODUCTION

1.1 THESIS OVERVIEW

Ovarian cancer is the most common gynecological malignancy and since it is often detected only after it has progressed to an invasive or metastatic stage, patients with ovarian cancer typically have a poor prognosis. Treatment options consist of aggressive cytoreductive surgery that is usually combined with radiation and drug combinations which include cisplatin or carboplatin (Gurney, Crowther et al. 1990; Conte, Bruzzone et al. 1991; Alberts, Green et al. 1992; Swenerton, Jeffrey et al. 1992; Greenlee, Hill-Harmon et al. 2001; Parmar, Ledermann et al. 2003). Although initially responsive to this treatment, ovarian cancer patients relapse frequently (in more than 60% of the cases) (Greenlee, Hill-Harmon et al. 2001). The relapsed disease is typically no longer responsive to platinum and these patients must then be treated with a second line of chemotherapeutic agents which at the present time includes drugs such as topotecan, oral etoposide, gemcitabine or liposomal doxorubicin (Ahmad and Gore 2004; Herzog 2004; Thigpen, Aghajanian et al. 2005). These treatments are not curative and there is a critical need to develop more effective treatment options for ovarian cancer patients both in the primary and relapsed setting. Work presented in this thesis was aimed at developing an effective treatment option that can benefit ovarian cancer patients that have relapsed following treatment with platinum containing drug combinations. The approach considers the use of drug combinations comprising agents that are already approved for use as single agents to treat recurrent ovarian cancer when front line therapy fails.

Historically drug combinations have evolved on the basis of clinical data obtained with agents known to exhibit some activity when used as single agents (Ramaswamy 2007)**.** The selection of drugs to be used in a combination regimen was further rationalized by identifying agents that exhibited different and perhaps complementary mechanisms of action as well as

different toxicity profiles. The goal was to achieve greater tumor cell kill which would then translate into significant increases in overall survival rates. Ideally, the outcomes would be achieved using drug doses that were well tolerated, but these combinations were still built around the concept that optimal treatment outcomes could only be achieved when the drugs were used at doses that engendered significant toxic side effects.

More recently there has been a great deal of interest in the identification of drug combinations that produce synergistic gains in therapeutic activity (Baek, Kim et al. 2006; Mayer, Harasym et al. 2006; Zimmermann, Lehar et al. 2007; Secord, Blessing et al. 2008; Bookman, Brady et al. 2009). Synergy has been defined in many ways but one of the most practical definitions is based on obtaining therapeutic benefits that are significantly better than those which could be expected on the basis of the activities of the agents when used alone. This definition highlights two points: 1) synergistic drug combinations could achieve therapeutic effects comparable to those achieved with the single agents, but at significantly lower and better tolerated drug doses; and 2) synergistic drug combinations could be administered at maximum tolerated doses to achieve treatment outcomes that are significantly better than what are currently achieved. Although the potential value of identifying synergistic drug combinations has been recognized for a long time (DeVita, Hellman et al. 1989; Schinazi 1991), the factors which influence drug:drug interactions leading to synergy are not well understood (Berenbaum 1989).

This thesis explores the role of drug exposure time on achieving synergistic drug:drug interactions. The drugs which were used, topotecan and doxorubicin, are known to have therapeutic effects as single agents when used to treat relapsed ovarian cancer. As described in Chapter 4, *in vitro* assays can be used to study drug:drug interactions where therapeutic effects are analyzed and compared to the effects achieved when the drugs are used alone. These cell

based screening assays provide an effective way to test for synergistic interactions and to address the question of whether exposure time plays an important role in generating synergistic effects. This question has not been considered prior to the work described in this thesis. Importantly, however, the studies outlined in this thesis also wanted to assess how exposure time influenced treatment outcomes in appropriate animal models of ovarian cancer.

It is more challenging to test this concept *in vivo*. One approach that has been used to achieve extended drug exposure time *in vivo* has involved the use of drug carriers. In fact one of the drugs approved for second line therapy of ovarian cancer is Doxil®, a lipid nanoparticle (LNP) formulation of doxorubicin (Martin 1998; Gabizon 2001). This drug carrier formulation of doxorubicin has several therapeutic benefits including reduced toxicity (Uziely, Jeffers et al. 1995), significantly longer circulation lifetimes (Martin 1998) and increased delivery of the doxorubicin to regions of tumor growth (Wu, Da et al. 1993; Yuan, Leunig et al. 1994). Clinical development of this LNP formulation was therefore based, in part, on evidence that increasing drug levels in regions of tumor growth for extended time periods can improve treatment outcomes. As indicated above, a key question to be addressed in this thesis was whether increasing drug levels of two agents for extended time periods would result in better treatment outcomes and whether the improvements achieved could be related to synergistic interactions. In order to address this question, research described in Chapter 3 of this thesis was also focused on development of a novel LNP of topotecan.

The rational for selecting topotecan included: i) topotecan is approved for use in the treatment of relapsed ovarian cancer (Broom 1996; Herzog 2002); ii) combinations of Doxil® and topotecan are being evaluated in ovarian cancer patients that have relapsed (Main, Bojke et al. 2006; Smith, Johnson et al. 2006), and iii) LNP formulations of topotecan have been

described in the literature and are being considered for use in the clinic (Drummond, Noble et al. ; Taggar, Alnajim et al. 2006; Dadashzadeh, Vali et al. 2008). Although other LNP formulations of topotecan have been described previously, original research described in this thesis adapted a novel formulation technology proven to be uniquely beneficial for irinotecan, a drug that is chemically related to topotecan (Ramsay, Alnajim et al. 2006). This LNP formulation of irinotecan relied on the use of copper to form a coordination complex with irinotecan and this complex formation was critical to achieving optimal drug retention in the LNP formulation following intravenous administration. Before this technology was to be adapted for use with topotecan, it was important to determine the minimum amount of copper that could be used to achieve improvements in drug retention. This research, described in Chapter 2, focused on irinotecan since there was a great deal already understood about formulation methodology and it was reasonable to assume that information gained by studying irinotecan would facilitate development of the formulation approach for topotecan. Given the availability of an optimized LNP topotecan formulation and the clinically useful LNP formulation of doxorubicin (Doxil®), it was possible to assess whether combination of the LNP formulations of topotecan and doxorubicin, that result in significant increases in drug exposure time, would provide treatment outcomes, as measured in murine models of ovarian cancer, that were significantly better than that which could be expected on the basis of the agents used alone.

To facilitate a better understanding of the research described in this thesis, a summary of current knowledge in several broad research areas; including: (i) ovarian cancer and its treatment options, (ii) combination chemotherapy in ovarian cancer, (iii) use of nanoparticulate drug carriers and liposomes as nano-carriers in ovarian cancer therapy; has been provided in this

introduction. In order to place this information into context, the overall working hypothesis for this thesis research and the thesis research objectives are provided below.

1.2 WORKING HYPOTHESES

Use of combination therapy in the treatment of patients with platinum refractory ovarian cancer will result in improved treatment outcomes if the combination is designed in a manner that achieves therapeutic synergy between the selected drugs which, when used alone, have proven therapeutic value as single agents in this patient population. Since the therapeutic effects of drugs used to treat cancer cells in tissue culture are highly dependent on drug concentration and exposure time, these variables will be critically important in achieving optimal therapeutic outcomes for the combinations when given to patients and synergistic interactions between selected drugs will also be dependent on drug concentration and exposure time. Drug carriers are important tools to achieve increased drug concentrations at sites of tumor growth over extended time periods and LNP formulations are a clinically viable drug carrier technology.

As such, it is hypothesized that an approved LNP formulation of doxorubicin referred to as Doxil® when used in combination with a LNP formulation of topotecan will achieve significantly better treatment outcomes when used to treat animal models of ovarian cancer. This combination will achieve improved therapeutic effects at lower drug doses that are better tolerated by patients and as such these combinations will be ideally suited for use in the context of emerging targeted therapies that will ultimately lead to a time when platinum refractory ovarian cancer is treated with curative intent.

1.3 SPECIFIC OBJECTIVES

Since irinotecan is a camptothecin derivative with similar structural and physico-chemical properties as that of topotecan, an aim of this thesis research was to determine whether

an optimized drug loading method used for irinotecan could be effectively used to prepare an optimized LNP formulation for topotecan. The therapeutic effects of this LNP topotecan formulation could then be assessed alone and in combination with Doxil® in relevant pre-clinical models of ovarian cancer. The specific aims of this thesis research included:

1. Determining the minimum concentration of copper required for preparation of a LNP formulation of irinotecan and to assess how the presence of copper influenced drug dissociation from the LNP following intravenous administration;
2. Using the information gained from the first aim to develop a LNP formulation of topotecan and to establish whether the resulting formulation is therapeutically superior to topotecan (administered in the clinically approved formulation) when used to treat pseudo orthotopic models of ovarian cancer;
3. Evaluating the effect of topotecan and doxorubicin (used alone and in combination) exposure time on ovarian cancer cell proliferation/viability and to analyze these data using the median effect methodology developed by Chou and Talalay to determine whether treatment effects could be due to synergistic drug-drug interactions; and
4. Evaluating the use of the LNP formulation of topotecan in combination with Doxil® for treatment of pseudo-orthotopic models of ovarian cancer.

1.4 OVARIAN CANCER

Ovarian cancer is the most common gynecologic cancer and the fifth most common lethal malignancy among women. The symptoms manifested in the early stages of the diseases are minimal or nonspecific. As a result, the disease is detected at later stages of disease development in most cases. This means that these patients have a poor prognosis as reflected by the very poor 5-year survival rate of 30% (Main, Bojke et al. 2006). For 2010, it is estimated that 2600

Canadian women will be diagnosed with new cases of ovarian cancer and 1750 women previously diagnosed with ovarian cancer will die due to disease progression (Canadian Cancer Encyclopedia from the Canadian Cancer Society).

1.4.1 Ovarian cancer classification and staging

Details regarding the origin and molecular events leading to development and progression of ovarian cancer are currently under extensive investigations. A better understanding of ovarian cancer is arising, in part, because of advances in gene sequencing technology that are essential to define the ovarian cancer genome. This information will, in turn, identify the genetic mutations that contribute to disease development as well as aid in the identification of disregulated intracellular pathways that combine to: i) promote an inability of these cancer cells to undergo programmed cell death, ii) lead to formation of new blood vessels essential for growth of the tumor, iii) promote survival following treatments by development of mechanisms of resistance, iv) promote invasion; and v) drive metastatic spread of the disease (Rudin and Thompsan 2002; Nelson, Tan et al. 2004; Dingli and Nowak 2006; Folkman 2008). With this understanding, new treatment options will emerge and patients will be treated in a manner that considers the unique features of the patient's own disease. Personalized medicines for ovarian cancer are already being considered (Kobel, Kalloger et al. 2008), but it is worth noting that the future of cancer treatment and development of personalized medicines depends on existing histological classification systems, refined with emerging molecular classification systems. Similarly, new molecularly targeted drugs designed to affect key drivers within the ovarian cancer cells are proving beneficial primarily in the context of existing treatment modalities. This is an important point to bear in mind when considering the research described in this thesis. Developing improvements in how existing drugs are currently used is as important as

efforts leading to the development of new, more selective and specific, drugs. In the majority of examples it would appear that targeted therapies are providing real clinical benefit only in the context of existing therapies.

Ovarian cancer is a heterogenous disease and thus should never be referred to in general terms when planning treatments. The World Health Organization (WHO) published a histological classification system for ovarian cancer based on the histogenesis of the normal ovary (Kaku, Ogawa et al. 2003). Accordingly, ovarian cancers can be categorized based on their origin into: i) epithelial tumors which originate from the surface epithelium of the ovary; ii) germ cell tumors which originate from the ovarian germ cells, and iii) stromal cell tumors which originate from cells of ovarian stroma. Of these, epithelial tumors are the most common and constitute more than two thirds of all ovarian tumors. Epithelial ovarian tumors, depending on further histological assessments, can be further sub-classified into serous, mucinous, endometrioid, clear cell, transitional, squamous cell, mixed epithelial tumors and undifferentiated carcinomas (Kaku, Ogawa et al. 2003). Adding biological attributes to this classification system allows for even greater refinement and clearly highlights why epithelial ovarian cancer should be considered as a diverse group of tumors with unique appearance and genetic features (Kurman and Shih Ie ; Kobel, Kalloger et al. 2008). This belies current treatment plans that rely on relatively uniform treatments for patients that clearly have a diverse set of diseases. What is common among these patients is the common site of diagnosis and the fact that these patients have a proliferative disorder that is life threatening.

As indicated above high-grade serous, clear cell, endometrioid, mucinous, and low-grade serous subtypes of ovarian cancer each represent different and unique diseases. Further subclassification of these diseases is now being pursued based on genetic events that correlate to

outcomes and/or response to treatments. Although exciting, these efforts are being partially undermined by the disease heterogeneity itself. Ovarian cancer subtypes may have unique markers that are difficult to use prognostically given the information known about the patient population being studied. If, for example, the cohort of ovarian cancer patients being evaluated comprises a mixture of different subtypes, then the power to define the utility of the measured feature is lost. This challenge could eventually be addressed through large cooperative groups that can examine genetic events in the context of large populations but even this will be a challenge given the lack of uniform treatment policies. In the mean time, patients with diverse diseases will have to be grouped based on rather simple features and these patients will continue to be treated in a manner that can offer best outcomes based on average responses in the population. This is why existing antiproliferative drugs will continue to have a broad beneficial effect for patients with ovarian cancer and the likely first benefits of genetic evaluations will be for those patients that exhibit biological/genetic features that accurately predict for poor treatment response.

Regardless, recent evidence (Kobel, Kalloger et al. 2008) suggested using a carefully selected set of biomarkers capable of differentiating ovarian cancer disease subtype so that outcomes will be most accurate for a given subtype regardless of the stage of disease at first diagnosis. This was an interesting outcome suggesting that accurate sub-classification needs to be done with staging in order to establish best patient management plans. The corollary to this is that staging in the absence of correct disease classification can lead to the generation of flawed treatment plans. Staging of ovarian cancer is done at the time surgery and requires a thorough evaluation of pelvic and abdominal peritoneal contents. The International Federation of Gynecology and Obstetrics adopted a staging system for ovarian carcinoma that is summarized

in Table 1.1 (see Chobanian and Dietrich 2008). 90% of stage I ovarian cancer patients can be effectively treated with the conventional surgery and chemotherapy; however, only 20% of ovarian cancers are detected at stage I because of the lack of effective screening methods (Bast, Hennessy et al. 2009). Most women diagnosed with ovarian cancer have a very poor prognosis because they are not diagnosed until stage III or IV. At these stages, the cancer usually is metastasized and spread to other parts of the body and this metastatic disease is inherently more difficult to treat than local disease.

Current methods of screening include transvaginal ultrasound (TVS), serum markers and the combination of two. Cancer antigen 125 (CA 125) is a commonly tested serum antigen capable of detecting epithelial cancers but it lacks the required sensitivity and specificity to be used as a single marker for the presence of an early stage disease (Helzlsouer, Bush et al. 1993; Chobanian and Dietrich 2008). Combination of TVS and CA 125 or long term monitoring of CA 125 can increase the specificity but only to a modest level. New markers are actively being developed in an effort to improve ovarian cancer screening. In June of 2010, the United States Food and Drug Administration (US-FDA) approved a chemiluminescent microparticle immunoassay that measures HE4 antigen (human epididymis protein 4) in serum. HE4 measurements can be used in combination with other clinical data to monitor recurrence or progression of epithelial ovarian cancer. It is expected that testing for both the CA125 and HE4 biomarkers could potentially improve the detection of ovarian cancer, particularly in its early stages, when treatment is most effective. It is already known that biomarkers can assist in sub-classifying a patients disease (Kobel, Kalloger et al. 2008) and in the future these and other biomarkers will be used along with surgical staging to help develop more patient specific treatment plans.

1.4.2 Ovarian cancer treatment at present and the need for better therapy

Ovarian cancer treatment requires a multi-faceted approach consisting of complete surgical staging and optimal cytoreductive surgery followed by radiation and/or chemotherapy (depending on the stage of the disease or tumor dissemination) (Guarneri, Piacentini et al. 2010). Comprehensive staging, as described in table 1.1, usually involves full assessment of abdomen and pelvis, random biopsies and lymph node dissection (except in Stage I). Staging is followed by optimal cytoreductive or 'debulking' surgery, which involves either complete removal of tumor (Stage I and II) or residual disease less than 1 cm (Stage III and IV). It has been shown in a number of studies that maximal cytoreduction is one of the most important prognostic factors influencing patient survival and that post-surgical residual tumor of less than 1 cm mass has a significant impact on patient outcome (Griffiths 1975; Allen, Heintz et al. 1995; Bristow, Tomacruz et al. 2002). In addition to residual mass, surgical outcome also depends on the surgical skills. An analysis of more than 3000 ovarian cancer patients showed that patients outcomes were significantly better when treated by gynaecologic oncologists or general gynaecologists when compared to general surgeons (Earle, Schrag et al. 2006).

The strategy of interval debulking may be used in patients who are not adequately debulked at the time of initial surgery or in whom an initial debulking surgery was not attempted. This strategy involves administering several courses (usually at least 3) of post-operative chemotherapy prior to optimal surgical treatment, which is then followed by more cycles of chemotherapy. A European randomized study showed that this approach improves outcome in patients with advanced cancer; however the results were not confirmed in a similar study performed in US (van der Burg, van Lent et al. 1995; Rose, Nerenstone et al. 2004). A meta-analysis of the two studies did not find conclusive evidence for survival benefit following

interval debulking but noted an apparent effect in patients whose primary surgery was either not performed by gynaecologic oncologists or was less extensive (Tangjitgamol, Manusirivithaya et al. 2009).

Post-operative chemotherapy is indicated in almost all patients with ovarian cancer. First line chemotherapy consists of platinum based compounds, cisplatin or carboplatin, in combination with paclitaxel (Jackel, Fuchs et al. 2002; Ozols, Bundy et al. 2003). Carboplatin is preferred over cisplatin as several randomized trials have shown that the drug is equally effective but offers an important advantage of lower incidence of non-hematological toxicities (particularly emesis, neurotoxicity and nephrotoxicity) (Gurney, Crowther et al. 1990; Conte, Bruzzone et al. 1991; Alberts, Green et al. 1992; Swenerton, Jeffrey et al. 1992). The carboplatin-paclitaxel combination has become a standard regimen and is routinely administered via intravenous (i.v.) route every 3 weeks (day 1 of 21-day cycle). In the recent years, various strategies to improve this first-line therapy have been explored, including addition of novel agents to the carboplatin-paclitaxel combination. However, most of these approaches have either failed to show a significant benefit on patient outcome or at best, demonstrated only a marginal improvement (Bertelsen, Jakobsen et al. 1987; 1992; Bookman, Brady et al. 2009).

Since relapsed disease is usually confined to the peritoneal cavity, intraperitoneal (i.p.) administration of chemotherapeutic agents has been evaluated to assess whether direct administration of these agents in the peritoneal cavity offers any advantages to standard i.v. administration. Several Phase III studies comparing i.v. administration to combined i.v.-i.p. administration as first-line therapy after initial surgery have shown that i.p. administration of agents like cisplatin and paclitaxel is superior to the i.v. route and is also associated with improvement in survival (Armstrong, Bundy et al. 2006; Elit, Oliver et al. 2007; Hess, Benham-

Hutchins et al. 2007). These results could be explained by higher local concentrations, increased tumor exposure times and reduced systemic toxicities with i.p. administration (Guarneri, Piacentini et al.2010). However, in spite of better outcomes, i.p. treatment has not yet become the standard route of therapy. It has been found that even though systemic toxicities are reduced with i.p. administration, treatment-related complications such as catheter complications, nausea, bowel perforation and abdominal discomfort are much higher, resulting in major patient compliance issues (Guarneri, Piacentini et al 2010.).

As indicated in the previous section, investigators are gaining a better understanding of the molecular features that influence ovarian cancer development and progression. Thus newly developed targeted agents are being tested in combination with existing chemotherapy. In several examples, existing targeted therapeutic agents already approved for use in humans have been studied in ovarian cancer patients with tumors that express the target. The results of these studies have thus far been disappointing, but this could be due to the fact that the targeted agents were not tested against an appropriately identified patient sub-population. Targeted agents such as trastuzumab (a monoclonal antibody targeted against HER2 or human epidermal growth factor receptor 2), EGFR (epidermal growth factor receptor) inhibitors and imatinib, a multikinase inhibitor, have showed some, but limited, clinical benefits in ovarian cancer (Bookman, Darcy et al. 2003; Schilder, Sill et al. 2005; Coleman, Broaddus et al. 2006). For example, erlotinib given in combination with carboplatin in a phase II study showed a 57% objective response in platinum sensitive patients but no response in platinum resistant patients (Hirte, Oza et al 2010). Cetuximab (a EGFR targeted monoclonal antibody) studied in combination with carboplatin showed 35% response rate in patients with EGFR positive tumors (Secord, Blessing et al. 2008). Gefitinib (a EGFR kinase inhibitor) combined with topotecan

showed no response rate in platinum resistant patients (Slomovitz, Coleman et al. 2006). Alternatively, when gefitinib was combined with vinorelbine and oxaplatin there was a 90% overall response in platinum sensitive recurrent ovarian cancer patients and 26% overall response in platinum resistant patients (Mavroudis, Efstathiou et al. 2004). In the case of trastuzumab recent studies suggest that benefits may be better realized in appropriately selected subtypes of the disease (McAlpine, Wiegand et al. 2009). These investigators previously established that women with mucinous carcinomas responded poorly to the standard treatment regimen of paclitaxel and carboplatin. They also demonstrated that a significant percentage (about 20%) of these patients overexpressed HER2 and it was this subpopulation that may receive the greatest benefits from use of trastuzumab therapy.

Bevacizumab is a vascular endothelial growth factor (VEGF) inhibitor that exhibited some activity when used as a single agent and showed significantly improved activity when combined with the chemotherapy (Burger, Sill et al. 2007). Another interesting strategy concerns interference of DNA repair through inhibition of poly-ADP-ribose polymerase (PARP). PARP promotes single strand break repairs in DNA and inhibitors of this enzyme have shown interesting activity in women with BRCA1 or BRCA2 mutations when used in combination with standard agents known to cause DNA damage, such as carboplatin (Farmer, McCabe et al. 2005). Importantly, a recent clinical study completed at the BC Cancer Agency indicated that the PARP inhibitor olaparib exhibited significant single agent activity in patients with high grade serous ovarian cancer. In patients with BRCA mutations the response rate was 41% (Gelmon, Hirte et al. 2010). This study needs to be expanded to include a larger patient population, but it clearly sets the stage for evaluating this PARP inhibitor in combination with carboplatin containing chemotherapy in selected ovarian cancer patient subpopulations.

In spite of advances in our understanding of ovarian cancer, the fact remains that relapsed ovarian cancer is not effectively treated. Tumor recurrence and emergence of platinum refractory/resistant disease results in a poor long-term overall survival rate for these patients (Armstrong 2002). Treatment options for the recurrent disease are dependent on the response rate to the first line treatment and the treatment free interval (Delgado, Oram et al. 1984). Depending on the response to the first line platinum-based therapy and the time of relapse, ovarian cancer patients are broadly categorized to have: i) Platinum-sensitive disease (those with a relapse-free interval of at least six months) which may show a good response if re-challenged with platinum-based chemotherapy (Parmar, Ledermann et al. 2003; Pfisterer, Plante et al. 2006); ii) Platinum-resistant disease (those who relapse within six months of first line therapy); and iii) Platinum-refractory disease (patients who progress on first line platinum therapy). With this information in hand, the development and evaluation of new cytotoxic agents and molecular targeted drugs has been pursued. Drugs such as topotecan, oral etoposide, gemcitabine, altretamine (hexamethylmelamine), vinorelbine and liposomal doxorubicin (Doxil®) have been used extensively as second-line chemotherapy. However, demonstrated response rates from these chemotherapeutic regimens remain low in the platinum refractory/resistant setting (Gordon, Fleagle et al. 2001) and only altretamine, gemcitabine, Doxil® and topotecan are currently approved by the US-FDA as single agents for treatment of ovarian cancer after a first -line therapy fails (Herzog 2004).

It is interesting to note that second line chemotherapy for refractory/resistant ovarian cancer typically involves the use of single agents (Broom 1996; Creemers, Bolis et al. 1996; Armstrong 2002; Horowitz, Hua et al. 2004; Burger, Sill et al. 2007) and this likely reflects the lack of clinical evidence supporting the benefits of the combinations and/or concerns about using

combinations where treatment toxicities may be a concern due to the poor health status of patients with advanced disease. As proposed in this thesis, development of drug combinations in which synergistic effects are achieved has the potential of being able to produce therapeutic results equal to that which can be achieved with a single agent, but at lower and better tolerated doses. The primary goal of agents used in secondary treatment is to provide disease control without compromising the patient's quality of life. Patients often experience multiple recurrences in spite of initial responses to second-line agents and, thus, there is a great need to develop more active chemotherapy treatments that are well tolerated and can be used in concert with emerging molecular targeted agents. This is one of the primary goals of this thesis research.

1.4.3 Topotecan and Doxil® and their use in ovarian cancer

As outlined above, topotecan and Doxil® have already been approved as single agents for use in the treatment of relapsed ovarian cancer. This thesis research has focused on these agents with the goal of establishing proof-of-concept data supporting the use of a combination of Doxil® with a LNP formulation of topotecan first described in this thesis research. When reviewing the results it is important to have an understanding of the active agents and their use as single agents, alone and in combination, for treatment of ovarian cancer.

1.4.3.1 Topotecan

Topotecan hydrochloride is a water soluble, semi-synthetic camptothecin analog. It is light yellow to greenish powder with a melting point in the range of 213-218ºC. The ultraviolet absorption spectrum of topotecan in methanol include maximas of 207, 224, 269, 296, 318, 332, 371, and 384 nm. It has a five ring heterocyclic structure with a α-hydoxylactone within its E-ring (Figure 1.5). The lactone form of the drug is active; however, like most camptothecins this E-ring hydrolyzes rapidly to a ring open form of the drug within minutes when maintained at

physiological pH (pH >7). Hydrolysis of lactone ring involves conversion of lactone to corresponding carboxylate. The opening of lactone ring results in the loss of *in vitro* activity and also diminished *in vivo* anticancer activity (Jaxel, Kohn et al. 1989). An intact lactone ring has been found to be essential for passive diffusion of this drug into cancer cells and also for its interaction with target topoisomerase I enzyme (Hsiang, Hertzberg et al. 1985; Hertzberg, Caranfa et al. 1989). Thus, strategies that can protect the intact lactone conformation of the E-ring should provide therapeutic benefits. As indicated later in this introduction, drug carrier formulations in general and LNP formulation in particular provide an approach that can help protect the lactone form of the drug from hydrolysis.

Topotecan, like the other water soluble camptothecin analog irinotecan, is a topoisomerase 1 inhibitor. Topoisomerase 1 (TOP1) (Hsiang, Hertzberg et al. 1985) is an essential enzyme present in higher eukaryotes that plays an important role during DNA replication process. During replication, DNA which exists as a supercoiled double helix unwinds to generate single strands that act as a template for synthesis of new strands. TOP1 forms a transient cleavable complex with DNA and introduces nicks in the DNA to relieve torsional stress that occurs when the helix unwinds. This is a transient complex and the enzyme is released allowing re-ligation of the new strand. Topotecan binds to the TOP1 nicked DNA complex and stabilizes it; thus preventing re-ligation of the nicked strand. This, in turn, promotes formation of irreversible double strand breaks (Hsiang, Hertzberg et al. 1985; Hsiang and Liu 1988; Hsiang, Lihou et al. 1989). The cytotoxic effects of topotecan, and other camptothecins analogues, are thus primarily obtained as the tumor cells go through the *s*-phase (DNA synthesis) of the cell cycle. *In vitro* studies have shown that cells in the *s*-phase are 100-1000 times more sensitive to camptothecins than when they are in G1 or G2 (Li, Fraser et al. 1972). As shown in Chapter 4 of

this thesis, the activity of topotecan is highly dependent on ensuring that the drug is available in its lactone form for extended time frames. This behavior highlights the need to develop optimized carrier formulations that can help stabilize the drug, while also engendering increases in drug exposure time following intravenous administration; a goal emphasized by the research summarized in Chapter 3.

Topotecan was approved in 1996 by the US-FDA and in 1997 by Health Canada for treatment of metastatic carcinoma of the ovary and relapsed small cell lung cancer after failure of first line chemotherapy. It is supplied as lyophilized powder (to be reconstituted) for injection under the brand name of Hycamtin®. Topotecan has also shown activity in gliomas, acute myelogenous leukemia, multiple myeloma, neuroblastoma, pancreatic cancer, retinoblastoma (Broom 1996). Recommended dose of Hycamtin is 1.5 mg/m^2 by i.v. infusion for 30 min daily for 5 consecutive days and this is repeated every 21 days. In the absence of tumor progression 4 courses of therapy are recommended. The drug can also be given in an orally active capsule form. The injectable topotecan formulations shows a terminal half-life of 2-3 hours following i.v. administration and its plasma area under the curve (AUC) has been observed to be dose proportional (Lorusso, Pietragalla et al. ; Chen, Lu et al. 2007). Plasma protein binding of topotecan is low (~35%) and this highlighted one of the unique properties of this camptothecin analog. More specifically, it is known that the lactone form of the parent drug camptothecin rapidly converts to the carboxylate form of the drug which exhibits strong binding to human serum albumin (Mi, Malak et al. 1995). Topotecan in its lactone or carboxylate form exhibits no association with human serum albumin and its activity against cells in culture is not influenced by the presence of serum albumin (Mi, Malak et al. 1995). Topotecan has been shown to undergo metabolism by liver microsomal enzymes to N-desmethyl topotecan. This form of the drug

exhibits reduced anti-tumor activity when compared to the parent compound (Rosing, van Zomeren et al. 1999). However, it should be noted that 25-90% of the administered topotecan dose is excreted in the urine from patients as the parent drug.

The dose-limiting toxicities of topotecan, whether given in the oral dosage form or as an intravenous drug, is myelosupression associated with neutropenia (97%), leukopenia (97%), anemia (89%), and thrombocytopenia (69%) that can have serious clinical consequences (ten Bokkel Huinink, Gore et al. 1997; Bookman, Malmstrom et al. 1998). Non-hematological toxicities are usually mild and easily managed in the clinic. These include nausea and vomiting, mucositis, alopecia, skin rash and fever (ten Bokkel Huinink, Gore et al. 1997; Bookman, Malmstrom et al. 1998).

1.4.3.2 Doxorubicin and Doxil® a LNP formulation of doxorubicin

Doxorubicin is known to be active in the treatment of ovarian cancer and its use in combination with cisplatin has been evaluated previously in this patient population (Vermorken, Harper et al. 1999). In fact a combination of cisplatin, adriamycin (aka doxorubicin) and prednisone (CAP cocktail) was used as a standard of care for a time period prior to the clinical development of taxanes. The use of doxorubicin introduced significant toxicities in patients and with the development of taxanes, such as paclitaxel, there was little incentive to revisit the role of doxorubicin in ovarian cancer until Doxil® became available (du Bois, Luck et al. 2000). Doxil® is a LNP formulation of doxorubicin that is better tolerated and is capable of exposing the ovarian cancer to the drug for extended time periods. Doxil® demonstrated better therapeutic effects when compared to free doxorubicin in multiple models of cancer and early in its clinical development, it provided robust therapeutic response rate (26%) in patients with refractory ovarian cancer (Muggia 1997).

1.4.3.2.1 Doxorubicin

Doxorubicin is an anthracycline antibiotic obtained from *Streptomyces peucetius*. Doxorubicin hydrochloride salt exists as hygroscopic, orange crystalline powder. It is soluble in water, methanol and aqueous alcohols and has a melting point between 204-205°C. Doxorubicin has an absorption maxima of 233, 252, 288, 479, 496 and 529 (Bouma, Beijnen et al. 1986). Doxorubicin molecule consists of an aglycone hydroxylated tetracycline quinine chromophore, and daunosamine sugar residue moieties in its structure (Minotti, Menna et al. 2004) (Figure 1.6). The anthracycline ring is lipophilic, but multiple hydroxyl groups adjacent to the amino sugar at the saturated end of the ring system produces a hydrophilic center. Doxorubicin has multiple pKa's: 1) the amino group in the sugar moiety (pK_1=8.15), 2) the phenolic group at C_6 (pK2=13.2), and 3) the phenolic group at C_{11} (pK_3=10.16) (Bouma, Beijnen et al. 1986; Fiallo, Tayeb et al. 1998). Variations in the chromophore groups lead to changes in the drug's absorption spectra (Bouma, Beijnen et al. 1986). For example, deprotonation of phenolic groups on chromophore can result in changes in UV, visible and circular dichroic spectra (Fiallo, Tayeb et al. 1998). pH or binding ions can also alter the absorption spectra of doxorubicin. Thus, doxorubicin in solution appears orange at pH 7, violet at pH 11 and blue at pH 13 (Fiallo, Tayeb et al. 1998). It also has a tendency to self associate due to the interactions between the planar aromatic rings of individual molecules. The molecule is amphoteric, with acidic phenolic groups and a basic sugar amino group. It is easily hydrolyzed *in vivo* to liberate the biologically active aglycone moiety.

Doxorubicin, like other anthracyclines, is known to interfere with a number of biochemical and biological functions within eukaryotic cells. For this reason the precise mechanism of doxorubicin's anticancer or anti-proliferative activity is not completely understood

even though the drug has been in clinical use for more than 40 years. Doxorubicin is known to form a complex with the DNA by intercalation of its planar rings between nucleotide base pairs and this adversely affects DNA synthesis, DNA-dependent RNA synthesis as well as protein synthesis (Swift, Rephaeli et al. 2006). Another notable mechanism of activity involves topoisomerase 2 (TOP2) inhibition. Like TOP1, TOP2 plays an important role during the process of DNA replication and synthesis. Doxorubicin acts by stabilizing the DNA-TOP2 complex after it has broken the DNA chain for replication. This prevents the DNA double helix from being resealed and thus stops the process of replication (Tewey, Rowe et al. 1984; Burden and Osheroff 1998,). Doxorubicin is also known to be involved in oxidation/reduction reactions to produce free radicals (Minotti, Menna et al. 2004), which can, in turn, react with molecular oxygen to generate highly reactive superoxide, hydroxyl radicals and hydrogen peroxide. Free radical formation has been implicated in doxorubicin cardiotoxicity (Minotti, Menna et al. 2004). Doxorubicin's cardiotoxicity may also be associated with its ability to bind cardiolipin which is an essential component of inner mitochondrial membrane in the heart tissue. Cardiolipin plays a key role in mitochondrial energy transduction (Nicolay, Timmers et al. 1984; Nicolay, Sautereau et al. 1988).

The cytotoxicity and/or anti-proliferative activity of doxorubicin may thus be a consequence of multiple therapeutic effects. Although this drug is most active during *s*-phase, its activity is often less cell cycle dependent because it can exert its action by multiple modes of action. Rapidly proliferating tissues such as tumour tissues (but also bone marrow, gastrointestinal and oral mucosa, hair follicles) are most sensitive to doxorubicin. Doxorubicin was initially approved by US-FDA in 1974 and is commonly used to treat various cancers including some leukemias, Hodgkin's lymphoma, cancers of the bladder, breast, stomach, lung,

ovaries, thyroid, soft tissue sarcoma, multiple myeloma, and others (Robert 1998; 2009). Doxorubicin is supplied in 10, 20 and 50 mg single dose vials either as lyophilized powder for reconstitution (Mayne Pharma), or a sterile solution (Novopharm and Pfizer). The recommended dose of doxorubicin is 50 mg/m^2 i.v. once every 4 weeks, but this is highly dependent on the cancer being treated and the stage of the disease (Robert 1998; 2009).

Doxorubicin exhibits a triphasic elimination behaviour following i.v. administration. The initial half-life is 8-30 min and this is associated with plasma elimination and tissue distribution. The second half-life is 1.5-10 h and this corresponds to metabolism in the liver. The terminal half-life is 24-48 h and this arises due to tissue re-distribution (Mross, Maessen et al. 1988). Doxorubicin is primarily metabolized to doxorubicinol, a therapeutically active metabolite (Cusack, Young et al. 1993) and other aglycones doxorubicinone and 7-deoxy-doxorubicinone. Doxorubicinol possess 10% of the activity of doxorubicin but is believed to play an important role in causing the cardiotoxic effects observed when doxorubicin is administered (Olson, Mushlin et al. 1988; Cusack, Young et al. 1993). As much as 40% of the administered dose of doxorubicin is excreted via bile (Riggs, Benjamin et al. 1977). As suggested above the dose-limiting toxicities of doxorubicin are cardiomyopathy, which can be life threatening. The cardiotoxic effects can be observed acutely, but are typically associated with chronic use of the drug and for this reason there is a cumulative dose limiting toxicity limiting the drug's use beyond a lifetime cumulative dose of 450 mg/m^2 (McEvoy 2005, Adriamycin-Product Monograph 2009). Acute toxicities are more typically myelosupression associated with leucopenia and thrombocytopenia (Adriamycin-Product Monograph 2009). Gastrointestinal toxicities are also severe when using doxorubicin and these are observed within 10 days of administration as nausea, vomiting, stomatitis, diarrhoea.

1.4.3.2.2 Doxil®

Doxil® is a trade name for the pegylated liposomal formulation available in USA. Outside of USA (including Canada), this formulation is marketed under the trade name Caelyx®. This drug was first approved for use in the treatment of AIDS related Kaposi's sarcoma and subsequently was tested in other disease indications including metastatic breast cancer and advanced ovarian cancer. This LNP formulation of doxorubicin was eventually approved for use in advanced ovarian cancer in 1995 by the US-FDA and in 1998 by Health Canada. The formulation arose from previous studies which demonstrated that the acute and chronic toxicity of doxorubicin could be ameliorated by presenting the drug encapsulated in liposomes. Additional details about liposomes and their use as drug carriers are provided in subsequent section, so here it should be sufficient to note that the liposomes used in the preparation of Doxil® exhibited two unique features at the time the product was developed. First, the lipid composition used contained N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phospho ethanolamine (MPEG-DSPE), a modified lipid that could extend the circulation lifetime of the liposomes following intravenous administration. Second, the lipid composition included a fully hydrogenated soy phosphatidylcholine (HSPC), a lipid that reduced the rate of doxorubicin dissociation from the liposomes following intravenous administration. It was demonstrated that the resulting formulation was less toxic than other liposomal formulation of doxorubicin and animal model data as well as clinical data demonstrated that Doxil® exhibited reduced cardiotoxicity as a consequence of its altered pharmacokinetic properties which minimize the exposure of drug to the heart. As noted by Gabizon in a 2001 review (Gabizon 2001), the toxicity profile of Doxil® was remarkably different than that observed following administration of free doxorubicin. Doxil® toxicity included dose-limiting mucocutaneous

toxicities (relating to the skin and a mucous membranes), but there was only mild myelosuppression and minimal alopecia. Importantly, little cardiac toxicity was observed. Interestingly, the single maximum tolerated dose (MTD) of Doxil® in humans (40 mg/m^2) was lower than that defined for the free drug (60 mg/m^2). Doxil® has proven to be an active agent in the treatments of AIDS-related Kaposi's sarcoma and now has an established role in the management of recurrent ovarian cancer. The potential of Doxil® in combination with other drugs is considerable, and the studies described in this thesis wanted to establish the therapeutic potential of Doxil® when it was used in combination with the LNP topotecan formulation described in Chapter 3.

1.4.3.3 Combinations of doxorubicin and topotecan for treating ovarian cancer.

There are several other combinations currently being tested to improve the treatment outcomes of recurrent ovarian cancer and as indicated in Section 1.4.2, overall observations indicate benefits of combination treatments over single agents in terms of improved survival and reduced toxicity. Interestingly, these studies highlight the fact that newly developed targeted agents will not replace the existing cytotoxic drugs, at least in the near future. These targeted agents will be useful in improving the activities of the existing drugs.

Doxil® is currently being evaluated in combination with several drugs to treat ovarian cancer. Doxil®-gemcitabine combinations, for example, have recently been tested in a phase II study which demonstrated improved survival at better tolerated doses. This combination is now going to be tested in a multi-centre phase III evaluation (Mirza, Lund et al. 2010). Another phase III study evaluated combination of Doxil® with trabectedin, a drug that can generate superoxide near DNA strands resulting in DNA breaks, and it was observed that the combination improved the progression free survival and overall response rate over that observed with Doxil® alone

(Monk, Herzog et al 2010.). Importantly in the context of this thesis research, Doxil® has also shown benefit when combined with topotecan in phase II study (Smith, Johnson et al. 2006; Verhaar-Langereis, Karakus et al. 2006). The later combination was of clinical interest because lab based research efforts found that the free forms of these drugs have synergistic effects when combined together against a series of primary tumor cell lines (Jonsson, Fridborg et al. 1998). These data were confirmed by the results summarized in Chapter 4, however the work described in this thesis was designed to better understand how drug exposure time influenced drug interactions between doxorubicin and topotecan. This combination is of interest for use in the treatment of relapsed ovarian cancer because it considers the combination of two established agents with proven single agent activity (e.g. topotecan and Doxil®). These drugs also have complementary mechanisms of action that target two major classes of enzymes involved in DNA topology and repair. As noted in the previous sections, topotecan acts by inhibiting TOP 1, the enzyme responsible for producing small single stranded nicks in DNA during replication (Alberts, Johnson et al. 2002). Doxorubicin's activity is due, in part, to the disruption of DNA and RNA synthesis and involves the inhibition of TOP 2, an enzyme responsible for the unraveling of two interlocked DNA circles (Alberts, Johnson et al. 2002). It is also worth noting that when cells are exposed to TOP 1 inhibitors studies have suggested a compensatory response that involves increases in TOP 2 levels (Nitiss, Rose et al. 1996). Similarly when cells are exposed to TOP 2 inhibitors, there can be increases in TOP 1 expression. Finally, it has already been established that topotecan/doxorubicin combinations achieve higher in vitro cytotoxicity, particularly following prolonged exposure to topotecan (Cheng, Chatterjee et al. 1994). These previous observation clearly support the concepts developed in this thesis.

1.5 DEVELOPING COMBINATION CHEMOTHERAPY: ACHIEVING SYNERGY

If drugs are required in the management of patients with cancer, then invariably multiple drugs are used to achieve optimal treatment outcomes (DeVita, Hellman et al. 1989). As indicated above for ovarian cancer, first line treatment frequently involves use of carboplatin (or cisplatin) in combination with paclitaxel (or docetaxel). Further, the future of chemotherapy for patients with ovarian cancer will continue to involve multiple drugs. As discussed in this thesis, the application of drug combinations should have benefits in patients with platinum refractory (relapsed) disease, but it is also anticipated that as new targeted drugs are identified for this patient population they will be used in combination with existing drug combinations in the first line and relapsed setting.

Classically drug combinations have been developed based on principles that considered drug resistance mechanisms, mechanisms of action and toxicity profiles (Berenbaum 1989; Ramaswamy 2007; Zimmermann, Lehar et al. 2007). Having stated this, it is very desirable to identify drugs that when used in combination against cancer produce therapeutic outcomes greater than that which would be expected on the basis of the activity of the individual drugs. This is referred to as synergy. When using two drugs that interact to produce synergistic effects there are numerous potential benefits for the patient being treated. First, if the drugs are used at maximum tolerated doses then one would expect to see treatment outcomes (time to progression, relapse free survival time, overall survival, etc.) to improve. Second, there is the possibility of achieving therapeutic effects comparable to those achieved with single agents, but at significantly lower drug doses. This can be assessed be estimating a dose reduction index (as discussed in section 1.5.1) and offers the potential for treating patients at drug doses that are much better tolerated. Of course there are potential toxicity concerns that arise when using

synergistic drug combinations. If the primary mechanisms of therapeutic effect which result in synergy are related to effects on cell proliferation, then one could imagine that synergistic drugs which impact tumor cell proliferation may also impact the proliferation of cells in the bone marrow. If this was the case then one would expect synergistic toxicity.

There are many strategies used to assess whether drugs, when used in combination, interact to produce additive effects, antagonistic effects or synergistic effects. In this thesis, drug-drug interactions have been analyzed using the median effect principle developed by Chou and Talalay (Chou and Talalay 1984; Chou 1991). When using this method to assess drug combination effects, a synergistic drug combination achieves therapeutic effects equal to that achievable with single agents, but at significantly lower, better tolerated, drug doses (Chou 1991). Antagonistic combinations, on the other hand, produce combined effects that are actually lower than those observed with the single agents and therefore such combinations should be carefully avoided. Although the definitions of synergy/antagonism are relatively well understood, the factors that influence synergy/antagonism are not. It is becoming evident that drug-drug interactions that produce synergistic (or antagonistic) effects are influenced by: (i) the specific and non-specific effects of the individual drugs, (ii) the drug combination dose which can be evaluated at one or more measured effect levels, (iii) drug-drug ratio and (iv) drug sequencing (Waterhouse, Gelmon et al. 2006). It is also argued in this thesis that drug interactions will be influenced by the length of time a target cell population is exposed to each drug in a given combination. These drug variables are relatively easy to control in the *in vitro* setting, but much more challenging to control in the *in vivo* setting. As outlined in subsequent sections, it is believed that drug carrier technologies offer the potential to achieve control over critical parameters important in maximizing the synergistic potential of drugs when used in

combination. This has recently been demonstrated for drug combinations that exhibit synergistic effects that are strongly dependent on drug-drug ratio. Thus, in the context of the work described in this thesis, synergy can be defined as effect elicited by combination that is greater than that achieved by single agents used at equivalent doses or the effect of combination that is equivalent or higher than achievable by single agents but at significantly lower doses. This thesis provides evidence, for the first time, that drug-drug interactions producing synergistic effects can be dependent on drug exposure time and that one way in which drug exposure time can be controlled is through the use of drug carrier technologies. Prior to discussing the drug carrier technologies useful in maximizing synergistic combination effects it is, however, important to provide an overview of the different methodologies used to analyze drug-drug interactions in terms of synergy, antagonism and additivity.

1.5.1 Assessment of drug-drug interactions

Selecting drugs and then assessing combination effects is a challenge when considering the preclinical and clinical development of drug combinations. It is believed that development of innovative drug delivery technology and their use in the creation of drug combination products will foster a closer examination of important variables influencing combination effects. In this regard the selection of suitable drugs followed by an assessment and validation of the factors that influence combination effects will be a crucial step in the development of new combination therapies. These factors must be screened preclinically and *in vitro* assays combined with well designed preclinical studies in animal models can be used to develop this understanding. The knowledge gained from these studies will result in combination products or combination protocols that have a high likelihood of proving to be effective in the clinic. Regardless, there are multiple ways to assess combination effects and defining the nature of these interactions cannot

be made easily because dose response curves are non-linear and therapeutic effects are influenced by the endpoint being measured and by the influence of toxicities on these measured therapeutic endpoints. For an unbiased assessment of interactions, investigators have relied on the use of mathematical models, but all models are limited by the experimental model being used and as noted in the following section, it is not possible to examine multiple variables in clinical trials. Therefore it will not be possible to "prove" that synergy is achieved for a specific combination in a clinical trial. Rather the clinical test will be based on standard measures of therapeutic activity achieved at defined doses.

As indicated, the work described here relies on the Median effect principle developed by Chou and Talalay (Chou and Talalay 1984; Chou 2006). There are several mathematical models available to study drug-drug interactions, some of these and the limitations associated are listed below:

i. Empirical method (Waterhouse, Kalra et al. 2008): A dose response curve is generated by plotting effect of single agent and a fixed ratio combination. Horizontal shift in the curve towards right indicates antagonism and to the left indicates synergism. This simple method is frequently used and can give a basic understanding about whether synergy or antagonism is occurring. However, this method is not quantitative and is rather misleading particularly when the effective doses of the drugs being used are substantially different (e.g. one capable of achieving activity in the nanomolar range while the other is active when used in the micromolar range).

ii. Summation of effects method (Cavalieri, Munhall et al. 1983; Abou-Issa, Koolemans-Beynen et al. 1989; Berenbaum 1989; Cassatella, Hartman et al. 1989; Clejan and Cederbaum 1989): The effect of combination is calculated by addition of the effect of

individual agents: $(E)_{1,2} = (E)_1 + (E)_2$. Effects ($(E)_{1,2}$) lesser than the calculated indicates antagonism where as a greater $(E)_{1,2}$ value indicates synergism. The problem with this method, as well as the fractional product method described below, is that they cannot produce accurate results over a broad range of effective doses (effect levels) unless the dose response curve for the drugs being evaluated are linear. Dose response curves, as indicated already, are typically non-linear. Even in the case of linear dose response curves; it is difficult to distinguish synergistic effect from additive effects.

iii. Fractional product method (Valeriote and Lin 1975): The expected effect of combination is the product of the single effects: $(E)_{1,2} = (E)_1 \text{ X } (E)_2$. If the effect is equal to the product, the combination is considered additive whereas a lesser value indicates antagonism and higher value indicates synergism. Although able to distinguish synergy from additive effect to some extent, this method is limited when assessing non-linear dose response curves. It can give accurate results if applied to an agent that produces exponential dose response curve, but as indicated above this is rarely the case.

iv. Isobologram method (Loewe 1953; Fraser 1972): This method is well established and has been used over the years by investigators. According to this method;

$$A_{comb}/A_E + B_{comb}/B_E = 1.$$

where A_E and B_E are the concentrations of drug A and drug B required to produce the same effect (E) whereas A_{comb} and B_{comb} are the concentrations required to produce the same effect when combined. Resultant values less than 1 indicate synergism; greater than 1 indicates antagonism, and equal to 1 indicates an additive effect. Graphically (Figure 1.1) an additive combination is represented by a point in a graph (comprising the dose of drug A on the X axis and drug B on the Y axis) that lies on a line joining Dm_a and Dm_b. Dm_a and Dm_b

represent the doses required to achieve 50% activity against a measured endpoint (e.g. cell proliferation) when using drugs A and B, respectively. Data points lying above and below the line represent antagonism or synergism, respectively. Concerns regarding the applicability of this method again lie in the fact that dose response curves are non-linear.

v. Median effect principle (MEP) method (Chou and Talalay 1984; Chou 1991; Chou 2006): This method described by Chou and Talalay is based on Michealis Menton enzyme kinetics and was the first method to address the fact that dose response curves were non-linear. According to this theory, median effect equation is defined as;

$$fa/fu = (D/D_m)^m$$

where fa is the fraction of cells affected by the treatment, fu (or 1-fa) is the fraction of cells unaffected, D is the concentration of drug and Dm is the concentration required to produce 50% of the required effect (e.g. the IC_{50} or EC_{50}). "m" represents a coefficient indicating the shape of the dose response curve (m=1, hyperbolic; m>1, sigmoidal; or m<1, flat sigmoidal). The derivations of this equation lead to an equation that defines a combination index (CI)

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

where CI >1, =1(± 0.2) and <1 indicates synergistic, additive and antagonistic interactions respectively. D_1 and D_2 represent doses of drugs 1 and 2 used in a combination to produce x% effect. $(D_x)_1$ and $(D_x)_2$ represent doses of drugs 1 and 2 when used alone in order to produce x% of the effect. The reason why the median effect method has become so popular within the current literature is due to the fact that there is a commercially available computer program, CompuSyn®, which can be used to analyze data from specific dose response curves and then subsequently provide calculated CI values. Graphically, (Figure 1.2-a) fa is plotted against dose to generate dose response curves used in a combination. Dm values are

calculated from these curves. Transformation of sigmoidal dose response curves into linear forms allows the program to generate median effect plots (Figure 1.2-b). This is done by plotting log (fa/fu) against log (dose). The slopes of linearized data represent the degree of sigmoidity of the dose response curve and Dm values can be calculated by taking anti-logs of the x intercepts.

Interestingly, the MEP methodology is built around the concept that combination effects need to be studied at fixed drug ratios and that the effect of these fixed drug ratio combinations must be determined over a broad range of effective doses. Thus CI values are determined at different effect levels, which can readily be assessed when plotted as Fa-CI plots (Figure 1.3). The analysis of dose response curves also provided investigators with a measure of the dose reduction index (DRI) which highlights the practical importance of identifying synergistic combinations: one can achieve therapeutic effects comparable to those achievable with the single agents, but at significantly reduced drug doses. This method dose not necessarily provides clear guidance in terms of drug ratio or what sequence the drugs should be added or whether the drugs should be added simultaneously. Isobologram analysis or combination index methods are not applicable in all situations, and these rely on the effect level; e.g. one isobologram is applicable to single effect level or one combination index describes only single dose pair. Therefore, in order to describe all possible interactions, analysis may need to be conducted at multiple effect levels (ED_{25}, ED_{50}, ED_{75} etc.).

vi. Response surface analysis (Greco, Bravo et al. 1995): This is a sophisticated modification of the most widely accepted methods that may address some of the limitations mentioned for isobologram or combination index methods. It considers three variables, the concentration of both drugs and the resulting biological effect and is used to build isoboloes at different

effect levels in three dimensions, thus creating a response surface and produces three dimensional picture of the drug interactions at multiple effect levels (Figure 1.4). Thus each measured effect is a response and thus a point that defines response surface. The surface of an additive combination is determined and used as a reference point. If actual combination doses produce synergistic effects then these points are located above the additive surface and similarly for an antagonistic combination, measured points are located below the additive surface.

Different models are used by different researchers in order to get a preliminary idea about the drug-drug interactions in *in vitro* settings, and no single model used is universally applicable in all possible situations and therefore have their own limitations. Finally, this can be stated about all models used to define combination effects, does not consider the influence of drug pharmacology or drug metabolism when determining combination effects.

1.5.2 Assessment of combination effects in the clinic

Although the MEP method to assess whether two drugs interact synergistically stresses the importance of evaluating effects over a broad range of drug doses using combinations at several drug-drug ratios, this assessment cannot be done practically in patients. Combination therapy in the clinic is typically developed by first establishing the recommended dose of one drug followed by subsequent addition of other drugs to the combination at increasing concentrations until the aggregated toxicity is considered limiting (Frei 1991; Tannock 1992; DeVita 1997). Clinically, factors such as drug dose, dosage form and dose frequency and sequence can be manipulated easily. However even when considering these simple parameters, their assessment in the context of Phase 2 or Phase 3 clinical trials designed to prove benefits in patients can take decades to complete. For this reason alone, the empirical approach to defining

whether two drugs should or should not be used in combination is fundamentally flawed. There are additional reasons:

(i) Clinical studies are reliant on use of drugs proven to be therapeutically active when used as a single agent and this limits the potential of using agents which may have therapeutic effects that are not necessarily observed as a "complete response" in a clinical setting yet could profoundly influence the effect of another drug; and

(ii) The clinical approach used to date does not take into consideration pharmacokinetic and biodistribution of the drugs used in the combination. The evidence now clearly shows that synergistic interactions for drugs used in combination are dependent on drug-drug ratio. This suggests that one must control the fate of both drugs biologically to ensure that the drugs access the target cell population at a defined drug-drug ratio. Since the pharmacokinetic and biodistribution of individual drugs are dependent on their individual chemistry and metabolism, it is likely impossible to define dosing methods to ensure that a specific drug ratio is achieved when the drugs are administered.

As implied above, a variety of approaches can be used to determine drug-drug interactions *in vitro* and the experimental conditions for *in vitro* assay systems can be easily controlled and standardized. *In vivo* studies using well defined animal models are more time consuming and expensive, but the principles used to assess whether drug combinations are synergistic (or antagonistic) are comparable to those used for *in vitro* studies; multiple drug ratios should be evaluated over a broad range of effective doses where dose response curves are determined in identical models for Drug A, Drug B and the Drug A/B combination. This requires a large number of animals and the studies are inherently more difficult to control. Therefore, *in vivo* studies of individual combinations are usually carried out only following careful *in vitro* screens

assessing a number of variables which are then used to guide the conduct of the *in vivo* studies. When considering patient populations, the problem of defining whether drug combinations act synergistically is compounded several fold. The variation in subject population with respect to age, sex, race, disease stage or subtype as well as the influence of previous treatments and use of co-medications cannot be easily studied preclinically. There are obvious ethical concerns with respect to treating patients with suboptimal doses of drugs used in a combination, yet one of the principles of developing synergistic drug combinations is based on the DRI which predicts that therapeutic effects can be achieved at substantially lower doses (depending on the drugs used in a given combination, dose reductions of 10 to 100 fold can be easily achieved) than those which could be achieved when using the agents alone. As already indicated, drug combinations in the clinic are often limited to drugs already approved for use in humans. Because of these and other complexities, it is important to rely on methods developed for *in vitro* and preclinical *in vivo* studies to identify combinations that have the potential to interact synergistically in the clinical setting and perhaps most importantly, to use the information gained by studying variables influencing synergistic interactions to help guide the development of dosage forms/methods that would provide the greatest potential for obtaining synergistic effects in the patient population being studied. One approach which is garnering significant attention concerns the use of drug delivery systems to deliver two or more drugs as part of a well defined combination product (Ramsay, Dos Santos et al. 2005). This thesis research is developed around the principle that drug carriers in general, and lipid-based formulations more specifically, can be used to define drug dosage forms that can control drug delivery parameters such that combination effects leading to synergy are attained in patients.

1.6 NANOPARTICULATE DRUG CARRIERS IN OVARIAN CANCER THERAPY

It is believed that drug delivery approaches reliant on nanotechnology will revolutionize the future of parenterally administered chemotherapy. Nanotechnology is a broad field, but when considered in terms of systemic drug delivery applications for patients it refers to particles with mean size ranges that are 100 nm or less and particulate formulations that incorporate one or more approved drugs or drug candidates (Bawa 2007). The value of using nanoparticulate formulations of drugs has been defined in many ways, but in general the application of nanotechnology is designed to maximize the therapeutic benefits of a drug while minimizing non-specific toxicities. When used for parenteral administration of drugs, these carriers can modify the pharmacokinetics and biodistribution of an associated drug minimizing its exposure to healthy cells or tissues while enhancing exposure to sites of disease. As an example, when nanoscaled delivery systems are given intravenously they can be "trapped" in the blood compartment depending on their size and design. This limits their distribution except within tissues with cells specifically designed to remove foreign particulate matter (e.g. cells of the MPS or mononuclear phagocytic cell system) and in regions where the blood vessel structure allows for movement of nanoparticles from the blood compartment to extravascular tissue; a process typically mediated through pores or gaps (typically between 100-600 nm diameter) within the associated vascular structures. When considering tumors, it is known that the blood vessel structure is poorly defined and permeable to nanoparticles (Maeda and Matsumura ; Suzuki, Hori et al. 1981; Skinner, Tutton et al. 1990; Hori, Suzuki et al. 1991; Maeda, Fang et al. 2003; Daruwalla, Greish et al. 2009). Further, lymphatic drainage from these sites of tumor growth is often compromised due to the lack of a well developed network of lymphatic vessels (Maeda and Matsumura ; Matsumura and Maeda 1986; Maeda 2001). When taken together, one

observes a localization of the nanoparticulate drug carriers to the region of tumor growth; an effect referred to as passive targeting. The enhanced permeability and retention (EPR) effect, first described by Maeda (Matsumura and Maeda 1986), is a widely accepted theory that explains passive targeting achieved by nanoparticulate formulations (Li, Miyamoto et al. 1993; Maeda 2001) (Figure 1.10). To achieve efficient passive targeting, in general, the nanoparticulate formulation must exhibit extended circulation lifetime.

Prior to defining the "EPR" effect it was well understood that tumors residing in the peritoneal cavity could spread (metastasize) via lymphatic dissemination (Chen and Lee 1983; Wu, Qu et al. 1986; Ransom, Patel et al. 1990; Eltabbakh and Mount 2002) and when disease burden was large lymphatic drainage could become compromised leading to a buildup of fluids within the peritoneal cavity. This understanding was used to define models for nanoparticle movement into and out of the peritoneal cavity (Nagy, Herzberg et al. 1989; Bally, Masin et al. 1994). These earlier studies taken in the context of what is understood about ovarian cancer would suggest that nanoparticulate formulations of drugs relevant for treatment of ovarian cancer are worth pursuing. As noted earlier in this introduction a major challenge achieving improved treatment outcomes for ovarian cancer patients is the occurrence of metastasis. Ovarian cancer can spread by direct seeding of cells into the peritoneal cavity where they can form additional cancers. Ovarian cancer metastasis in the peritoneal cavity is not limited by any anatomical or physiological barrier. More specifically, peritoneal metastatic lesions can give rise to ascitic tumor cells growing in fluid that is constantly entering into the peritoneal cavity from the blood (plasma) compartment. Alternatively the cells can spread via lymphatic dissemination to the regional draining lymph nodes. Importantly, regardless of disease subtype or stage, both tumor size and accumulation of ascites are correlated to poor survival (Bast, Hennessy et al. 2009). For

this and other reasons intraperitoneal therapy has been studied as an appropriate treatment option for patients with ovarian cancer. Even though this route of treatment can provide meaningful improvements in treatment outcomes, intraperitoneal therapy has not become a standard care in treating ovarian cancer. This is because of treatment related issues associated with catheter complications (infection, bowel perforation, obstruction etc.) and patient compliance. Toxicities associated with high drug absorption within tissues and organs within the peritoneal cavity are also a problem. These concerns, however, have the potential to be mitigated through use of nanopharticulate drug formulations.

Use of nanoparticles can be a very effective strategy to deliver therapeutically efficacious doses of chemotherapeutic drugs to the peritoneal cavity. Carefully designed long circulating nanoparticles can be used to carry and deliver active and intact forms of drugs to the peritoneal cavity when administered i.v (Bally, Masin et al. 1994; Kim, Gao et al. 2009). When administered i.p., nanoparticles can prevent the nonspecific absorption of drugs within the cavity and thereby can help reduce toxicity concerns. Different types of nanoparticulate carriers are currently under investigation to treat ovarian cancer. These include lipid based carriers, such as liposomes (see section 1.6.1) (Mangala, Han et al. 2009), as well as polymeric nanoparticles (Huang, Zugates et al. 2009), micelles (Kim, Gao et al. 2009), dendrimers (Yellepeddi, Kumar et al. 2009), magnetic nanoparticles (Scarberry, Dickerson et al. 2010), polymer conjugates (Markman 2004), peptide conjugates (Scarberry, Dickerson et al. 2008), etc. Specific examples include Doxil™, the liposomal formulation of doxorubicin described earlier (see section 1.4.3.2.2). The FDA approved Doxil™ for second line treatment in relapsed ovarian cancer and this has helped to establish the potential for liposomal nanoparticles to provide clinical benefit in treating this disease. Other liposomal formulations have also been examined preclinically. A

lipid based formulation of a siRNA targeting EphA2 (ephrin type-A receptor 2, a tyrosine kinase receptor) has shown significant anti-tumor activity in pre-clinical models of ovarian cancer (Mangala, Han et al. 2009). Additional examples include doxorubicin loaded polymeric micelles that have shown significant activity in suppressing growth of multidrug resistant ovarian cancer in pre-clinical animal models (Kim, Gao et al. 2009); micellar formulation of pactitaxel prepared using a dendrimer formulation method has demonstrated improved antitumor efficacy when compared to docetaxel when used to treat mice with established SKOV-3 ovarian cancer (Luo, Hiao et al. 2010). Chitosan-phospholipid hybrid formulation methods have been used to deliver docetaxel to the peritoneal cavity and these released docetaxel in a sustained manner in the peritoneal region following i.p. administration with no significant toxicity. This formulation showed significant tumor inhibition in a murine xenografts model of human ovarian adenocarcinoma (Zahedi, De Souza et al. 2009). Poly(β-amino ester) polymer nanoparticles were used to administer diphtheria toxin (an inhibitor of protein sysnthesis and expression of which in tumor cells results in their death) to s.c. xenografts and directly to the peritoneal cavity of mice bearing primary and metastatic ovarian tumors (Huang, Zugates et al. 2009). This treatment resulted in significant tumor reduction and prolongation of survival compared to control and no significant systemic toxicity was observed. Paclitaxel nano-conjugate with a biodegradable polymer of glutamic acid showed promising clinical activity in pre-treated ovarian cancer patients with improvement in therapeutic activity and toxicity profile as compared to free paclitaxel (Markman 2004). Folate receptor targeted doxorubicin loaded pH sensitive micellar formulation showed tumor selective accumulation and intracellular delivery that resulted in significant tumor suppression in multi-drug resistant ovarian tumor xenografts mouse models (Kim, Gao et al. 2009).

As suggested there are a wide variety of nanoparticulate drug carrier technologies available and all are suitable for development of drug candidates for use in the treatment of patients with ovarian cancer. These formulations can be considered for intravenous or intraperitoneal use. For this thesis research the focus has been on development and application of lipid-based drug carrier technology (more specifically liposomes) and intravenous routes of administration. The latter decision was reached primarily on the basis of current clinical practice which continues to place emphasis on intravenous treatment over intraperitoneal administration. As suggested above, this is due in part to increased risks of infection associated with the intraperitoneal route of administration, concerns about patient compliance and the fact that drug available for intraperitoneal treatment have poorly understood safety profiles when given via this route. The emphasis on lipid-based delivery systems is due in part to the fact that the FDA has already approved a lipid based drug formulation for use in the treatment of relapsed ovarian cancer. Further the clinical development path for lipid-based formulations is relatively straight forward and this is a reflection of the relatively advanced state of this nanotechnology platform. This hopefully becomes self-evident within the following sections.

1.6.1 Liposomes: a lipid-based nanoparticulate drug delivery platform

With the advent of clinically approved liposomal formulations of doxorubicin (Doxil® and Myocet®) and daunorubicin (DaunoXome®), the potential of liposomal carriers to improve the therapeutic activity of anticancer drugs was established. As summarized in Table 1.2, there are now many FDA approved liposomal formulations in the market and these are used for a range of disease indications. This history helps to establish the fact that lipid-based formulation approaches are pharmaceutically viable and methods exist for the scaled manufacturing of products that can meet the rigorous chemistry and manufacturing requirements of regulatory

bodies. It needs to be emphasized, however, that no two liposomal products are identical and each formulation presents unique issues that must be addressed when developing products for human use. This is reflected by the work summarized in Chapter 2 of this thesis which addresses a unique concern related to the manufacturing process being used for the preparation of an optimal lipid-based nanoparticulate formulation of the water soluble camptothecin irinotecan and how this influenced the development of a formulation of topotecan (Chapter 3) for use in the treatment of ovarian cancer alone (Chapter 3) and in combination with Doxil® (Chapter 4). Before describing these projects in detail, a brief overview of liposomes and their use as lipid-based nanoparticulate formulations is provided here.

1.6.1.1 Liposome structure and composition

Liposomes are spherical structures that are spontaneously formed upon hydration of dried lipids in aqueous solutions. These were first described by Bangham *et al*. in the 1960s (Bangham, Standish et al. 1965). Liposomes can be made from natural or semi-synthetic phospholipids that adopt bilayered membrane structures when hydrated. Depending on the number of bilayers present in a structure, liposomes have been referred to as unilamellar, oligolamellar or multilamellar vesicles. Unilamellar and oligolamellar liposomes can be further sub-classified based on size into small or large vesicles (Table 1.3).

Phospholipids are amphiphilic molecules with hydrophilic head group which may or may not be charged and hydrophobic fatty acid acyl chains that vary in length (according to number of carbon atoms in the chain) and saturation (number of double bonds within a given acyl chain (Figure 1.7). When put in an aqueous solution the phospholipids spontaneously orient themselves such that the hydrophilic headgroup faces the aqueous solutions while the hydrophobic acyl chains become arranged in a manner that maximizes their interaction. The resulting spherical

structures encompass central aqueous compartments with inner and outer compartments separated by bilayer membranes as shown in Figure 1.8-A. Since the bilayer structure is adopted when certain lipids are hydrated, liposomes have been used as a means to model the physical and chemical properties of cell membranes (Frezard and Garnier-Suillerot 1991; Ricchelli, Jori et al. 1991). However, they have also played an important role as drug delivery systems as first postulated by Gregory Gregoriadis (Gregoriadis 1993).

It is now well established that hydrophilic drugs can be incorporated into the central aqueous compartments of a liposomal structure and lipophilic compounds can be incorporated into the hydrophobic regions within each lipid bilayer. The physico-chemical characteristics of liposomal drug delivery formulation are determined by the nature and chemistry of lipids being used to prepare the liposomes. For example, the phospholipid head group and/or the length and degree of saturation of its associated acyl chains can influence the amount of drug that becomes associated with the carrier and, as outlined below, can influence the dissociation of the drug from the liposome. Since selected drugs have the potential to partition into the bilayer or localize at the phospholipid interface, it is also possible that the drug itself can influence its own association/dissociation properties. Choline-containing phospholipids (PC) are commonly used when preparing liposomes for drug delivery applications. This phospholipid headgroup is zwitterionic and neutral at physiological pH. Other phospholipids that have been used in the preparation of liposomal nanoparticulate formulations include anionic lipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidic acid (PA). Phosphatidylethanolamines (PE) have also been used, particularly in formulations that are designed to be fusogenic as select PEs when hydrated can be triggered to adopt a non-bilayer structure (Vance 2002) and as a result can fuse with nearby associated cell membranes or result

in a complete destabilization of the liposomal structure with associated loss of encapsulated contents (Bailey and Cullis 1997; Vance 2002; Kunisawa, Masuda et al. 2005). Cholesterol is another commonly used lipid when preparing liposomes. This sterol intercalates into the phospholipid bilayer parallel to the phospholipid hydrocarbon chains (Yeagle 1985, Subczynski, Wisniewska et al. 1994). The close proximity of planar sterol ring system tends to order the hydrocarbon chains. Cholesterol insertion into the bilayer does not alter the conformation of the phospholipid head group (Yeagle 1985). The importance of this lipid in developing liposomal nanoparticulate drug formulations was based on studies demonstrating its role in decreasing membrane permeability (enhancing drug retention) (Chapman 1975; Needham, McIntosh et al. 1988) and for increasing the stability of liposomes when exposed to plasma proteins (Kirby, Clarke et al. 1980; Semple, Chonn et al. 1996).

As suggested above, lipid composition can affect the amount of drug association with a liposomal formulation as well as the drug dissociation rate. In reference to the latter, the thermotropic phase behavior of phospholipids can be used to help guide selection of lipids to be used when preparing a liposomal formulation. Phospholipids exhibit two types of temperature dependent phase changes that are characterized by glass transition temperature (T_c) and melting temperature (T_m) respectively. Below the Tc, a phospholipid bilayer exists in a gel state where the acyl chains are arranged in tightly packed and in highly ordered form (Figure 1.9). Molecular motion of the acyl chains is at a minimum in the gel phase and therefore permeability of the lipid membrane is at its minimum in this phase. Increasing the temperature of the bilayer above the T_c leads to an increased disorder as well as molecular motion in the acyl chains. This phase is called the liquid crystalline state. Although disordered, lipid molecules are still confined in a two dimensional plane when in the liquid crystalline phase (Figure 1.9).

Lipid membrane permeability to small molecules and ions is relatively higher in the liquid crystalline state when compared to the gel state, but permeability is highly dependent on the properties of the encapsulated drug. Phospholipids have a characteristic T_c that is dependent on multiple factors. Increases in acyl chain length generally increases T_c. Lipids with unsaturated acyl chains tend to have a lower T_c when compared to saturated lipids. As indicated above cholesterol is a common constituent of most liposomal nanoparticulate drug formulations and addition of cholesterol exerts a significant effect on the measured phase behavior of the lipid bilayer. Cholesterol modulates membrane fluidity around the phospholipids transition temperature; an effect that depends on the amount of cholesterol being incorporated (Chapman 1975). Below the T_c, cholesterol alters lipid packing, changes acyl chain configuration from tilted to vertical and this leads to an increase in bilayer width (Needham and Nunn 1990) and a reduction in acyl chain order, i.e. an associated increase in lipid membrane permeability at temperatures below the T_c. At temperatures above T_c, cholesterol has condensing effect on the lipid bilayer (Needham, McIntosh et al. 1988; Needham and Nunn 1990) and increases the order of the acyl chains. In this context cholesterol decreases membrane permeability. When cholesterol is incorporated in a phospholipid bilayer in amounts above 30 mole% the gel to liquid crystalline phase transition is eliminated, however the impact of the acyl chains and cholesterol on membrane permeability are still governed by the T_c of the phospholipid. For the studies described here the liposomal nanoparticulate formulations used were prepared of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; 55 mol%) which has a T_c of 54^{o}C and contained 45 mol% cholesterol. As noted in Chapters 2 and 3, formulations prepared of this lipid composition were prepared at temperatures above 55^{o}C and drug loading rates (see subsequent sections for details) were fastest when temperatures were above the T_c of DSPC. In the case of

Doxil™, the formulation is prepared with a hydrogenated soyPC (T_c = 53°C) and cholesterol, as well as a small amount of a DSPE that has been chemically modified on the amine group with polyethylene glycol (average MW of 2000). The PEG modified lipid in this formulation was designed to enhance the circulation lifetime of the liposomal structure (see below). The general methods used to prepare these liposomes and subsequently load them with the specified drugs are described in the following sections.

1.6.1.2 Liposomes preparation

A variety of methods are available to prepare liposomes and most involve the generation of large structures that are subsequently processed to generate liposome sizes that are suitable for the indicated application. For intravenous applications and drug formulations designed to treat cancer, the optimal mean size diameter is between 50 and 400 nm, most preferentially 100 nm; a size that takes advantage of the EPR effect described in section 1.6.3. Some of the methods commonly used for laboratory scale production of liposomes have been described elsewhere (Bangham, Hill et al. 1974; Szoka and Papahadjopoulos 1978; Lichtenberg and Barenholz 1988; Gregoriadis 1993) and most of these have been adapted in some form for larger scaled production of drug formulations candidates developed for use in humans. The studies described in this thesis produced MLVs using simple lipid hydration methods. This is equivalent to the methods first described by Bangham (Bangham, Standish et al. 1965; Bangham, Hill et al. 1974; Gregoriadis 1993) where lipids are dissolved in an organic solvent (e.g. chloroform) and then the solvent is removed by evaporation to produce a thin film. The lipid film is then dried further under high vacuum to completely remove residual solvents. The resulting film is then hydrated with vigorous vortexing using the aqueous buffer of choice. Hydration is usually carried out at a temperature that is above the T_c of the bulk phospholipid species being used. This methodology

can be modified further to include multiple freezing and thawing cycles in order to achieve better hydration of the lipid film and to enhance equilibrium solute distribution (Oshawa, Miura et al. 1985; Oshawa, Miura et al. 1985). Although suitable for laboratory scale, this methodology is rarely used when scaling production to volumes of >500 mL. This is largely due to difficulties in subsequent processing of these MLVs to produce smaller structures suitable for intravenous application. In this case procedures that rely on use of lipids solubilized in ethanol are used. An ethanol injection method for production of liposomes was described previously (Batzri and Korn 1973). In this method lipids were dissolved in ethanol and the resulting solution was then rapidly injected in large quantities of aqueous buffer to produce MLVs (Gregoriadis 1993). When added to the aqueous solution the liposomes are spontaneously generated. These are well hydrated structures that are smaller than those generated using the direct hydration of dried lipid films. These MLVs are much easier to process to form smaller sizes and this method is now most commonly employed for large scale production of liposomal formulations. Other methods to prepare large MLVs have been described and include the ether injection method that is similar to ethanol injection method (Lichtenberg and Barenholz 1988; Gregoriadis 1993); but since ether cannot be used in many lab settings and it is not appropriate for scaled manufacturing this method is not commonly used. A subtle variation of the solvent based methods described above involves the reverse phase evaporation method; a method that involves mixing of a solution of lipids in organic solvents and the aqueous buffer followed by sonication to produce water in oil emulsion (Szoka and Papahadjopoulos, 1978). Subsequently, the organic solvent is slowly removed under reduced pressure to produce a viscous gel (pudding) and continued removal of organic solvent eventually leads to formation of MLVs. This methodology has been used with some success to create MLV precursors in a large scale, but it is limited to solvents that are

considered appropriate for the production of products for use in humans. Another method relied on the use of detergents to solubilise lipids (Razin 1972), where subsequent removal of the detergent generated liposomal structures. This method is rarely used today since it is almost impossible to remove residual detergent and encapsulation efficiency of liposomes obtained is also small. Finally, a recently developed method for production liposomes without the use of any organic solvents or chemicals has been described (Mozafari 2005). This method involves hydration of lipids in an aqueous buffer followed by heating in the presence of glycerol (3% v/v) at temperatures up to 120°C. Since glycerol is physiologically acceptable excipient it is not necessary to remove it from the liposomes. Glycerol can also help to increase the stability of lipid vesicles by preventing aggregation (Mozafari 2005) and it can also be used as a cryo-protective agent if the liposomal formulations need to be stored frozen.

As indicated already, all the methods described above generate MLVs that are heterogeneous with respect to size and lamellarity. These MLVs need to be processed further to generate formulation with more uniform size distributions. The method that has been most generally accepted in the literature involves extrusion of the MLV precursors through membranes that have single channel pores of defined size. This method was originally described by Szoka et al. (Olson, Hunt et al. 1979) and was used to prepare LUVs from MLVs prepared using the reverse phase evaporation methodology described above. Hope et al. (Hope, Bally et al. 1985) adapted this methodology for use with MLVs prepared by hydration methods where the primary change involved increasing the pressure used to force the MLV precursor through the polycarbonate membranes which exhibited defined pore size. This method was applicable to almost any MLV precursor and could be used with lipid concentrations as high as 400 mg/mL (Mayer, Hope et al. 1986). This general methodology has now been adapted to produce LUVs at

a scale ranging from 1 mL to volumes > 10 L (LIPEX extruder – Northern Lipids, Vancouver). Other methods have been described to prepare unilamellar vesicles from MLV precursors, such as French pressure cell (Hamilton, Goerke et al. 1980), sonication (Johnson, Bangham et al. 1971; Huang, Zugates et al. 2009), and microfluidization (Mayhew, Lazo et al. 1984). In general these methods size reduce MLV precursors to limit sized SUVs and thus typically generate solutions containing a mixture of large liposomes and SUVs. The proportion of SUVs in the solution is dependent on the processing parameters and time. Since SUVs are not generally used for drug delivery purposes, these methods are not commonly used. Extrusion methods were used in this thesis research to generate LUVs from MLV precursors that were generated following hydration of a dried lipid film. The MLVs were extruded under high pressure through polycarbonate filters with defined pore sizes such that the resulting liposomes exhibited a mean diameter ranging from 80 to 120 nm.

1.6.1.3 Drug loading into liposomes

The methods for preparing liposomes described above have not considered how to prepare liposomes with an associated drug. In general there are two methods used to achieve this. The first involves addition of the drug to the solvents or aqueous solutions used when preparing the MLV precursors; this method is typically referred to as the passive drug loading procedure. The second involves addition of the drug to liposomes that have been processed to achieve a more uniform size distribution; this method is typically referred to as the active drug loading procedure. There are some subtle variations on these two general themes as described in the following paragraphs.

For the passive loading method the hydrophobic or hydrophilic drug to be loaded is mixed with the lipids during film preparation or added to the aqueous buffer during film

hydration. The former approach relies on the ability of the drug to partition into the lipid bilayers and therefore is suitable for lipophilic compounds e.g. Amphotericin B (Madden, Harrigan et al. 1990). The composition of the lipid bilayer and the drug's ability to partition into the hydrophobic region of the bilayer determines the encapsulation efficiency in this method. Encapsulation efficiency is a measure of the amount of drug associated with the final liposomal product when compared to the amount of drug added during the manufacturing of the liposome. The amount of drug which can be associated with the liposomal membrane can be limited because the drug itself alters the properties of the lipid bilayer. For example in the case of cyclosporine, only about 2 mol% of this hydrophobic drug can partition into the liposomal membrane (PC/Chol) (Ouyang, Choice et al. 1995). Having indicated this, the formulation can be manufactured such that 100% of the added drug becomes associated with the liposomes, representing an encapsulation efficiency of 100%.

For hydrophilic drugs the active agent is included in the aqueous buffer used to hydrate the lipids (or mix with the solvent solubilized lipids). In this example of passive encapsulation, the efficiency of encapsulation is dependent on the aqueous trapped volume of the liposomes produced and the liposomal lipid concentration. SUVs have very small aqueous trapped volumes (0.2 µl/µmole lipid) (Szoka and Papahadjopoulos 1980), thus the potential for efficient encapsulation is very low. Even if prepared at high lipid concentrations (400 mM) the maximum encapsulation efficiency achievable would be 8%. For 100 nm LUVs the trapped volume can be as high as 2 to 2.5 µl/µmole lipid and for this reason higher encapsulation efficiencies (up to 80% at lipid concentrations of 400 mM) can be achieved (Mayer, Hope et al. 1985). It is challenging to work with liposomes prepared at high lipid concentrations, and when 80% of the aqueous solution is encapsulated typically a gel is obtained. Thus, from a practical perspective,

most investigators work at lipid concentrations of 100 mM or less, thus the encapsulation efficiencies under these conditions are frequently less than 30%. In many cases 70% loss of added drug is considered unacceptable and for this reason other methods were developed to efficiently encapsulate drugs within the aqueous core of the liposome, methods referred to as active drug loading.

Active loading methods have been shown to result in a greater than predicted drug-to-lipid ratios based on theoretical models which would suggest that the amount of drug loading would be directly proportional to the magnitude of the pH gradient (Mayer, Bally et al. 1986; Lasic, Ceh et al. 1995). Encapsulation efficiencies as high as 99.9% are not uncommon with these methods. Perhaps more importantly, the active drug loading methods can be designed to accumulate drug levels inside the liposomes that exceed the solubility of the drug. This leads to drug precipitation that is dependent in part on the encapsulated salts present in the liposomal core (Madden, Harrigan et al. 1990; Cheung, Sun et al. 1998; Abraham, Edwards et al. 2004; Ramsay, Alnajim et al. 2006; Dicko, Tardi et al. 2007; Drummond, Noble et al. 2009).

Active loading methods have been shown to result in a greater than predicted drug-to-lipid ratios based on theoretical models which would suggest that the amount of drug loading would be directly proportional to the magnitude of the pH gradient (Mayer, Bally et al. 1986; Lasic, Ceh et al. 1995). Encapsulation efficiencies as high as 99.9% are not uncommon with these methods. Perhaps more importantly, the active drug loading methods can be designed to accumulate drug levels inside the liposomes that exceed the solubility of the drug. This leads to drug precipitation that is dependent in part on the encapsulated salts present in the liposomal core (Madden, Harrigan et al. 1990; Cheung, Sun et al. 1998; Abraham, Edwards et al. 2004; Ramsay, Alnajim et al. 2006; Dicko, Tardi et al. 2007; Drummond, Noble et al. 2009).

More recently, divalent metal ions have been shown to stabilize encapsulated drug by forming complexes (Abraham, Edwards et al. 2002; Ramsay, Alnajim et al. 2006). Divalent metals like Mn^{+2}, Mg^{+2} and Cu^{+2} have been shown to form co-ordination complexes with drugs like anthracyclines and camptothecins (Cheung, Sun et al. 1998; Abraham, Edwards et al. 2002; Chiu, Abraham et al. 2005; Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006). Formation of co-ordination complexes has been used to drive accumulation of drugs inside preformed liposomes. This was demonstrated for a formulation of doxorubicin where the drug was encapsulated in liposomes containing magnesium (Cheung, Sun et al. 1998; Abraham, Edwards et al. 2002; Chiu, Abraham et al. 2005). Complex formation in this case involved deprotonation of the anthraquinone moiety resulting in the formation of six membered chelate compound (Abraham, Edwards et al. 2002). Interestingly, doxorubicin loading occurs independent of a transmembrane pH gradient. It was speculated that drug release rates for such formulations would be dependent on the dissociation rate of drug-metal complex. More recently, this strategy has been effectively used to load camptothecin analogs like irinotecan and topotecan into liposomes (Abraham, Edwards et al. 2004; Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006; Dicko, Tardi et al. 2007). Drugs capable of forming such co-ordination complexes may include groups such as alcohols, esters, carboxylic acids, amines or ketones.

It should be noted that liposomes prepared using divalent metals in combination with an initial transmembrane pH gradient (maintained with the help of a divalent metal ionophore) were found to retain drugs better than those formulations which relied solely on use of metal complexation (Abraham, Edwards et al. 2002; Ramsay, Alnajim et al. 2008). Most interestingly, when the pH gradient was combined with a selected metal (copper) there were additional unexpected improvements in drug retention when compared to formulations prepared with other

metals (e.g. magnesium, manganese) (Ramsay, Alnajim et al. 2008). Typically, liposomes are prepared using aqueous solution of copper sulfate as an internal buffer (pH 3.5) and are suspended in a neutral buffer (pH 7.5) to create an initial pH gradient across liposomal lipid bilayer (Figure 1.8-B). Weakly basic drugs (e.g. topotecan, irinotecan) are added externally to this suspension at the time of drug loading and this mixture is incubated at required temperature. Depending on the physicochemical characteristics of the drug and lipid composition of liposomes, neutral form of the drug permeates through liposomal bilayer, enters acidic core of liposomes and becomes either protonated or has a tendency to form co-ordination complex with the copper ions. Thus, protonated or complexed form of the drug being less lipid permeable is retained inside the liposomes. This method was further developed in this thesis research and used to define a novel formulation of topotecan that exhibited significant activity when used to treat models of ovarian cancer (Chapter 3).

1.6.2 Drug retention

The biological activity of liposomal nanoparticulate formulations is influenced by many factors but is now well understood that one of the most important factors concerns how fast the drug is released from the formulation following administration. Obviously, once a drug is encapsulated it is important that the drug remains encapsulated until it is ready for administration. This is a storage parameter that must be well characterized in order for the product candidate to be viable as a pharmaceutical for use in humans. Long term storage of drug loaded liposomes typically involves the use of lower temperature (Drummond, Hayes et al. 2007), which reduces drug release rates from the liposomes or the generation of frozen products that maintain their attributes when thawed and prepared for administration. Alternatively, some

formulations which rely on active drug loading methods are prepared as kits where the preformed liposomes are loaded with the drug just prior to administration (e.g. Myocet®).

Drug retention during storage is a distinct problem from that which deals with optimal drug retention attributes following administration. The rate of drug release from liposomes upon administration can significantly influence the efficacy as well as the toxicity of the associated drug (Mayer, Cullis et al. 1994; Lim, Masin et al. 1997). It is obvious to suggest that if the associated drug is lost instantaneously from the drug carrier following administration then the drug's activity would be indistinguishable from the drug administered in the absence of the carrier. If the drug release rate is too slow, on the other hand, then the concentration of free drug at the disease site may be too low to have a therapeutic effect. This was clearly demonstrated for liposomal nanoparticulate formulations of mitoxantrone (Lim, Masin et al. 1997) and for vincristine (Johnston, Semple et al. 2006). Quantitative measures of drug-to-lipid ratio in plasma samples obtained following administration is a commonly used method to estimate drug release rates (Boman, Mayer et al. 1993; Webb, Harasym et al. 1995; Drummond, Hayes et al. 2007); albeit this method is flawed in that it makes the assumption that free drug is rapidly eliminated and all the drug measured in the plasma is associated with the liposomal carrier.

1.6.3 Benefits for formulating anticancer drugs in liposomal nanoparticles

There are a number of potential benefits that can be achieved when using liposomal nanoparticulate forms of anticancer drugs. Liposomes can increase solubility of certain compounds; as exemplified by paclitaxel formulations (Schmitt-Sody, Strieth et al. 2003, (Zhigaltsev, Winters et al.). Liposome encapsulation can be used to protect a drug from degradation or inactivation under physiological conditions. This is perhaps best exemplified by the camptothecins which are prone to conversion to an inactive form of the drug at neutral pH

(Burke 1992; Wall, Wani et al. 1993). This inactivation can be prevented by associating the drug with the liposomal lipid bilayer or by encapsulating the drug in an aqueous environment that stabilize the drug in its active form (Burke and Gao 1994). This form of protection is emphasized in Chapters 2 and 3, where the water soluble camptothecin derivatives irinotecan and topotecan are protected in their active forms within a low pH environment inside the liposomes. As already noted, when a drug is administered as a liposomal nanoparticulate formulation, the drug's biodistribution is now partially dictated by the carrier. For this reason liposomes can limit the biodistribution of drugs to certain tissues while increasing their distribution to disease tissues like cancer through the EPR effect. For cardiotoxic drugs such as doxorubicin, liposomal encapsulation reduces the drugs distribution to cardiac tissue (Kanter, Bullard et al. 1993; Gabizon 2001; Gabizon, Shmeeda et al. 2003) while increasing distribution to sites of cancer growth (Mayer, Bally et al. 1990)**.** This benefit is illustrated by the Doxil® formulation used in chapter 4 of this thesis.

The pharmacokinetics and biodistribution of the encapsulated drug is dictated by the composition of the liposomal formulation which controls the rate at which the associated drug is released from the liposome (as noted in the previous section). Thus liposomes can be engineered to release associated drugs at optimal rates (Lim, Masin et al. 1997; Zhigaltsev, Maurer et al. 2005). As emphasized in chapter 3 and 4, a formulation of irinotecan that exhibits improved drug retention and improved therapeutic effects has been described based on use of a pH gradient loading technology in combination with encapsulated copper which can complex the encapsulated drug; a formulation approach that works well for topotecan (Chapter 3).

It should be noted that the biodistribution, pharmacokinetics and pharmacological effects (toxicity and efficacy) of liposomal drugs are also governed by many biological factors. As

already indicated, the enhanced permeation and retention (EPR) is a widely accepted theory that explains passive targeting achieved by nanoparticulate formulations such as liposomes (Li, Miyamoto et al. 1993; Maeda 2001). To achieve efficient passive delivery of liposomal drugs at the required site, prolong circulation is a pre-requisite for the formulation. Liposome circulation lifetime is influenced by lipid composition (Dapergolas and Gregoriadis 1977; Davis and Gregoriadis 1979) which influence serum protein binding (Black and Gregoriadis 1976; Semple, Chonn et al. 1996; Johnstone, Masin et al. 2001; Allen, Dos Santos et al. 2002), which in turn influence the recognition of the liposomal nanoparticulate formulation by cells of the mononuclear phagocytic cell system (MPS). Liposome circulation longevity is also influenced by particle size (Allen and Everest 1983) where it can be generally stated that smaller particles exhibit longer circulation lifetimes than larger particles. Having stated this it is known that particles with diameters below 600 nm can extravasate in to the tumor interstitial spaces through its associated leaky tumor vasculature. For optimal EPR effects, the size of liposomes must also be large enough to bypass renal clearance (>40 kDa) (Li, Miyamoto et al. 1993; Maeda 2001). The size of the liposomal nanoparticulate formulation is also important in avoiding distribution through the sinusoids in spleen and liver; where the nanoparticulates can interact with phagocytic cells in the liver (Kupffer cells) and spleen. To date investigators have defined the optimal liposome size range to be between 50 and 400 nm, more preferably around 100 nm; a size range that helps to retain the liposomes in the circulation for extended periods and facilitates passive targeting to tumors via the EPR effect. Surface modification of liposomes with polyethylene glycol (PEG) can help to ensure enhanced circulation lifetimes for certain liposomal formulations, particularly when administered at low doses (Allen and Chonn 1987; Gabizon and Papahadjopoulos 1988; Allen, Hansen et al. 1991). The PEG lipid helps to prevent surface-

surface interaction that can cause the formulations to aggregate or associate non-specifically with other membranes (Allen, Dos Santos et al. 2002). "PEGylated" liposomes have been referred to as Stealth® liposomes (Figure 1.11) and the Doxil® formulation used in chapter 4 of this thesis incorporates a PEG modified DSPE lipid, where the average molecular weight of the PEG polymer is 2000.

A final benefit of use of liposomal nanoparticulate drug formulations concerns the fact that they can readily be functionalized to facilitate target cell specific delivery. In this context, it has been proposed that following passive targeting to sites of tumor growth, the carrier can be actively targeted through the use of a surface associated targeting ligand. These ligands would include receptor specific molecules that would facilitate binding to cell associated receptors such as the high affinity folate receptor (Pan and Lee 2004; Wu, Liu et al. 2006) or the EGFR (Drummond, Noble et al. ; Mamot, Drummond et al. 2003); two examples that are of interest in the context of ovarian cancer since it has been suggested that over 90% of all ovarian cancers overexpress the high affinity folate receptor (Markert, Lassmann et al. 2008) and the EGFR is overexpressed in up to 60% of ovarian epithelial malignancies (Stewart, Owens et al. 1992; Niikura, Sasano et al. 1997; Lafky, Wilken et al. 2008). Alternatively active targeting of liposomal nanoparticles can be promoted by incorporating antibodies to specific antigens that are overexpressed by the tumor cells, as exemplified by the EGFR specific antibody cetuximab. Antibody modified liposomal formulations have been referred to as immunoliposomes (Drummond, Noble et al. 2010; Blanco, Kessinger et al. 2009) (Figure 1.11). Although these active targeting approaches are being examined pre-clinically and clinically with varying degree of success, active targeting approaches were not considered in this thesis research. The primary reason for this concern the fact that a comprehensive understanding of the formulations prepared

in the absence of a targeting ligand needs to be established prior to considering whether active targeting provides additional benefits as measured by enhanced therapeutic effects at lower, less toxic, doses.

1.7 SUMMARY

The main objective of this thesis project was to develop a new and effective treatment strategy against recurrent ovarian cancer using a combination of chemotherapeutic drugs that are approved for use in the treatment of relapsed disease. The combination of Doxil®, a liposomal nanoparticulate formulation of doxorubicin already approved for use in patients with relapsed ovarian cancer, and a newly described liposomal nanoparticulate formulation of topotecan (TopophoreC™) showed significant efficacy in two different pre-clinical models of recurrent ovarian carcinoma (Chapter 4). An important advantage of this combination is that improved therapeutic efficacy was achieved at lower and better tolerated therapeutic doses of the two agents. It is hoped that such a combination can be rapidly developed for clinical assessment and such a combination may provide the foundation from which newer targeted drugs can be introduced in an effort to define well tolerated drug combinations that can be used with curative intent.

Table 1.1: Staging of ovarian cancer by International Federation of Gynecology and Obstetrics

Stage	Sub-stage	Tumor confinement
I		Tumor confined to the ovaries
	I A	Growth limited to one ovary, no surface growth, intact capsule
	I B	Growth involving both ovaries
	I C	Growth on surface, capsule rupture, malignant ascites
II		Growth on both ovaries with pelvic extension
	II A	Extension to the uterus and fallopian tubes
	II B	Involvement of other pelvic tissues
	II C	Surface involvement, capsule rupture, malignant tumor
III		Peritoneal implants beyond pelvis including small omentum or bowel. Positive lymph nodes.
	III A	Seeding to abdominal peritoneal surfaces
	III B	$\leq$ 2 cm implants
	III C	$\geq$ 2 cm implants
IV		Growth involving one or both ovaries with distant metastasis and parenchymal liver metastasis.

Table 1.2: Liposomal products approved by FDA

Product	Therapeutic Agent	Use	Company	Year of Approval
Abelcet	Amphotericin B	Systemic fungal infections	Enzon	1995/1996
DaunoXome	Daunorubicin	AIDS-related Kaposi's sarcoma	Gilead	1996
Ambisome	Amphotericin B	Systemic fungal infections	Gilead	1997
Amphotec	Amphotericin B	Systemic fungal infections	Alza Corp.	1997
Doxil	Doxorubicin	AIDS-related Kaposi's sarcoma/ovarian and breast cancer	Alza Corp./Schering-Plough	1995/1999
DepoCyt	Cytarabine	Lymphomatous meningitis	SkyePharma/Enzon	1999
Myocet	Doxorubicin	Metastatic breast cancer	Elan Corp.	2000
Visudyne	Verteporfin	Age-related macular degeneration	QLT/Novartis Opthalmics	2000

Table 1.3: Liposome classification according to size and lamellae.

Liposome type	Size (nm)	No. of lamellae
Small unilamellar vesicles (SUVs)	<50 nm	1
Large unilamellar vesicles (LUVs)	>50 nm < 200 nm	1
Oligolamellar vesicles	>200<1000	2 to 5
Multilamellar vesicles	>1000	numerous

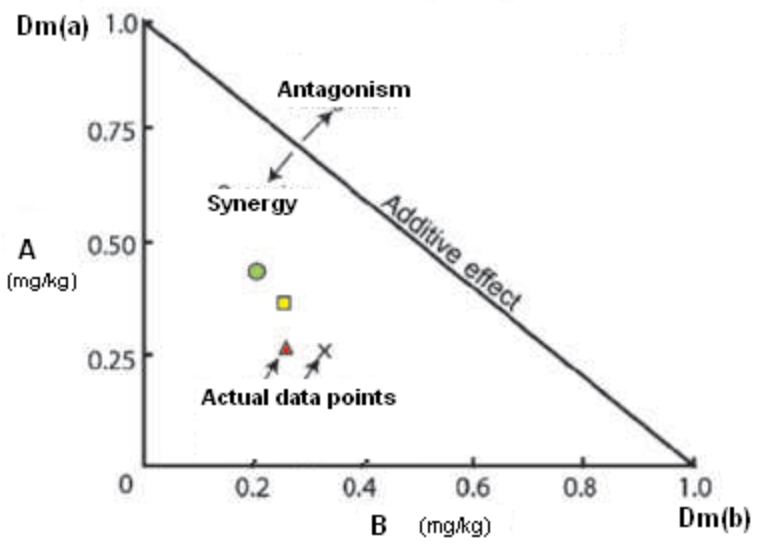


Figure 1.1: Schematic representation of isobologram for drugs A and B. (Figure adapted from Chou 2010)

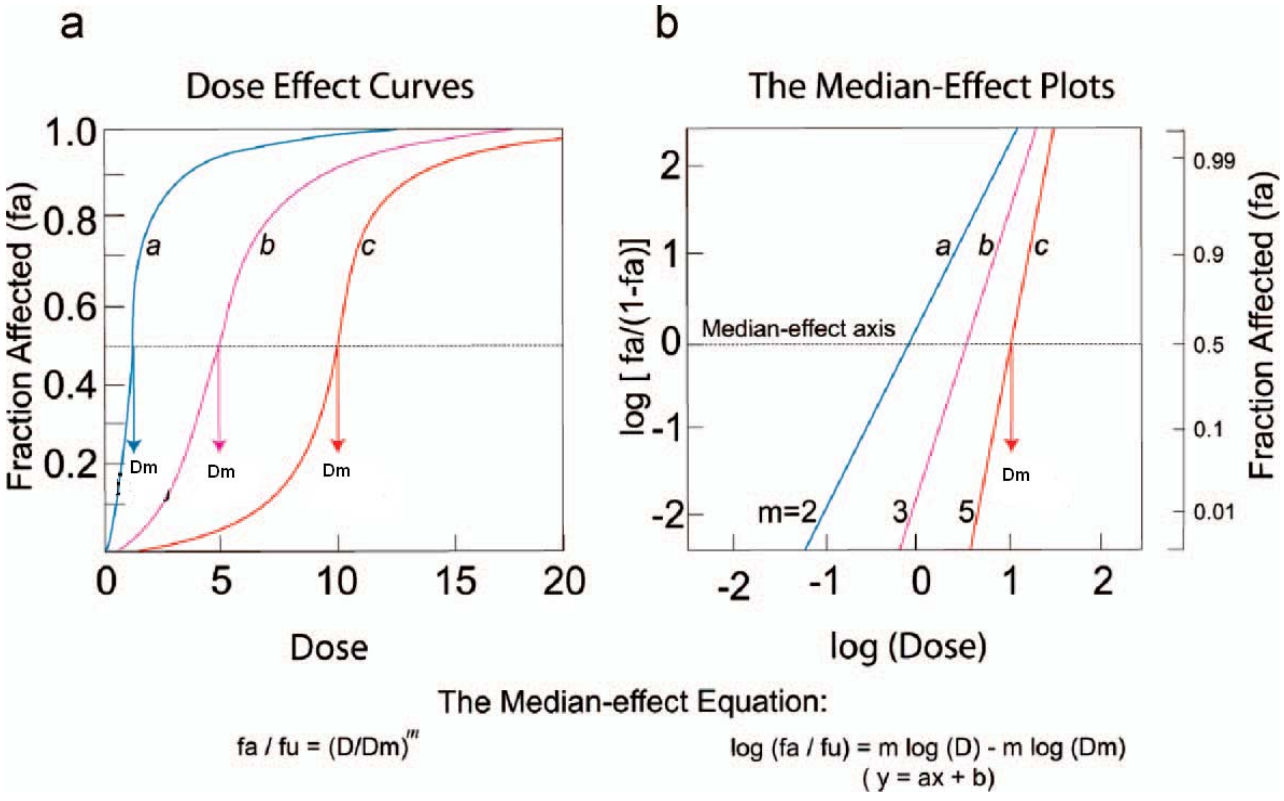


Figure 1.2: (a) Representative dose response curves for drugs a, b and c and (b) their transformation into corresponding median-effect plots. (Figure adapted from Chou 2008)

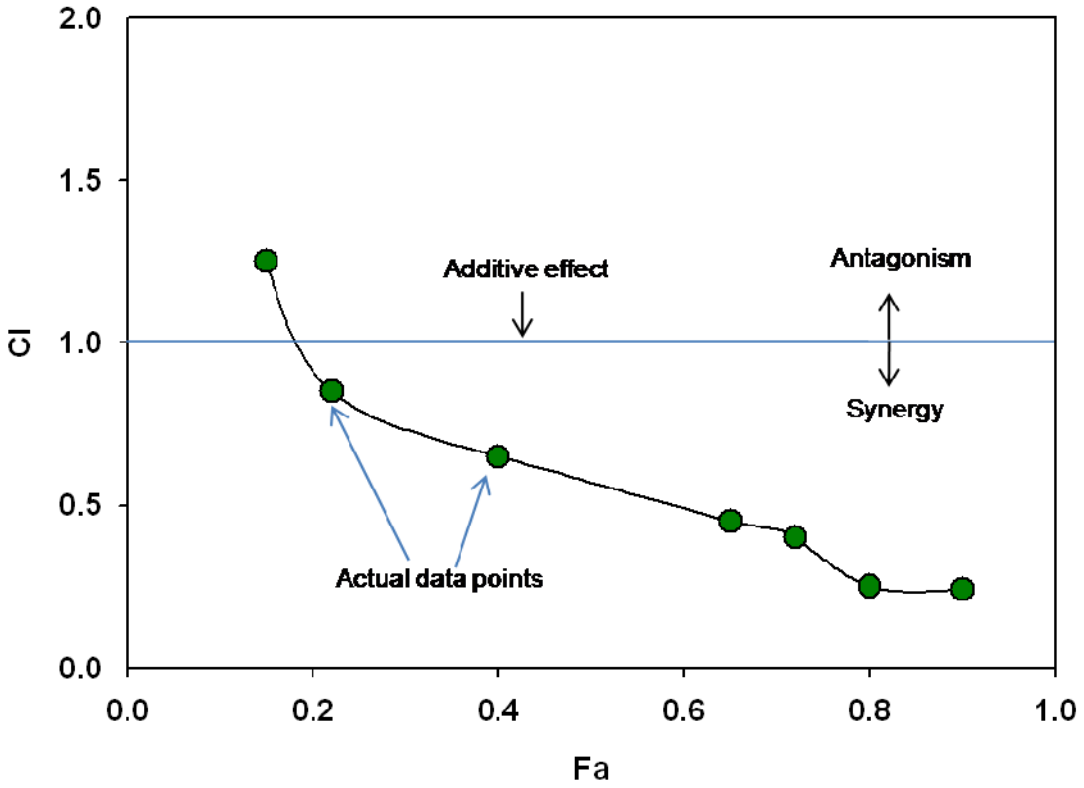


Figure 1.3: Representative Fa-CI plot generated with the help of MEP method of determining drug-drug interactions. (Figure adapted from Chou 2008)

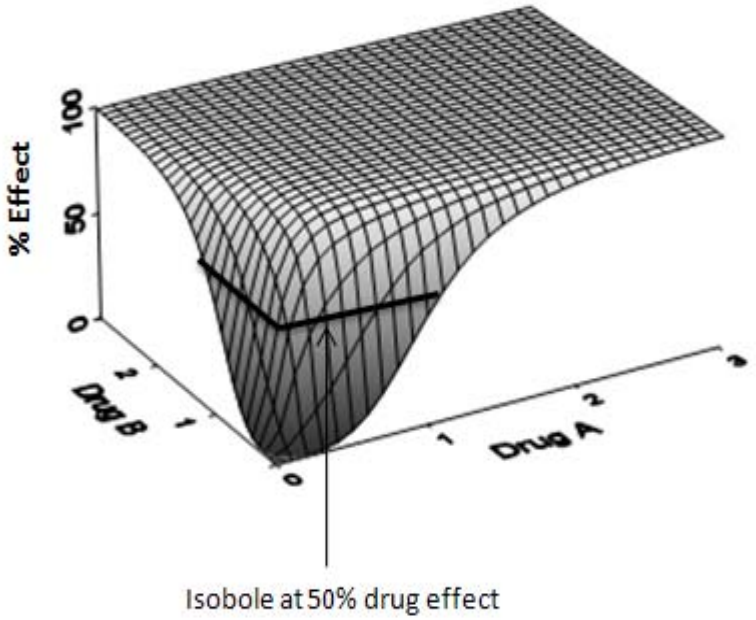


Figure 1.4: Schematic showing three dimensional response surface for drug combination containing drug A and drug B

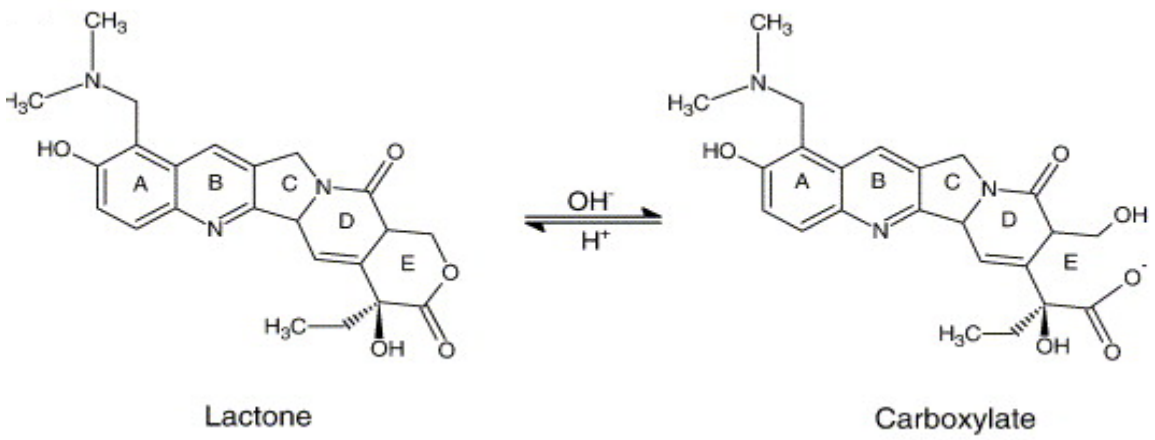
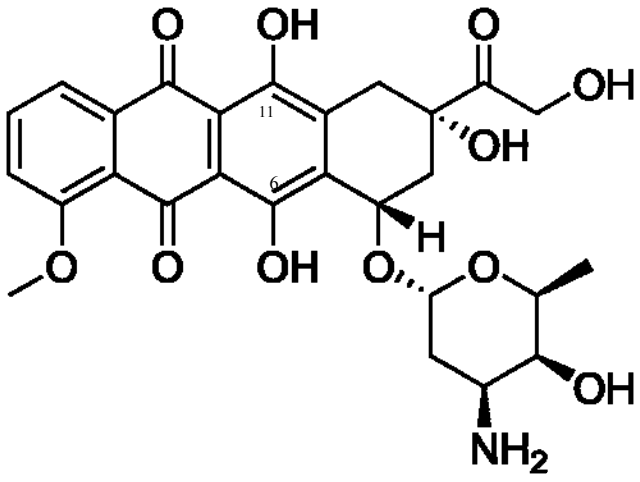


Figure 1.5: Chemical structure of topotecan hydrochloride



$C_{27}H_{29}NO_{11}$ **. HCl**

Figure 1.6: Chemical structure of doxorubicin hydrochloride

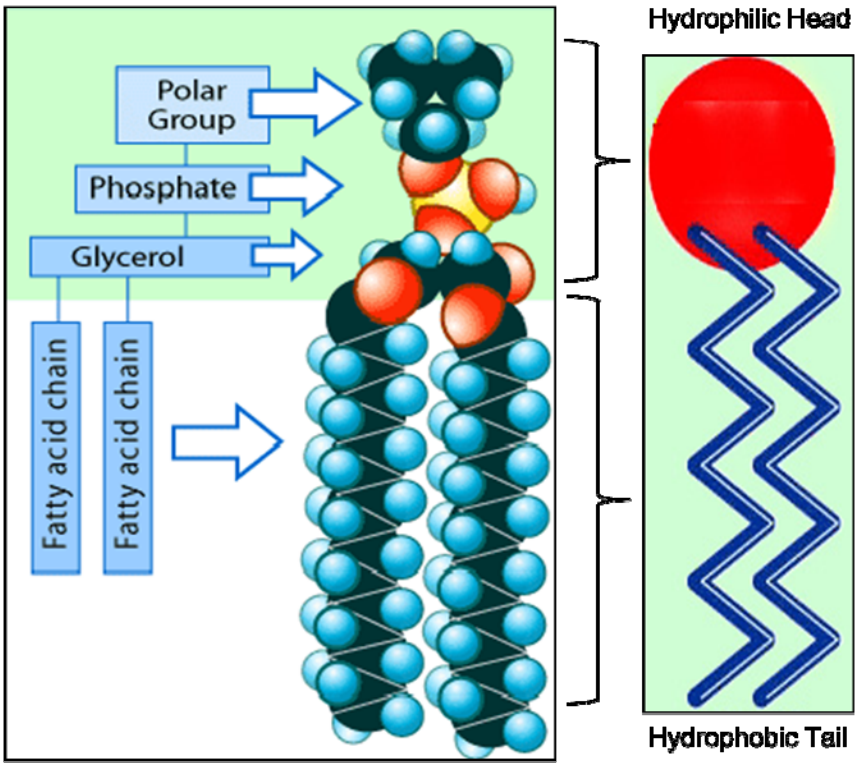
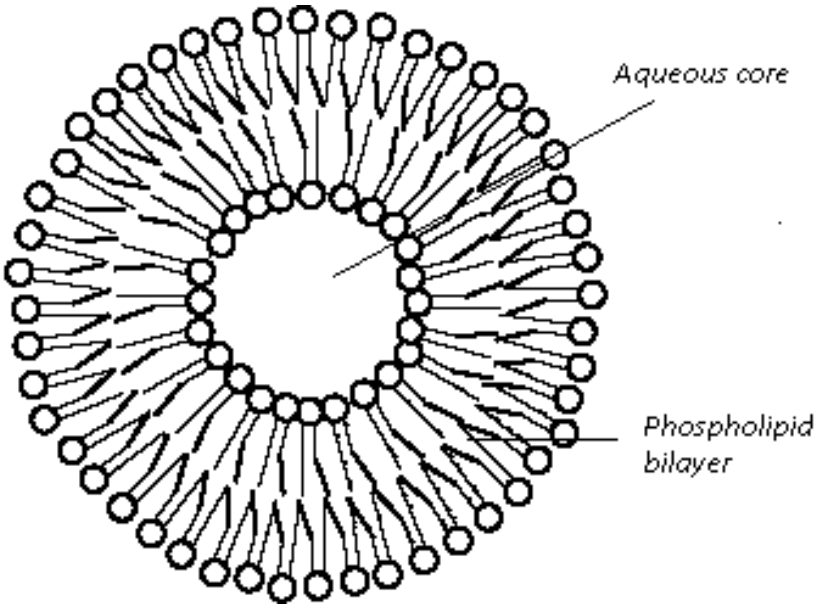


Figure 1.7: A schematic representation of a phospholipid molecule. (Figure adapted from http://www.bioteach.ubc.ca/Bio-industry/Inex/.)

(A)



(B)

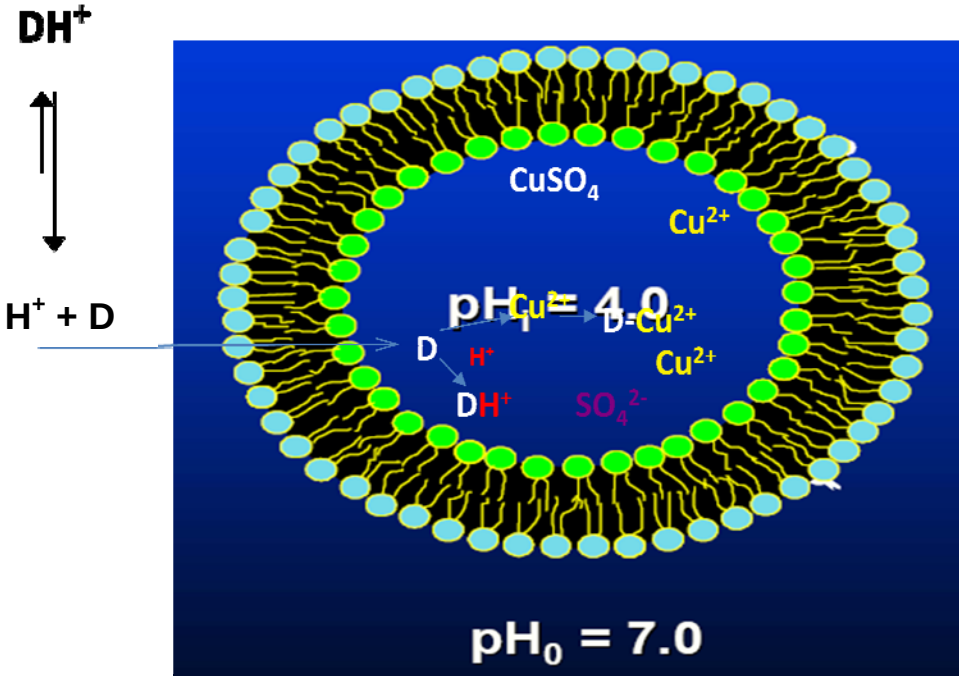


Figure 1.8: (A) Schematic representation of a liposomal vesicle and (B) Schematic showing process of active drug loading into liposomes prepared using aqueous buffer copper sulfate (pH 3.5-4.0) and suspended in a neutral buffer (pH 7.5) to create a transmembrane pH gradient, D=Drug.

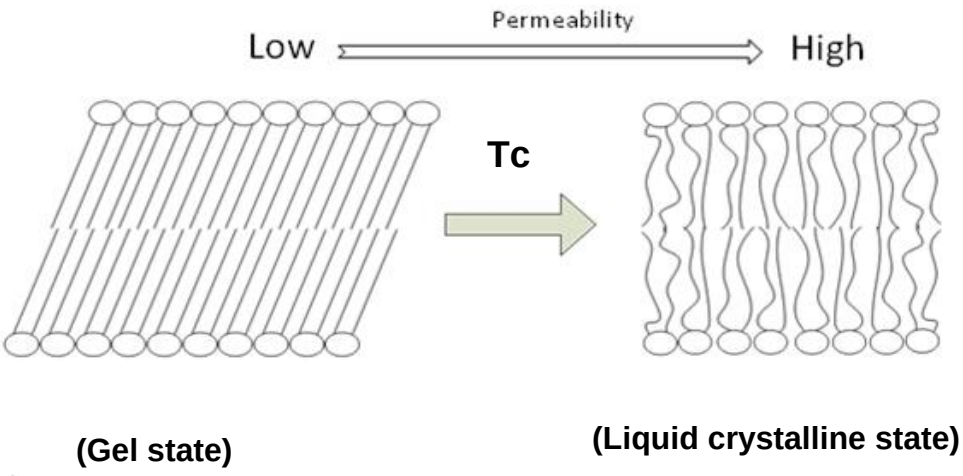


Figure 1.9: Schematic representation of Gel-to-liquid crystalline phase transition of a lipid bilayer. (Tc denotes transition temperature).

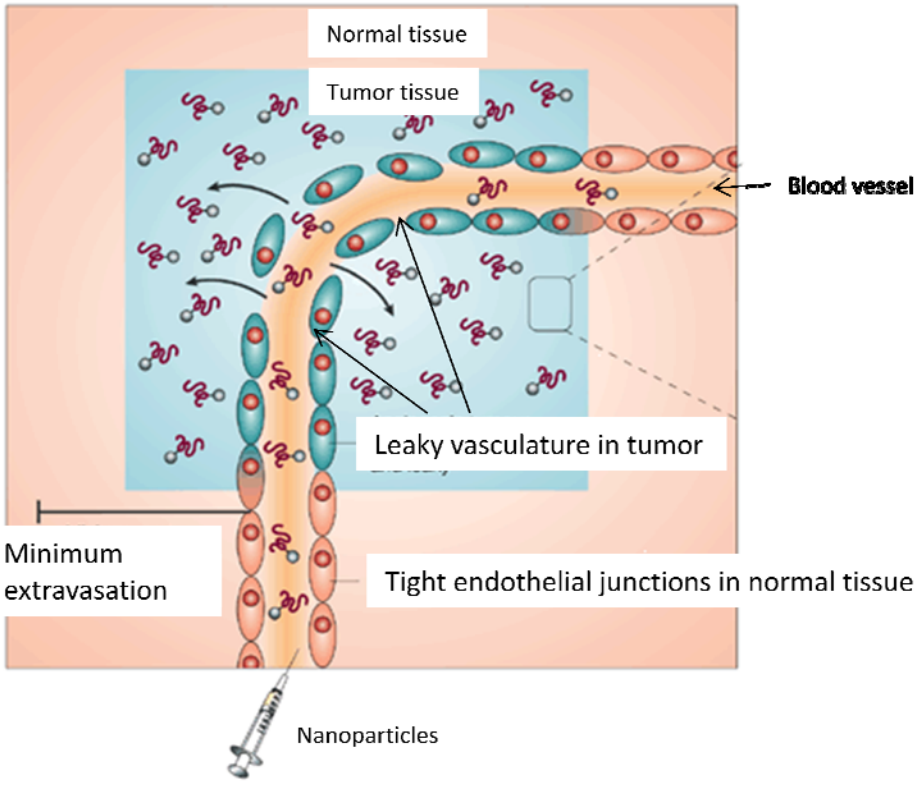


Figure 1.10: Schematic representation of passive tumor targeting with enhanced permeation and retention (EPR) effect. (Image adapted from Nature Reviews Drug Discovery 2, (May 2003))

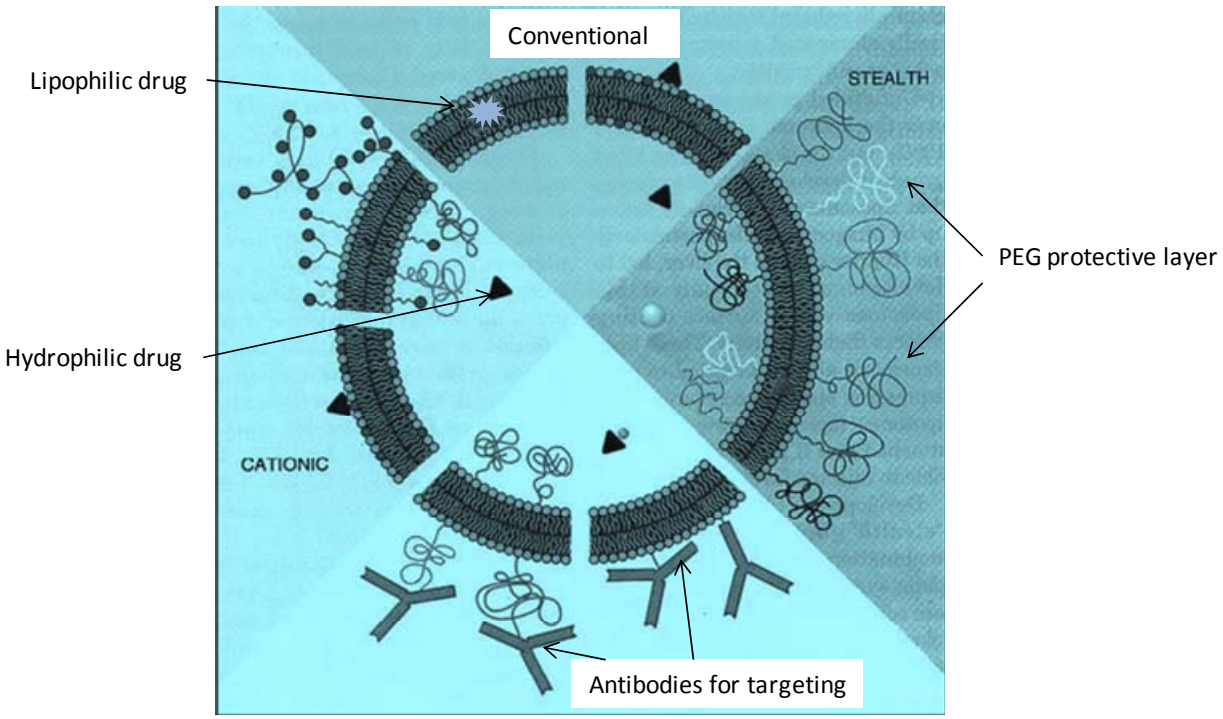


Figure 1.11: Surface modification of liposomes for enhanced stability or active targeting. (Image adapted from http://www.ntnu.edu/physics/medphys/drugdelivery)

1.8 REFERENCES

(2009). Adriamycin (Doxorubicin Hydrochloride) - Product Monograph. P. C. Inc. Kirkland.

Abou-Issa, H., Koolemans-Beynen, A., Minton, J. P. and Webb, T. E. (1989). "Synergistic interaction between 13-cis-retinoic acid and glucarate: activity against rat mammary tumor induction and MCF-7 cells." Biochem Biophys Res Commun **163**(3): 1364-9.

Abraham, S. A., Edwards, K., Karlsson, G., Hudon, N., Mayer, L. D. and Bally, M. B. (2004). "An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes." J Control Release **96**(3): 449-61.

Abraham, S. A., Edwards, K., Karlsson, G., MacIntosh, S., Mayer, L. D., McKenzie, C. and Bally, M. B. (2002). "Formation of transition metal-doxorubicin complexes inside liposomes." Biochim Biophys Acta **1565**(1): 41-54.

Ahmad, T. and Gore, M. (2004). "Review of the use of topotecan in ovarian carcinoma." Expert Opin Pharmacother **5**(11): 2333-40.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P.(2002). Molecular Biology of the Cell. New York, USA, Garland Science.

Alberts, D. S., Green, S., Hannigan, E. V., O'Toole, R., Stock-Novack, D., Anderson, P., Surwit, E. A., Malvlya, V. K., Nahhas, W. A. and Jolles, C. J. (1992). "Improved therapeutic index of carboplatin plus cyclophosphamide versus cisplatin plus cyclophosphamide: final report by the Southwest Oncology Group of a phase III randomized trial in stages III and IV ovarian cancer." J Clin Oncol **10**(5): 706-17.

Allen, C., Dos Santos, N., Gallagher, R., Chiu, G. N., Shu, Y., Li, W. M., Johnstone, S. A., Janoff, A. S., Mayer, L. D., Webb, M. S. and Bally, M. B. (2002). "Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol)." Biosci Rep **22**(2): 225-50.

Allen, D. G., Heintz, A. P. and Touw, F. W. (1995). "A meta-analysis of residual disease and survival in stage III and IV carcinoma of the ovary." Eur J Gynaecol Oncol **16**(5): 349-56.

Allen, T. M. and Chonn, A. (1987). "Large unilamellar liposomes with low uptake into the reticuloendothelial system." FEBS Lett **223**(1): 42-6.

Allen, T. M. and Everest, J. M. (1983). "Effect of liposome size and drug release properties on pharmacokinetics of encapsulated drug in rats." J Pharmacol Exp Ther **226**(2): 539-44.

Allen, T. M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991). "Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo." Biochim Biophys Acta **1066**(1): 29-36.

Armstrong, D. K., Bundy, B., Wenzel, L., Huang, H. Q., Baergen, R., Lele, S., Copeland, L. J., Walker, J. L. and Burger, R. A. (2006). "Intraperitoneal cisplatin and paclitaxel in ovarian cancer." N Engl J Med **354**(1): 34-43.

Armstrong, D. K. (2002). "Relapsed ovarian cancer: challenges and management strategies for a chronic disease." Oncologist **7 Suppl 5**: 20-8.

Aung, T. T., Davis, M. A., Ensminger, W. D. and Lawrence, T. S. (2000). "Interaction between gemcitabine and mitomycin-C in vitro." Cancer Chemother Pharmacol **45**(1): 38-42.

Baek, J. H., Kim, J. G., Jeon, S. B., Chae, Y. S., Kim, D. H., Sohn, S. K., Lee, K. B., Choi, Y. J., Shin, H. J., Chung, J. S., Cho, G. J., Jung, H. Y. and Yu, W. (2006). "Phase II study of capecitabine and irinotecan combination chemotherapy in patients with advanced gastric cancer." Br J Cancer **94**(10): 1407-11.

Bailey, A. L. and Cullis, P. R. (1997). "Membrane fusion with cationic liposomes: effects of target membrane lipid composition." Biochemistry **36**(7): 1628-34.

Bally, M. B., Masin, D., Nayar, R., Cullis, P. R. and Mayer, L. D. (1994). "Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumor-bearing mice." Cancer Chemother Pharmacol **34**(2): 137-46.

Bally, M. B., Mayer, L. D., Loughrey, H., Redelmeier, T., Madden, T. D., Wong, K., Harrigan, P. R., Hope, M. J. and Cullis, P. R. (1988). "Dopamine accumulation in large unilamellar vesicle systems induced by transmembrane ion gradients." Chem Phys Lipids **47**(2): 97-107.

Bangham, A. D., Hill, M. W. and Miller, N. G. A, Eds. (1974). Membranes in Membrane Biology. Ney York, Plenum.

Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965). "Diffusion of univalent ions across the lamellae of swollen phospholipids." J Mol Biol **13**(1): 238-52.

Bast, R. C., Jr., Hennessy, B. and Mills, G. B. (2009). "The biology of ovarian cancer: new opportunities for translation." Nat Rev Cancer **9**(6): 415-28.

Batist, G., Ramakrishnan, G., Rao, C. S., Chandrasekharan, A., Gutheil, J., Guthrie, T., Shah, P., Khojasteh, A., Nair, M. K., Hoelzer, K., Tkaczuk, K., Park, Y. C. and Lee, L. W. (2001). "Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer." J Clin Oncol **19**(5): 1444-54.

Batzri, S. and Korn, E. D. (1973). "Single bilayer liposomes prepared without sonication." Biochim Biophys Acta **298**(4): 1015-9.

Bawa, R. (2007). "Patents and nanomedicine." Nanomedicine (Lond) **2**(3): 351-74.

Berenbaum, M. C. (1989). "What is synergy?" Pharmacol Rev **41**(2): 93-141.

Bertelsen, K., Jakobsen, A., Andersen, J. E., Ahrons, S., Pedersen, P. H., Kiaer, H., Arffmann, E., Bichel, P., Boestofte, E., Stroyer, I. and et al. (1987). "A randomized study of cyclophosphamide and cis-platinum with or without doxorubicin in advanced ovarian carcinoma." Gynecol Oncol **28**(2): 161-9.

Betageri, G., Jenkins, S.A., Parsons, D.L. (1993). Liposomes Drug Delivery Systems., Technomic Publishing Company Inc.

Black, C. D. and Gregoriadis, G. (1976). "Interaction of liposomes with blood plasma proteins." Biochem Soc Trans **4**(2): 253-6.

Blanco, E., Kessinger, C. W., Sumer, B. D. and Gao, J. (2009). "Multifunctional micellar nanomedicine for cancer therapy." Exp Biol Med (Maywood) **234**(2): 123-31.

Boman, N. L., Mayer, L. D. and Cullis, P. R. (1993). "Optimization of the retention properties of vincristine in liposomal systems." Biochim Biophys Acta **1152**(2): 253-8.

Bookman, M. A., Brady, M. F., McGuire, W. P., Harper, P. G., Alberts, D. S., Friedlander, M., Colombo, N., Fowler, J. M., Argenta, P. A., De Geest, K., Mutch, D. G., Burger, R. A., Swart, A. M., Trimble, E. L., Accario-Winslow, C. and Roth, L. M. (2009). "Evaluation of new platinum-based treatment regimens in advanced-stage ovarian cancer: a Phase III Trial of the Gynecologic Cancer Intergroup." J Clin Oncol **27**(9): 1419-25.

Bookman, M. A., Darcy, K. M., Clarke-Pearson, D., Boothby, R. A. and Horowitz, I. R. (2003). "Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group." J Clin Oncol **21**(2): 283-90.

Bookman, M. A., Malmstrom, H., Bolis, G., Gordon, A., Lissoni, A., Krebs, J. B. and Fields, S. Z. (1998). "Topotecan for the treatment of advanced epithelial ovarian cancer: an open-label phase II study in patients treated after prior chemotherapy that contained cisplatin or carboplatin and paclitaxel." J Clin Oncol **16**(10): 3345-52.

Bouma, J., Beijnen, J. H., Bult, A. and Underberg, W. J. (1986). "Anthracycline antitumour agents. A review of physicochemical, analytical and stability properties." Pharm Weekbl Sci **8**(2): 109-33.

Bristow, R. E., Tomacruz, R. S., Armstrong, D. K., Trimble, E. L. and Montz, F. J. (2002). "Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis." J Clin Oncol **20**(5): 1248-59.

Broom, C. (1996). "Clinical studies of topotecan." Ann N Y Acad Sci **803**: 264-71.

Burden, D. A. and Osheroff, N. (1998). "Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme." Biochim Biophys Acta **1400**(1-3): 139-54.

Burger, R. A., Sill, M. W., Monk, B. J., Greer, B. E. and Sorosky, J. I. (2007). "Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: a Gynecologic Oncology Group Study." J Clin Oncol **25**(33): 5165-71.

Burke, T.G. et al. (1992). "Liposomal stabilization of camptothecin's lactone ring." J. Am. Chem. Soc. **114**: 8318-8319.

Burke, T. G. and Gao, X. (1994). "Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine." J Pharm Sci **83**(7): 967-9.

Carter, W. H., Jr. and Wampler, G. L. (1986). "Review of the application of response surface methodology in the combination therapy of cancer." Cancer Treat Rep **70**(1): 133-40.

Cassatella, M. A., Hartman, L., Perussia, B. and Trinchieri, G. (1989). "Tumor necrosis factor and immune interferon synergistically induce cytochrome b-245 heavy-chain gene expression and nicotinamide-adenine dinucleotide phosphate hydrogenase oxidase in human leukemic myeloid cells." J Clin Invest **83**(5): 1570-9.

Chapman, D. (1975). "Fluidity and phase transitions of cell membranes." Biomembranes **7**: 1-9.

Cavalieri, E., Munhall, A., Rogan, E., Salmasi, S. and Patil, K. (1983). "Syncarcinogenic effect of the environmental pollutants cyclopenteno[cd]pyrene and benzo[a]pyrene in mouse skin." Carcinogenesis **4**(4): 393-7.

Chen, J., Lu, Q. and Balthasar, J. P. (2007). "Mathematical modeling of topotecan pharmacokinetics and toxicodynamics in mice." J Pharmacokinet Pharmacodyn **34**(6): 829-47.

Chen, S. S. and Lee, L. (1983). "Incidence of para-aortic and pelvic lymph node metastases in epithelial carcinoma of the ovary." Gynecol Oncol **16**(1): 95-100.

Cheng, M. F., Chatterjee, S. and Berger, N. A. (1994). "Schedule-dependent cytotoxicity of topotecan alone and in combination chemotherapy regimens." Oncol Res **6**(6): 269-79.

Cheung, B. C., Sun, T. H., Leenhouts, J. M. and Cullis, P. R. (1998). "Loading of doxorubicin into liposomes by forming Mn2+-drug complexes." Biochim Biophys Acta **1414**(1-2): 205-16.

Chiu, G. N., Abraham, S. A., Ickenstein, L. M., Ng, R., Karlsson, G., Edwards, K., Wasan, E. K. and Bally, M. B. (2005). "Encapsulation of doxorubicin into thermosensitive liposomes via complexation with the transition metal manganese." J Control Release **104**(2): 271-88.

Chobanian, N. and Dietrich, C. S., 3rd (2008). "Ovarian cancer." Surg Clin North Am **88**(2): 285-99, vi.

Chou, T. (1991). Synergism and Antagonism in Chemotherapy. San Diego, Academic Press.

Chou, T. C. (2006). "Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies." Pharmacol Rev **58**(3): 621-81.

Chou, T. C. and Talalay, P. (1984). "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors." Adv Enzyme Regul **22**: 27-55.

Clejan, L. and Cederbaum, A. I. (1989). "Synergistic interactions between NADPH-cytochrome P-450 reductase, paraquat, and iron in the generation of active oxygen radicals." Biochem Pharmacol **38**(11): 1779-86.

Coleman, R. L., Broaddus, R. R., Bodurka, D. C., Wolf, J. K., Burke, T. W., Kavanagh, J. J., Levenback, C. F. and Gershenson, D. M. (2006). "Phase II trial of imatinib mesylate in patients with recurrent platinum- and taxane-resistant epithelial ovarian and primary peritoneal cancers." Gynecol Oncol **101**(1): 126-31.

Conte, P. F., Bruzzone, M., Carnino, F., Chiara, S., Donadio, M., Facchini, V., Fioretti, P., Foglia, G., Gadducci, A., Gallo, L. and et al. (1991). "Carboplatin, doxorubicin, and cyclophosphamide versus cisplatin, doxorubicin, and cyclophosphamide: a randomized trial in stage III-IV epithelial ovarian carcinoma." J Clin Oncol **9**(4): 658-63.

Creemers, G. J., Bolis, G., Gore, M., Scarfone, G., Lacave, A. J., Guastalla, J. P., Despax, R., Favalli, G., Kreinberg, R., Van Belle, S., Hudson, I., Verweij, J. and Ten Bokkel Huinink, W. W. (1996). "Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study." J Clin Oncol **14**(12): 3056-61.

Cusack, B. J., Young, S. P., Driskell, J. and Olson, R. D. (1993). "Doxorubicin and doxorubicinol pharmacokinetics and tissue concentrations following bolus injection and continuous infusion of doxorubicin in the rabbit." Cancer Chemother Pharmacol **32**(1): 53-8.

Cullis, P. R., Hope, M. J., Bally, M. B., Madden, T. D., Mayer, L. D. and Fenske, D. B. (1997). "Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles." Biochim Biophys Acta **1331**(2): 187-211.

Dadashzadeh, S., Vali, A. M. and Rezaie, M. (2008). "The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats." Int J Pharm **353**(1-2): 251-9.

Dapergolas, G. and Gregoriadis, G. (1977). "The effect of liposomal lipid composition on the fate and effect of liposome-entrapped insulin and tubocurarine [proceedings]." Biochem Soc Trans **5**(5): 1383-6.

Daruwalla, J., Greish, K., Malcontenti-Wilson, C., Muralidharan, V., Iyer, A., Maeda, H. and Christophi, C. (2009). "Styrene maleic acid-pirarubicin disrupts tumor microcirculation and enhances the permeability of colorectal liver metastases." J Vasc Res **46**(3): 218-28.

Davis, C. and Gregoriadis, G. (1979). "The effect of lipid composition on the stability of liposomes in vivo [proceedings]." Biochem Soc Trans **7**(4): 680-2.

Delgado, G., Oram, D. H. and Petrilli, E. S. (1984). "Stage III epithelial ovarian cancer: the role of maximal surgical reduction." Gynecol Oncol **18**(3): 293-8.

DeVita, V. (1997). Principles of cancer management: chemotherapy. Philadelphia.

DeVita, VT Jr, Hellman, S. and Rosenberg, SA, Eds. (1989). Principles of Chemotherapy. Cancer Principles and Practice of Oncology. Philadelphia, J. B. Lippincott.

Dicko, A., Tardi, P., Xie, X. and Mayer, L. (2007). "Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes." Int J Pharm **337**(1-2): 219-28.

Dingli, D. and Nowak, M. A. (2006). "Cancer biology: infectious tumour cells." Nature **443**(7107): 35-6.

Drummond, D. C., Hayes, M. E., Noble, C. O. and Kirpotin, D. B., Eds. (2007). Intraliposomal trapping agents for improving in vivo liposomal drug formulation stability. Liposome Technology. New York, Informa Healthcare.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Connolly-Ingram, C., Gabriel, B. S., Hann, B., Liu, B., Park, J. W., Hong, K., Benz, C. C., Marks, J. D. and Kirpotin, D. B. (2010). "Development of a highly stable and targetable nanoliposomal formulation of topotecan." J Control Release **141**(1): 13-21.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Park, J. W., Ou, C. J., Tseng, Y. L., Hong, K. and Kirpotin, D. B. (2009). "Improved pharmacokinetics and efficacy of a highly stable nanoliposomal vinorelbine." J Pharmacol Exp Ther **328**(1): 321-30.

du Bois, A., Luck, H. J., Pfisterer, J., Meier, W. and Bauknecht, T. (2000). "[Anthracyclines in therapy of ovarian carcinoma: a systematic review of primary and 2nd-line therapy after platinum]." Zentralbl Gynakol **122**(5): 255-67.

Earle, C. C., Schrag, D., Neville, B. A., Yabroff, K. R., Topor, M., Fahey, A., Trimble, E. L., Bodurka, D. C., Bristow, R. E., Carney, M. and Warren, J. L. (2006). "Effect of surgeon specialty on processes of care and outcomes for ovarian cancer patients." J Natl Cancer Inst **98**(3): 172-80.

Elit, L., Oliver, T. K., Covens, A., Kwon, J., Fung, M. F., Hirte, H. W. and Oza, A. M. (2007). "Intraperitoneal chemotherapy in the first-line treatment of women with stage III epithelial ovarian cancer: a systematic review with metaanalyses." Cancer **109**(4): 692-702.

Eltabbakh, G. H. and Mount, S. L. (2002). "Lymphatic spread among women with primary peritoneal carcinoma." J Surg Oncol **81**(3): 126-31.

Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N., Johnson, D. A., Richardson, T. B., Santarosa, M., Dillon, K. J., Hickson, I., Knights, C., Martin, N. M., Jackson, S. P., Smith, G. C. and Ashworth, A. (2005). "Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy." Nature **434**(7035): 917-21.

Fiallo, M. M., Tayeb, H., Suarato, A. and Garnier-Suillerot, A. (1998). "Circular dichroism studies on anthracycline antitumor compounds. Relationship between the molecular structure and the spectroscopic data." J Pharm Sci **87**(8): 967-75.

Folkman, J., Ed. (2008). Tumor Angiogenesis: from Bench to Bedside. Tumor Angiogenesis. New York, Springer Berlin Heidelberg.

Fraser, TR (1972). "The antagonism between the actions of active substances." British Medical Journal **2**: 485-487.

Frei, E. (1991). Clinical studies of combination therapy for cancer. San Diego, Academic Press.

Frezard, F. and Garnier-Suillerot, A. (1991). "DNA-containing liposomes as a model for the study of cell membrane permeation by anthracycline derivatives." Biochemistry **30**(20): 5038-43.

Gabizon, A. A. (2001). "Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy." Cancer Invest **19**(4): 424-36.

Gabizon, A. and Papahadjopoulos, D. (1988). "Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors." Proc Natl Acad Sci U S A **85**(18): 6949-53.

Gabizon, A., Shmeeda, H. and Barenholz, Y. (2003). "Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies." Clin Pharmacokinet **42**(5): 419-36.

Gelmon, KA, Hirte, HW, Robidoux, A, Tonkin, KS, Tischkowitz, M, Swenerton, K, Huntsman, D, Carmichael, J., Macpherson, E and Oza, AM (2010). Can we define tumors that will respond to PARP inhibitors? A phase II correlative study of olaparib in advanced serous ovarian cancer and triple-negative breast cancer., J Clin Oncol **28:** 15s.

Gordon, A. N., Fleagle, J. T., Guthrie, D., Parkin, D. E., Gore, M. E. and Lacave, A. J. (2001). "Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan." J Clin Oncol **19**(14): 3312-22.

Greco, W. R., Bravo, G. and Parsons, J. C. (1995). "The search for synergy: a critical review from a response surface perspective." Pharmacol Rev **47**(2): 331-85.

Greenlee, R. T., Hill-Harmon, M. B., Murray, T. and Thun, M. (2001). "Cancer statistics, 2001." CA Cancer J Clin **51**(1): 15-36.

Gregoriadis, G., Ed. (1993). Liposome Technology. Liposomes Preparation and Related Techniques. Boca Raton, Florida, CRC Press.

Griffiths, C. T. (1975). "Surgical resection of tumor bulk in the primary treatment of ovarian carcinoma." Natl Cancer Inst Monogr **42**: 101-4.

Guarneri, V., Piacentini, F., Barbieri, E. and Conte, P. F. "Achievements and unmet needs in the management of advanced ovarian cancer." Gynecol Oncol **117**(2): 152-8.

Gurney, H., Crowther, D., Anderson, H., Murphy, D., Prendiville, J., Ranson, M., Mayor, P., Swindell, R., Buckley, C. H. and Tindall, V. R. (1990). "Five year follow-up and dose delivery analysis of cisplatin, iproplatin or carboplatin in combination with cyclophosphamide in advanced ovarian carcinoma." Ann Oncol **1**(6): 427-33.

Hamaguchi, T., Kato, K., Yasui, H., Morizane, C., Ikeda, M., Ueno, H., Muro, K., Yamada, Y., Okusaka, T., Shirao, K., Shimada, Y., Nakahama, H. and Matsumura, Y. (2007). "A phase I and pharmacokinetic study of NK105, a paclitaxel-incorporating micellar nanoparticle formulation." Br J Cancer **97**(2): 170-6.

Hamilton, R. L., Jr., Goerke, J., Guo, L. S., Williams, M. C. and Havel, R. J. (1980). "Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique." J Lipid Res **21**(8): 981-92.

Hanahan, D., Bergers, G. and Bergsland, E. (2000). "Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice." J Clin Invest **105**(8): 1045-7.

Haran, G., Cohen, R., Bar, L. K. and Barenholz, Y. (1993). "Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases." Biochim Biophys Acta **1151**(2): 201-15.

Harasym T. O., P.G. Tardi, M. B., A. Janoff (2006). Fixed drug ratio liposome formulations of combination cancer therapeutics. New York, informa healthcare.

Helzlsouer, K. J., Bush, T. L., Alberg, A. J., Bass, K. M., Zacur, H. and Comstock, G. W. (1993). "Prospective study of serum CA-125 levels as markers of ovarian cancer." JAMA **269**(9): 1123-6.

Hertzberg, R. P., Caranfa, M. J. and Hecht, S. M. (1989). "On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme-DNA complex." Biochemistry **28**(11): 4629-38.

Herzog, T. J. (2002). "Update on the role of topotecan in the treatment of recurrent ovarian cancer." Oncologist **7 Suppl 5**: 3-10.

Herzog,T.J. (2004). "Recurrent ovarian cancer: how important is it to treat to disease progression?" Clin Cancer Res **10**(22): 7439-49.

Hess, L. M., Benham-Hutchins, M., Herzog, T. J., Hsu, C. H., Malone, D. C., Skrepnek, G. H., Slack, M. K. and Alberts, D. S. (2007). "A meta-analysis of the efficacy of intraperitoneal cisplatin for the front-line treatment of ovarian cancer." Int J Gynecol Cancer **17**(3): 561-70.

Hirte, H., Oza, A., Swenerton, K., Ellard, S. L., Grimshaw, R., Fisher, B., Tsao, M. and Seymour, L. "A phase II study of erlotinib (OSI-774) given in combination with carboplatin in patients with recurrent epithelial ovarian cancer (NCIC CTG IND.149)." Gynecol Oncol.

Hope, M. J., Bally, M.B., Webb, G and Cullis, P. R. (1985). "Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential." Biochim Biophys Acta **812**: 55-65.

Hori, K., Suzuki, M., Tanda, S., Saito, S., Shinozaki, M. and Zhang, Q. H. (1991). "Fluctuations in tumor blood flow under normotension and the effect of angiotensin II-induced hypertension." Jpn J Cancer Res **82**(11): 1309-16.

Horowitz, N. S., Hua, J., Gibb, R. K., Mutch, D. G. and Herzog, T. J. (2004). "The role of topotecan for extending the platinum-free interval in recurrent ovarian cancer: an in vitro model." Gynecol Oncol **94**(1): 67-73.

Hovi, T., Williams, S. C. and Allison, A. C. (1975). "Divalent cation ionophore A23187 forms lipid soluble complexes with leucine and other amino acids." Nature **256**(5512): 70-2.

Hsiang, Y. H., Hertzberg, R., Hecht, S. and Liu, L. F. (1985). "Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I." J Biol Chem **260**(27): 14873-8.

Hsiang, Y. H., Lihou, M. G. and Liu, L. F. (1989). "Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin." Cancer Res **49**(18): 5077-82.

Hsiang, Y. H. and Liu, L. F. (1988). "Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin." Cancer Res **48**(7): 1722-6.

Huang, Y. H., Zugates, G. T., Peng, W., Holtz, D., Dunton, C., Green, J. J., Hossain, N., Chernick, M. R., Padera, R. F., Jr., Langer, R., Anderson, D. G. and Sawicki, J. A. (2009). "Nanoparticle-delivered suicide gene therapy effectively reduces ovarian tumor burden in mice." Cancer Res **69**(15): 6184-91.

Jackel, R., Fuchs, M., Raff, T. and Wiedemann, B. (2002). "[Drug-induced toxic epidermal necrolysis with involvement of the intestinal and respiratory tract. A case report]." Anaesthesist **51**(10): 815-9.

Jaxel, C., Kohn, K. W., Wani, M. C., Wall, M. E. and Pommier, Y. (1989). "Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity." Cancer Res **49**(6): 1465-9.

Johnson, S. M., Bangham, A. D., Hill, M. W. and Korn, E. D. (1971). "Single bilayer liposomes." Biochim Biophys Acta **233**(3): 820-6.

Johnston, M. J., Semple, S. C., Klimuk, S. K., Edwards, K., Eisenhardt, M. L., Leng, E. C., Karlsson, G., Yanko, D. and Cullis, P. R. (2006). "Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations." Biochim Biophys Acta **1758**(1): 55-64.

Johnstone, S. A., Masin, D., Mayer, L. and Bally, M. B. (2001). "Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages." Biochim Biophys Acta **1513**(1): 25-37.

Jonsson, E., Fridborg, H., Nygren, P. and Larsson, R. (1998). "Synergistic interactions of combinations of topotecan with standard drugs in primary cultures of human tumor cells from patients." Eur J Clin Pharmacol **54**(7): 509-14.

Kaku, T., Ogawa, S., Kawano, Y., Ohishi, Y., Kobayashi, H., Hirakawa, T. and Nakano, H. (2003). "Histological classification of ovarian cancer." Med Electron Microsc **36**(1): 9-17.

Kanter, P. M., Bullard, G. A., Ginsberg, R. A., Pilkiewicz, F. G., Mayer, L. D., Cullis, P. R. and Pavelic, Z. P. (1993). "Comparison of the cardiotoxic effects of liposomal doxorubicin (TLC D-99) versus free doxorubicin in beagle dogs." In Vivo **7**(1): 17-26.

Kanzawa, F., Koizumi, F., Koh, Y., Nakamura, T., Tatsumi, Y., Fukumoto, H., Saijo, N., Yoshioka, T. and Nishio, K. (2001). "In vitro synergistic interactions between the cisplatin analogue nedaplatin and the DNA topoisomerase I inhibitor irinotecan and the mechanism of this interaction." Clin Cancer Res **7**(1): 202-9.

Kerbel, R. and Folkman, J. (2002). "Clinical translation of angiogenesis inhibitors." Nat Rev Cancer **2**(10): 727-39.

Kim, D., Gao, Z. G., Lee, E. S. and Bae, Y. H. (2009). "In vivo evaluation of doxorubicin-loaded polymeric micelles targeting folate receptors and early endosomal pH in drug-resistant ovarian cancer." Mol Pharm **6**(5): 1353-62.

Kirby, C., Clarke, J. and Gregoriadis, G. (1980). "Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro." Biochem J **186**(2): 591-8.

Kobel, M, Kalloger, SE, Boyd, N, Mckinney, S, Mehl, E, Palmer, C, Leung, S, Bowen, NJ, Lonescu, DN, Rajput, A, Prentice, LM, Miller, D, Santos, J, Swenerton, K, Gilks, CB and Huntsman, D (2008). "Ovarian Carcinoma Subtypes Are Different Diseases: Implications for Biomarker Studies." PLOS Medicine **5**(12).

Kunisawa, J., Masuda, T., Katayama, K., Yoshikawa, T., Tsutsumi, Y., Akashi, M., Mayumi, T. and Nakagawa, S. (2005). "Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release." J Control Release **105**(3): 344-53.

Kurman, R. J. and Shih Ie, M. "The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory." Am J Surg Pathol **34**(3): 433-43.

Lafky, J. M., Wilken, J. A., Baron, A. T. and Maihle, N. J. (2008). "Clinical implications of the ErbB/epidermal growth factor (EGF) receptor family and its ligands in ovarian cancer." Biochim Biophys Acta **1785**(2): 232-65.

Lasic, D. D., Ceh, B., Stuart, M. C., Guo, L., Frederik, P. M. and Barenholz, Y. (1995). "Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery." Biochim Biophys Acta **1239**(2): 145-56.

Li, C. J., Miyamoto, Y., Kojima, Y. and Maeda, H. (1993). "Augmentation of tumour delivery of macromolecular drugs with reduced bone marrow delivery by elevating blood pressure." Br J Cancer **67**(5): 975-80.

Li, L. H., Fraser, T. J., Olin, E. J. and Bhuyan, B. K. (1972). "Action of camptothecin on mammalian cells in culture." Cancer Res **32**(12): 2643-50.

Lichtenberg, D. and Barenholz, Y. (1988). "Liposomes: preparation, characterization, and preservation." Methods Biochem Anal **33**: 337-462.

Lim, H. J., Masin, D., Madden, T. D. and Bally, M. B. (1997). "Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone." J Pharmacol Exp Ther **281**(1): 566-73.

Loewe, S. (1953). "The problem of synergism and antagonism of combined drugs." Arzneimittelforschung **3**(6): 285-90.

Lorusso, D., Pietragalla, A., Mainenti, S., Masciullo, V., Di Vagno, G. and Scambia, G. "Review role of topotecan in gynaecological cancers: current indications and perspectives." Crit Rev Oncol Hematol **74**(3): 163-74.

Luo, J., Hiao, K., Li, Y., Lee, J., Shi, L., Tan, Y., Xing, L., Cheng, R. H., Liu, G. Y. and Lam, K. S. (2010). "Well-Defined, Size-Tunable, Multifunctional Micelles for Efficient Paclitaxel Delivery for Cancer Treatment." Bioconjugate Chemistry **21**: 1216-24.

Madden, T. D., Harrigan, P. R., Tai, L. C., Bally, M. B., Mayer, L. D., Redelmeier, T. E., Loughrey, H. C., Tilcock, C. P., Reinish, L. W. and Cullis, P. R. (1990). "The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey." Chem Phys Lipids **53**(1): 37-46.

Maeda, H. (2001). "The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting." Adv Enzyme Regul **41**: 189-207.

Maeda, H. (2001). "SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy." Adv Drug Deliv Rev **46**(1-3): 169-85.

Maeda, H., Fang, J., Inutsuka, T. and Kitamoto, Y. (2003). "Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications." Int Immunopharmacol **3**(3): 319-28.

Maeda, H. and Matsumura, Y. (2010). "EPR effect based drug design and clinical outlook for enhanced cancer chemotherapy." Adv Drug Deliv Rev.

Main, C., Bojke, L., Griffin, S., Norman, G., Barbieri, M., Mather, L., Stark, D., Palmer, S. and Riemsma, R. (2006). "Topotecan, pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line or subsequent treatment of advanced ovarian cancer: a systematic review and economic evaluation." Health Technol Assess **10**(9): 1-148.

Main, C., Bojke, L., Griffin, S., Norman, G., Barbieri, M., Mather, L., Stark, D., Palmer, S. and Riemsma, R. (2006). "Topotecan, pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line or subsequent treatment of advanced ovarian cancer: a systematic review and economic evaluation." Health Technol Assess **10**(9): 1-132 iii-iv.

Mamot, C., Drummond, D. C., Greiser, U., Hong, K., Kirpotin, D. B., Marks, J. D. and Park, J. W. (2003). "Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells." Cancer Res **63**(12): 3154-61.

Mangala, L. S., Han, H. D., Lopez-Berestein, G. and Sood, A. K. (2009). "Liposomal siRNA for ovarian cancer." Methods Mol Biol **555**: 29-42.

Markert, S., Lassmann, S., Gabriel, B., Klar, M., Werner, M., Gitsch, G., Kratz, F. and Hasenburg, A. (2008). "Alpha-folate receptor expression in epithelial ovarian carcinoma and non-neoplastic ovarian tissue." Anticancer Res **28**(6A): 3567-72.

Markman, M. (2004). "Improving the toxicity profile of chemotherapy for advanced ovarian cancer: a potential role for CT-2103." J Exp Ther Oncol **4**(2): 131-6.

Martin, FM, Ed. (1998). Clinical pharmacology and anti-tumor efficacy of Doxil (pegylated liposomal doxorubicin). Medical Applications of Liposomes. Amsterdam, Elsevier.

Matsumura, Y. and Maeda, H. (1986). "A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs." Cancer Res **46**(12 Pt 1): 6387-92.

Mavroudis, D., Efstathiou, E. and Polyzos, A. (2004). A phase I–II trial of gefitinib in combination with vinorelbine and oxaliplatin as salvage therapy in women with advanced ovarian cancer (AOC). ASCO Annual Meeting Proceedings.

Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986). "Vesicles of variable sizes produced by a rapid extrusion procedure." Biochim Biophys Acta **858**(1): 161-8.

Mayer, L. D., Bally, M. B., Cullis, P. R., Wilson, S. L. and Emerman, J. T. (1990). "Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor." Cancer Lett **53**(2-3): 183-90.

Mayer, L.D., Cullis, P. R. and Bally, M. B. (1994). "The use of thransmebrane pH gradient-driven drug encapsulation in the pharmacodynamic evaluation of liposomal doxorubicin. ." Journal of Liposome Research **4**(1): 529-553.

Mayer, L. D., Harasym, T. O., Tardi, P. G., Harasym, N. L., Shew, C. R., Johnstone, S. A., Ramsay, E. C., Bally, M. B. and Janoff, A. S. (2006). "Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice." Mol Cancer Ther **5**(7): 1854-63.

Mayer, L. D., Bally, M. B., Hope, M. J. and Cullis, P. R. (1985). "Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential." Biochim Biophys Acta **816**(2): 294-302.

Mayer, L. D., Bally, M. B., Hope, M. J. and Cullis, P. R. (1986). "Techniques for encapsulating bioactive agents into liposomes." Chem Phys Lipids **40**(2-4): 333-45.

Mayer, L. D., Hope, M. J., Cullis, P. R. and Janoff, A. S. (1985). "Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles." Biochim Biophys Acta **817**(1): 193-6.

Mayhew, E., Lazo, R., Vail, W. J., King, J. and Green, A. M. (1984). "Characterization of liposomes prepared using a microemulsifier." Biochim Biophys Acta **775**(2): 169-74.

McEvoy, G. K. (2005). AHFS 2005 Drug Information. . American Society of Health-System Pharmacists, Inc. . G. K. McEvoy. Bethesda, Maryland.

McAlpine, J. N., Wiegand, K. C., Vang, R., Ronnett, B. M., Adamiak, A., Kobel, M., Kalloger, S. E., Swenerton, K. D., Huntsman, D. G., Gilks, C. B. and Miller, D. M. (2009). "HER2 overexpression and amplification is present in a subset of ovarian mucinous carcinomas and can be targeted with trastuzumab therapy." BMC Cancer **9**: 433.

McGuire, W. P., Hoskins, W. J., Brady, M. F., Kucera, P. R., Partridge, E. E., Look, K. Y., Clarke-Pearson, D. L. and Davidson, M. (1996). "Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer." N Engl J Med **334**(1): 1-6.

Messerer, C. L., Ramsay, E. C., Waterhouse, D., Ng, R., Simms, E. M., Harasym, N., Tardi, P., Mayer, L. D. and Bally, M. B. (2004). "Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer." Clin Cancer Res **10**(19): 6638-49.

Mi, Z., Malak, H. and Burke, T. G. (1995). "Reduced albumin binding promotes the stability and activity of topotecan in human blood." Biochemistry **34**(42): 13722-8.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004). "Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity." Pharmacol Rev **56**(2): 185-229.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004). "Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity." Pharmacol Rev **56**(2): 185-229.

Mirza, M. R., Lund, B., Lindegaard, J. C., Keldsen, N., Mellemgaard, A., Christensen, R. D. and Bertelsen, K. "A phase II study of combination chemotherapy in early relapsed epithelial ovarian cancer using gemcitabine and pegylated liposomal doxorubicin." Gynecol Oncol.

Moghimi, S. M. and Patel, H. M. (1988). "Tissue specific opsonins for phagocytic cells and their different affinity for cholesterol-rich liposomes." FEBS Lett **233**(1): 143-7.

Monk, B. J., Herzog, T. J., Kaye, S. B., Krasner, C. N., Vermorken, J. B., Muggia, F. M., Pujade-Lauraine, E., Lisyanskaya, A. S., Makhson, A. N., Rolski, J., Gorbounova, V. A., Ghatage, P., Bidzinski, M., Shen, K., Ngan, H. Y., Vergote, I. B., Nam, J. H., Park, Y. C., Lebedinsky, C. A. and Poveda, A. M. "Trabectedin plus pegylated liposomal Doxorubicin in recurrent ovarian cancer." J Clin Oncol **28**(19): 3107-14.

Mozafari, M. R. (2005). "Liposomes: an overview of manufacturing techniques." Cell Mol Biol Lett **10**(4): 711-9.

Mross, K., Maessen, P., van der Vijgh, W. J., Gall, H., Boven, E. and Pinedo, H. M. (1988). "Pharmacokinetics and metabolism of epidoxorubicin and doxorubicin in humans." J Clin Oncol **6**(3): 517-26.

Muggia, F. M. (1997). "Clinical efficacy and prospects for use of pegylated liposomal doxorubicin in the treatment of ovarian and breast cancers." Drugs **54 Suppl 4**: 22-9.

National Cancer Institute of Canada (2005). "Statistics Canada, Provincial/Territorial Cancer Registries." Canadian Cancer Statistics

Nagy, J. A., Herzberg, K. T., Masse, E. M., Zientara, G. P. and Dvorak, H. F. (1989). "Exchange of macromolecules between plasma and peritoneal cavity in ascites tumor-bearing, normal, and serotonin-injected mice." Cancer Res **49**(19): 5448-58.

Needham, D., McIntosh, T. J. and Evans, E. (1988). "Thermomechanical and transition properties of dimyristoylphosphatidylcholine/cholesterol bilayers." Biochemistry **27**(13): 4668-73.

Needham, D. and Nunn, R. S. (1990). "Elastic deformation and failure of lipid bilayer membranes containing cholesterol." Biophys J **58**(4): 997-1009.

Nelson, D. A., Tan, T. T., Rabson, A. B., Anderson, D., Degenhardt, K. and White, E. (2004). "Hypoxia and defective apoptosis drive genomic instability and tumorigenesis." Genes Dev **18**(17): 2095-107.

Nicolay, K., Sautereau, A. M., Tocanne, J. F., Brasseur, R., Huart, P., Ruysschaert, J. M. and de Kruijff, B. (1988). "A comparative model membrane study on structural effects of membrane-active positively charged anti-tumor drugs." Biochim Biophys Acta **940**(2): 197-208.

Nicolay, K., Timmers, R. J., Spoelstra, E., Van der Neut, R., Fok, J. J., Huigen, Y. M., Verkleij, A. J. and De Kruijff, B. (1984). "The interaction of adriamycin with cardiolipin in model and rat liver mitochondrial membranes." Biochim Biophys Acta **778**(2): 359-71.

Niikura, H., Sasano, H., Sato, S. and Yajima, A. (1997). "Expression of epidermal growth factor-related proteins and epidermal growth factor receptor in common epithelial ovarian tumors." Int J Gynecol Pathol **16**(1): 60-8.

Nishiyama, N., Okazaki, S., Cabral, H., Miyamoto, M., Kato, Y., Sugiyama, Y., Nishio, K., Matsumura, Y. and Kataoka, K. (2003). "Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice." Cancer Res **63**(24): 8977-83.

Nitiss, J. L., Rose, A., Sykes, K. C., Harris, J. and Zhou, J. (1996). "Using yeast to understand drugs that target topoisomerases." Ann N Y Acad Sci **803**: 32-43.

Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J. and Papahadjopoulos, D. (1979). "Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes." Biochim Biophys Acta **557**(1): 9-23.

Olson, R. D., Mushlin, P. S., Brenner, D. E., Fleischer, S., Cusack, B. J., Chang, B. K. and Boucek, R. J., Jr. (1988). "Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol." Proc Natl Acad Sci U S A **85**(10): 3585-9.

Oshawa, T, Miura, H and Harada, K (1985). "Evaluation of a new liposome preparation technique, the freeze-thawing method, using L-asparaginase as a model drug.
." Chemical and Pharmaceutical Bulletin **33**(7): 2916-23.

Oshawa, T, Miura, H and Harada, K (1985). "Studies on the effect of water-soluble additives and on the encapsulation mechanism in liposome preparation by the freeze-thawing method. ." Chemical and Pharmaceutical Bulletin **33**(12): 5474.

Ouyang, C., Choice, E., Holland, J., Meloche, M. and Madden, T. D. (1995). "Liposomal cyclosporine. Characterization of drug incorporation and interbilayer exchange." Transplantation **60**(9): 999-1006.

Ozols, R. F., Bundy, B. N., Greer, B. E., Fowler, J. M., Clarke-Pearson, D., Burger, R. A., Mannel, R. S., DeGeest, K., Hartenbach, E. M. and Baergen, R. (2003). "Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study." J Clin Oncol **21**(17): 3194-200.

Pan, X. and Lee, R. J. (2004). "Tumour-selective drug delivery via folate receptor-targeted liposomes." Expert Opin Drug Deliv **1**(1): 7-17.

Parmar, M. K., Ledermann, J. A., Colombo, N., du Bois, A., Delaloye, J. F., Kristensen, G. B., Wheeler, S., Swart, A. M., Qian, W., Torri, V., Floriani, I., Jayson, G., Lamont, A. and Trope, C. (2003). "Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial." Lancet **361**(9375): 2099-106.

Pavillard, V., Kherfellah, D., Richard, S., Robert, J. and Montaudon, D. (2001). "Effects of the combination of camptothecin and doxorubicin or etoposide on rat glioma cells and camptothecin-resistant variants." Br J Cancer **85**(7): 1077-83.

Pfisterer, J., Plante, M., Vergote, I., du Bois, A., Hirte, H., Lacave, A. J., Wagner, U., Stahle, A., Stuart, G., Kimmig, R., Olbricht, S., Le, T., Emerich, J., Kuhn, W., Bentley, J., Jackisch, C., Luck, H. J., Rochon, J., Zimmermann, A. H. and Eisenhauer, E. (2006). "Gemcitabine plus carboplatin compared with carboplatin in patients with platinum-sensitive recurrent

ovarian cancer: an intergroup trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG." J Clin Oncol **24**(29): 4699-707.

Raitanen, M., Rantanen, V., Kulmala, J., Helenius, H., Grenman, R. and Grenman, S. (2002). "Supra-additive effect with concurrent paclitaxel and cisplatin in vulvar squamous cell carcinoma in vitro." Int J Cancer **100**(2): 238-43.

Ramaswamy, S. (2007). "Rational design of cancer-drug combinations." N Engl J Med **357**(3): 299-300.

Ramsay, E., Alnajim, J., Anantha, M., Taggar, A., Thomas, A., Edwards, K., Karlsson, G., Webb, M. and Bally, M. (2006). "Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation." Pharm Res **23**(12): 2799-808.

Ramsay, E., Alnajim, J., Anantha, M., Zastre, J., Yan, H., Webb, M., Waterhouse, D. and Bally, M. (2008). "A novel liposomal irinotecan formulation with significant anti-tumour activity: use of the divalent cation ionophore A23187 and copper-containing liposomes to improve drug retention." Eur J Pharm Biopharm **68**(3): 607-17.

Ramsay, E. C., Anantha, M., Zastre, J., Meijs, M., Zonderhuis, J., Strutt, D., Webb, M. S., Waterhouse, D. and Bally, M. B. (2008). "Irinophore C: a liposome formulation of irinotecan with substantially improved therapeutic efficacy against a panel of human xenograft tumors." Clin Cancer Res **14**(4): 1208-17.

Ramsay, E. C., Dos Santos, N., Dragowska, W. H., Laskin, J. J. and Bally, M. B. (2005). "The formulation of lipid-based nanotechnologies for the delivery of fixed dose anticancer drug combinations." Curr Drug Deliv **2**(4): 341-51.

Ransom, D. T., Patel, S. R., Keeney, G. L., Malkasian, G. D. and Edmonson, J. H. (1990). "Papillary serous carcinoma of the peritoneum. A review of 33 cases treated with platin-based chemotherapy." Cancer **66**(6): 1091-4.

Razin, S. (1972). "Reconstruction of biological membranes." Biochim Biophys Acta **265**(2): 241-96.

Rose, P. G., Nerenstone, S., Brady, M. F., Clarke-Pearson, D., Olt, G., Rubin, S. C., Moore, D. H. and Small, J. M. (2004). "Secondary surgical cytoreduction for advanced ovarian carcinoma." N Engl J Med **351**(24): 2489-97.

Rosing, H., van Zomeren, D. M., Doyle, E., ten Bokkel, W. W., Schellens, J. H., Bult, A. and Beijnen, J. H. (1999). "Quantification of topotecan and its metabolite N-desmethyltopotecan in human plasma, urine and faeces by high-performance liquid chromatographic methods." J Chromatogr B Biomed Sci Appl **727**(1-2): 191-203.

Ricchelli, F., Jori, G., Gobbo, S. and Tronchin, M. (1991). "Liposomes as models to study the distribution of porphyrins in cell membranes." Biochim Biophys Acta **1065**(1): 42-8.

Riggs, C. E., Jr., Benjamin, R. S., Serpick, A. A. and Bachur, N. R. (1977). "Bilary disposition of adriamycin." Clin Pharmacol Ther **22**(2): 234-41.

Robert, J., Ed. (1998). Anthracyclines. A clinician's guide to chemotherapy pharmacokinetics and pharmacodynamics. Baltimore, Williams & Wilkins.

Rudin, CM and Thompsan, CB, Eds. (2002). Apoptosis and Cancer. The genetic basis of human cancer. New York, McGraw-Hill, Medical Pub. Division.

Saltz, L. (2000). "Irinotecan-based combinations for the adjuvant treatment of stage III colon cancer." Oncology (Williston Park) **14**(12 Suppl 14): 47-50.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., Maroun, J. A., Ackland, S. P., Locker, P. K., Pirotta, N., Elfring, G. L. and Miller, L. L. (2000). "Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group." N Engl J Med **343**(13): 905-14.

Scarberry, K. E., Dickerson, E. B., McDonald, J. F. and Zhang, Z. J. (2008). "Magnetic nanoparticle-peptide conjugates for in vitro and in vivo targeting and extraction of cancer cells." J Am Chem Soc **130**(31): 10258-62.

Scarberry, K. E., Dickerson, E. B., Zhang, Z. J., Benigno, B. B. and McDonald, J. F. "Selective removal of ovarian cancer cells from human ascites fluid using magnetic nanoparticles." Nanomedicine **6**(3): 399-408.

Schinazi, RF, Ed. (1991). Combined chemotherapeutic modalities for viral infections:rationale and clinical potential. . Synergism **and** Antagonism in Chemotherapy,. New York, Academic Press.

Schilder, R. J., Sill, M. W., Chen, X., Darcy, K. M., Decesare, S. L., Lewandowski, G., Lee, R. B., Arciero, C. A., Wu, H. and Godwin, A. K. (2005). "Phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma and evaluation of epidermal growth factor receptor mutations and immunohistochemical expression: a Gynecologic Oncology Group Study." Clin Cancer Res **11**(15): 5539-48.

Schmitt-Sody, M., Strieth, S., Krasnici, S., Sauer, B., Schulze, B., Teifel, M., Michaelis, U., Naujoks, K. and Dellian, M. (2003). "Neovascular targeting therapy: paclitaxel encapsulated in cationic liposomes improves antitumoral efficacy." Clin Cancer Res **9**(6): 2335-41.

Secord, A. A., Blessing, J. A., Armstrong, D. K., Rodgers, W. H., Miner, Z., Barnes, M. N., Lewandowski, G. and Mannel, R. S. (2008). "Phase II trial of cetuximab and carboplatin in relapsed platinum-sensitive ovarian cancer and evaluation of epidermal growth factor receptor expression: a Gynecologic Oncology Group study." Gynecol Oncol **108**(3): 493-9.

Semple, S. C., Chonn, A. and Cullis, P. R. (1996). "Influence of cholesterol on the association of plasma proteins with liposomes." Biochemistry **35**(8): 2521-5.

Skinner, S. A., Tutton, P. J. and O'Brien, P. E. (1990). "Microvascular architecture of experimental colon tumors in the rat." Cancer Res **50**(8): 2411-7.

Slomovitz, B. M., Coleman, R. L., Levenback, C., Jung, M., Gershenson, D. M. and Wolf, J. (2006). Phase I study of weekly topotecan and gefitinib in patients with platinum-resistant ovarian, peritoneal, or fallopian tube cancer. ASCO Annual Meeting Proceedings.

Smith, S. M., Johnson, J. L., Niedzwiecki, D., Eder, J. P., Canellos, G., Cheson, B. D., Bartlett, N. L., Cancer and Leukemia Group, B. (2006). "Sequential doxorubicin and topotecan in relapsed/refractory aggressive non-Hodgkin's lymphoma: results of CALGB 59906." Leuk Lymphoma **47**(8): 1511-7.

Stewart, C. J., Owens, O. J., Richmond, J. A. and McNicol, A. M. (1992). "Expression of epidermal growth factor receptor in normal ovary and in ovarian tumors." Int J Gynecol Pathol **11**(4): 266-72.

Subczynski, W. K., Wisniewska, A., Yin, J. J., Hyde, J. S. and Kusumi, A. (1994). "Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol." Biochemistry **33**(24): 7670-81.

Suzuki, M., Hori, K., Abe, I., Saito, S. and Sato, H. (1981). "A new approach to cancer chemotherapy: selective enhancement of tumor blood flow with angiotensin II." J Natl Cancer Inst **67**(3): 663-9

.

Swenerton, K., Jeffrey, J., Stuart, G., Roy, M., Krepart, G., Carmichael, J., Drouin, P., Stanimir, R., O'Connell, G., MacLean, G. and et al. (1992). "Cisplatin-cyclophosphamide versus carboplatin-cyclophosphamide in advanced ovarian cancer: a randomized phase III study of the National Cancer Institute of Canada Clinical Trials Group." J Clin Oncol **10**(5): 718-26.

Swift, L. P., Rephaeli, A., Nudelman, A., Phillips, D. R. and Cutts, S. M. (2006). "Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death." Cancer Res **66**(9): 4863-71.

Szoka, F., Jr. and Papahadjopoulos, D. (1980). "Comparative properties and methods of preparation of lipid vesicles (liposomes)." Annu Rev Biophys Bioeng **9**: 467-508.

Taggar, A. S., Alnajim, J., Anantha, M., Thomas, A., Webb, M., Ramsay, E. and Bally, M. B. (2006). "Copper-topotecan complexation mediates drug accumulation into liposomes." J Control Release **114**(1): 78-88.

Tangjitgamol, S., Manusirivithaya, S., Laopaiboon, M. and Lumbiganon, P. (2009). "Interval debulking surgery for advanced epithelial ovarian cancer." Cochrane Database Syst Rev(2): CD006014.

Tannock, I. (1992). New York, McGraw-Hill.

ten Bokkel Huinink, W., Gore, M., Carmichael, J., Gordon, A., Malfetano, J., Hudson, I., Broom, C., Scarabelli, C., Davidson, N., Spanczynski, M., Bolis, G., Malmstrom, H., Coleman, R., Fields, S. C. and Heron, J. F. (1997). "Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer." J Clin Oncol **15**(6): 2183-93.

Thigpen, J. T., Aghajanian, C. A., Alberts, D. S., Campos, S. M., Gordon, A. N., Markman, M., McMeekin, D. S., Monk, B. J. and Rose, P. G. (2005). "Role of pegylated liposomal doxorubicin in ovarian cancer." Gynecol Oncol **96**(1): 10-8.

Tardi, P., Choice, E., Masin, D., Redelmeier, T., Bally, M. and Madden, T. D. (2000). "Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models." Cancer Res **60**(13): 3389-93.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D. and Liu, L. F. (1984). "Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II." Science **226**(4673): 466-8.

Uchino, H., Matsumura, Y., Negishi, T., Koizumi, F., Hayashi, T., Honda, T., Nishiyama, N., Kataoka, K., Naito, S. and Kakizoe, T. (2005). "Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats." Br J Cancer **93**(6): 678-87.

Uziely, B., Jeffers, S., Isacson, R., Kutsch, K., Wei-Tsao, D., Yehoshua, Z., Libson, E., Muggia, F. M. and Gabizon, A. (1995). "Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies." J Clin Oncol **13**(7): 1777-85.

Valeriote, F. and Lin, H. (1975). "Synergistic interaction of anticancer agents: a cellular perspective." Cancer Chemother Rep **59**(5): 895-900.

Vance, D.E., Ed. (2002). Biochemistry of Lipids Lipoproteins and Membranes. Boston, Elsevier.

van der Burg, M. E., van Lent, M., Buyse, M., Kobierska, A., Colombo, N., Favalli, G., Lacave, A. J., Nardi, M., Renard, J. and Pecorelli, S. (1995). "The effect of debulking surgery after induction chemotherapy on the prognosis in advanced epithelial ovarian cancer. Gynecological Cancer Cooperative Group of the European Organization for Research and Treatment of Cancer." N Engl J Med **332**(10): 629-34.

Vermorken, J. B., Harper, P. G. and Buyse, M. (1999). "The role of anthracyclines in epithelial ovarian cancer." Ann Oncol **10 Suppl 1**: 43-50.

Verhaar-Langereis, M., Karakus, A., van Eijkeren, M., Voest, E. and Witteveen, E. (2006). "Phase II study of the combination of pegylated liposomal doxorubicin and topotecan in platinum-resistant ovarian cancer." Int J Gynecol Cancer **16**(1): 65-70.

Wall, M. E., Wani, M. C., Nicholas, A. W., Manikumar, G., Tele, C., Moore, L., Truesdale, A., Leitner, P. and Besterman, J. M. (1993). "Plant antitumor agents. 30. Synthesis and structure activity of novel camptothecin analogs." J Med Chem **36**(18): 2689-700.

Waterhouse, D, Kalra, J , Verreault, M, Ramsay, E, Dragowska, W, Yapp, D, Webb, M, Chiu, G and Bally, M (2008). Nanotechnology as an Enabling Approach to the Development of Fixed-Dose Combination Products for Treating Cancer, American Scientific Publishers.

Waterhouse, D. N., Gelmon, K. A., Klasa, R., Chi, K., Huntsman, D., Ramsay, E., Wasan, E., Edwards, L., Tucker, C., Zastre, J., Wang, Y. Z., Yapp, D., Dragowska, W., Dunn, S., Dedhar, S. and Bally, M. B. (2006). "Development and assessment of conventional and targeted drug combinations for use in the treatment of aggressive breast cancers." Curr Cancer Drug Targets **6**(6): 455-89.

Webb, M. S., Harasym, T. O., Masin, D., Bally, M. B. and Mayer, L. D. (1995). "Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models." Br J Cancer **72**(4): 896-904.

Wu, J., Liu, Q. and Lee, R. J. (2006). "A folate receptor-targeted liposomal formulation for paclitaxel." Int J Pharm **316**(1-2): 148-53.

Wu, N. Z., Da, D., Rudoll, T. L., Needham, D., Whorton, A. R. and Dewhirst, M. W. (1993). "Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumor tissue." Cancer Res **53**(16): 3765-70.

Wu, P. C., Qu, J. Y., Lang, J. H., Huang, R. L., Tang, M. Y. and Lian, L. J. (1986). "Lymph node metastasis of ovarian cancer: a preliminary survey of 74 cases of lymphadenectomy." Am J Obstet Gynecol **155**(5): 1103-8.

Yeagle, P. L. (1985). "Cholesterol and the cell membrane." Biochim Biophys Acta **822**(3-4): 267-87.

Yellepeddi, V. K., Kumar, A. and Palakurthi, S. (2009). "Biotinylated poly(amido)amine (PAMAM) dendrimers as carriers for drug delivery to ovarian cancer cells in vitro." Anticancer Res **29**(8): 2933-43.

Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D. and Jain, R. K. (1994). "Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft." Cancer Res **54**(13): 3352-6.

Zahedi, P., De Souza, R., Piquette-Miller, M. and Allen, C. (2009). "Chitosan-phospholipid blend for sustained and localized delivery of docetaxel to the peritoneal cavity." Int J Pharm **377**(1-2): 76-84.

Zhigaltsev, I. V., Maurer, N., Akhong, Q. F., Leone, R., Leng, E., Wang, J., Semple, S. C. and Cullis, P. R. (2005). "Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention." J Control Release **104**(1): 103-11.

Zhigaltsev, I. V., Winters, G., Srinivasulu, M., Crawford, J., Wong, M., Amankwa, L., Waterhouse, D., Masin, D., Webb, M., Harasym, N., Heller, L., Bally, M. B., Ciufolini, M. A., Cullis, P. R. and Maurer, N. "Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles." J Control Release **144**(3): 332-40.

Zimmermann, G. R., Lehar, J. and Keith, C. T. (2007). "Multi-target therapeutics: when the whole is greater than the sum of the parts." Drug Discov Today **12**(1-2): 34-42.

Zoli, W., Ricotti, L., Tesei, A., Barzanti, F. and Amadori, D. (2001). "In vitro preclinical models for a rational design of chemotherapy combinations in human tumors." Crit Rev Oncol Hematol **37**(1): 69-82.

2. THE ROLE OF THE TRANSITION METAL COPPER AND THE IONOPHORE A23187 IN THE DEVELOPMENT OF IRINOPHORE C™

[1]A version of this chapter has been accepted for publication in "Pharmaceutical Research" as:

Patankar, N., Ramsay, E., Anantha, M., Waterhouse, D. and Bally, M. (2010). "The role of the transition metal copper and the ionophore A23187 in the development of Irinophore C™ ".

2.1 SUMMARY

A lipid nanoparticle (LNP) irinotecan formulation was recently described that exhibits significant therapeutic potential. This formulation, referred to as Irinophore C™, relies on the ability of copper to complex irinotecan within the liposome, thereby effectively "trapping" the drug. The resulting formulation exhibits surprising improvements in drug retention that could not have been predicted on the basis of previously described formulation approaches. This formulation is now being developed for assessment in clinical trials and part of this development program has involved a careful evaluation of critical drug loading parameters. The studies presented here were designed to determine the optimum concentration of copper required for the effective encapsulation and retention of irinotecan into liposomes. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (CH) (55:45 mol%) liposomes were formulated using buffers containing various concentrations of copper or manganese and irinotecan loading was determined in the presence and absence of A23187, a divalent metal ionophore. The rate and extent of irinotecan encapsulation was determined and subsequently the rate of irinotecan release from the liposomes was assessed *in vivo*. The amount of copper retained inside liposomes following irinotecan loading and the effect of copper on membrane permeability was determined. Results indicated that efficient (>98% encapsulation) irinotecan loading (to a drug-to-lipid ratio of 0.2) can be achieved using encapsulated copper concentrations as low as 50 mM. However irinotecan release was dependent on the concentration of copper used to facilitate drug loading, with a minimum concentration of 300 mM required for optimal drug retention. Importantly, the presence of copper was shown to increase membrane permeability as measured by using sucrose as a membrane permeable marker. This data provided an explanation as to why irinotecan loading rates are enhanced in the presence of formulations prepared with copper but is

counterintuitive given the improved drug retention attributes of Irinophore C™. It is speculated that the Irinophore C™ formulation exhibits improved drug retention when compared to formulations prepared with manganese due to generation of a complex between copper and irinotecan.

2.2 INTRODUCTION

The success of clinically approved liposomal formulations of anthracyclines (Batist, Ramakrishnan et al. 2001; Gordon, Fleagle et al. 2001) such as Doxil®/Caelyx®, have highlighted the potential of lipid nanoparticle (LNP) formulations to improve the therapeutic activity of selected anticancer drugs. However, the clinical development of preclinically promising LNP formulations has proven challenging. This is due to several reasons including the fact that preclinical data obtained with optimized LNP formulations has not effectively predicted activities in patients. Clinical development of Irinophore C™, a liposomal formulation of irinotecan discussed in this chapter is currently being pursued. Numerous LNP formulations of irinotecan are currently under investigation (Haran, Cohen et al. 1993; Emerson 2000; Messerer, Ramsay et al. 2004; Ramsay, Alnajim et al. 2006; Dicko, Tardi et al. 2007; Tardi, Gallagher et al. 2007; Ramsay, Anantha et al. 2008) and the success or failure of these formulations will depend on a comprehensive understanding of formulation parameters as well as biological activities. Irinophore C™ exhibited substantial therapeutic effects in multiple models of cancer (Ramsay, Anantha et al. 2008). The mechanisms governing the therapeutic activity of Irinophore C™ involve:

i) Stabilization of irinotecan into its active lactone form (Ramsay, Alnajim et al. 2006; Ramsay, Alnajim et al. 2008),

ii) Enhanced irinotecan delivery to sites of tumor growth (Ramsay, Anantha et al. 2008)

iii) Increased plasma concentration over extended time periods of irinotecan as well as its more active metabolite SN-38 (Ramsay, Alnajim et al. 2008; Ramsay, Anantha et al. 2008) , and

iv) The ability of this LNP irinotecan formulation to promote tumor vasculature normalization (Baker, Lam et al. 2008).

A substantial amount of information has been collected to better understand the biological activities of Irinophore C^{TM} and this database continues to expand as the toxicity of the formulation has recently entered formal evaluation in pre-clinical safety studies. The studies reported here serves to supplement the biological knowledge base with information on how various formulation parameters influence the physical properties of Irinophore C™.

Irinophore C^{TM} utilizes a pH gradient drug loading methodology combined with encapsulated divalent metals capable of forming coordination complexes with irinotecan (Ramsay, Alnajim et al. 2006). It is now well established that divalent metals can, even in the absence of a pH gradient, facilitate the encapsulation of selected anticancer drugs with chemical groups capable of forming coordination complexes with transition metals trapped inside liposomal vesicles (Cheung, Sun et al. 1998; Kamidate, Hashimoto et al. 2002; Abraham, Edwards et al. 2004; Messerer, Ramsay et al. 2004; Dos Santos, Waterhouse et al. 2005; Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006; Dicko, Tardi et al. 2007). When assessing the role of transmembrane pH gradients on this loading process, research suggested that copper exhibited a distinct advantage over manganese, another metal commonly employed to facilitate encapsulation in terms of drug retention (Messerer, Ramsay et al. 2004; Taggar, Alnajim et al. 2006; Tardi, Gallagher et al. 2007). Transmembrane pH gradients can be created in a number of ways: i) preparing liposomes using acidic aqueous buffers (Cullis, Hope et al.

1997; Abraham, Edwards et al. 2004), ii) use of aqueous solution of ammonium, sulfate (Haran, Cohen et al. 1993) or iii) by preparing liposomes with aqueous solutions of monovalent or divalent metal ions in combination with an appropriate transmembrane ionophore (Abraham, Edwards et al. 2004; Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006). This third method was used to develop Irinophore C™ where the transmembrane ionophore calcimycin (also known as calcium ionophore or A23187) was added to liposomes with encapsulated metal solutions. The pH gradient is created when encapsulated divalent metals are transported out of the liposome in exchange for protons present in the external buffer. As indicated above, studies with various divalent metals demonstrated that copper was able to facilitate drug loading of topotecan (Taggar, Alnajim et al. 2006) or irinotecan (Ramsay, Alnajim et al. 2006) even in the absence of a transmembrane pH gradient. However, in the presence of a pH gradient there were surprising improvements in drug retention (Ramsay, Alnajim et al. 2006). Based on these observations, Irinophore C™ has been developed using formulation methods that rely on both the transmembrane pH and encapsulated copper. However, the exact mechanism behind the unique role of copper is not very well understood.

It can be argued that this formulation should be developed using the least amount of copper required to achieve optimal drug retention attributes. Although copper is known to be an essential element regulating various physiological processes in humans (Burkitt 2001; Davis 2003), if it is present in excess of cellular needs it has the potential to produce toxicities. These may involve free radical production and direct oxidation of lipids, protein and DNA (Strausak, Mercer et al. 2001; Valko, Morris et al. 2005). Excess copper has also been shown to have roles in the development of neurodegenerative diseases (Valko, Morris et al. 2005). The primary objective of this study was to determine the minimum concentration of copper that can be used

within the liposomes in order to efficiently encapsulate irinotecan into pre-formed liposomes while generating a formulation that exhibits optimal drug retention parameters following intravenous administration. In addition, the ability of copper to alter membrane permeability was evaluated. The results suggested that copper unexpectedly, enhances membrane permeability as measured by sucrose permeability. However a defined amount of copper must be retained within the liposomes after irinotecan loading in order to achieve improved drug retention.

2.3 MATERIALS AND METHODS

2.3.1 Chemicals and reagents

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 3H-cholesterylhexadecyl ether ([3H]-CHE), ^{14}C-sucrose and Pico-Fluor40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Multi-use floating dialysis bags (Dispo-Dialyzer®) were purchased from Spectrum Labs (USA). All other chemicals used were analytical or HPLC grade. The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH) and all other chemicals (reagent grade) were purchased from Sigma-Aldrich (Oakville, On, Canada).

2.3.2 Liposome preparation

Large unilamellar vesicles (LUVs) were prepared by extrusion using DSPC and CH. Briefly, DSPC and CH were weighed, dissolved in chloroform individually and then mixed such that the final mole ratio of the two lipids is 55:45. A non-exchangeable and non-metabolizable lipid marker ^{3}H-CHE (5 μCi/100 μmol total lipid) was used to label the liposomes. This solution was then dried to a thin film with the help of a gentle stream of nitrogen gas. The residual chloroform was removed by placing the lipid film under high vacuum for at least 3h. The dried lipid films were hydrated at 65°C by mixing with one of the following solutions: (i) 300 mM

copper sulfate (Cu-300) (pH 3.5); (ii) 300 mM manganese sulfate (Mn-300) (pH 3.5); or (iii) different combinations of copper sulfate and manganese sulfate such that final metal concentration is 300 mM. Following hydration, samples were subjected to five freeze (liquid nitrogen) and thaw (65^{o}C) cycles (Mayer, Hope et al. 1986). The multilamellar vesicles (MLVs) obtained were extruded ten times through stacked polycarbonate filters (0.1 micron pore size) at 65°C using an Extruder^{TM} (Northern Lipids, Vancouver, BC, Canada). The size of the LUVs generated using this method was determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). The external buffer of LUVs was exchanged with sucrose (300 mmol/L), HEPES (20 mmol/L) and EDTA (15 mmol/L) (SHE buffer, pH 7.5) by running the sample through a Sephadex G-50 column equilibrated with the buffer. Liposomal lipid concentration was determined by measuring ^{3}H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer). Resultant liposomes contained the metal solution (unbuffered, pH 3.5) inside and were suspended in SHE buffer (pH 7.5).

2.3.3 Preparation of ion gradient and irinotecan loading

Prior to drug loading, the liposome solution was incubated at 30°C for 30 min in the presence or absence of A23187 (0.5 μg per 1 mg lipid). Irinotecan loading was carried out by incubating the required concentration of irinotecan hydrochloride solution (to achieve a target drug-to-lipid molar ratio ranging from 0.05 to 0.40, depending on the experiment) with the liposomal suspension at 50°C. 100 μL aliquots were removed at specific time interval and placed onto 1 mL Sephadex G-50 spin columns pre-equilibrated with phosphate buffer saline (PBS, pH 7.5). These columns were spun at 680x g for 3 min and liposomes collected in the void volume were analyzed for irinotecan and lipid concentrations. Lipid concentrations were measured by scintillation counting as above. Irinotecan concentrations were determined by spectrophotometric

measurement of absorbance at 370 nm (Agilent/Hewlett Packard, model: 8453, Agilent Technologies. Mississauga, ON, Canada). Briefly, an aliquot of the samples collected from the spin columns was adjusted to 100 μL followed by addition of 900 μL Triton X-100 (1%). This mixture was heated in a water bath at >90°C until the cloud point of the detergent was reached. Subsequently the samples were cooled to room temperature and the absorbance was read and compared against a standard curve of known irinotecan concentrations.

2.3.4 Measurement of copper concentration

Concentration of copper present inside the liposomes was determined using atomic absorption spectrometry (AA) (AANALYST 600 PerkinElmer Instruments, Woodbridge, ON). This instrument is equipped with THGA furnace with an AS-800 Autosampler. A hollow cathode lamp (Cu-LUMINA.HCL) was used as a light source for copper detection. Briefly, liposomes were prepared and irinotecan loaded as described above. At specified time points (5, 10, 20, 30 and 60 min) 100 μL aliquots were removed and placed onto 1 mL Sephadex G-50 spin columns equilibrated with PBS. Liposomes were collected in the void volume after spinning the columns at 680 x g for 3 min. The resulting samples were analyzed for lipid as described above and for copper as follows. Aliquots were diluted in nitric acid to achieve a final nitric acid concentration of 0.1%. A portion of this sample was injected into the analysis chamber of the AA where it was aspirated and atomized. Absorbance was determined at 325 nm. Concentration of copper from the samples was determined against a freshly prepared standard curve.

2.3.5 *In vivo* plasma elimination studies

A single dose (40 mg/kg irinotecan) of the specified liposomal formulation was injected i.v. into 20 to 25 g female Balb/c mice (Taconic, Hudson, NY). Four mice were used per time point and blood samples were collected via cardiac puncture after the mice were terminated by

CO_2 asphyxiation. Blood was immediately placed into EDTA-containing microtainers (Becton Dickinson, NJ) and stored on ice until they could be centrifuged at 2500 rpm for 15 min to separate plasma from blood cells. The concentration of liposomal lipid (^{3}H-CHE) in the plasma was determined by scintillation counting and concentration of irinotecan was determined by HPLC. HPLC was conducted using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump and controller and a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by diluting into 100% ice cold methanol. 10 µL of diluted sample was injected onto a Waters Symmetry Shield RP C_{18} cartridge column (100 Å, particle size 5 µm; 250 x 4.6 mm I.D., Waters). A two-solvent mobile phase consisted of mobile phase A (75 mM ammonium acetate, 7.5 mM tetra-butylammoniumbromide, pH 6.4 adjusted with glacial acetic acid) and mobile phase B (100% acetonitrile), with an isocratic mixture of 78% A: 22% B. Each sample was run for 14 minutes at a flow rate of 1.0 mL/min. The drug-to-lipid ratio was estimated from these data. These animal studies were completed as per animal care protocol reviewed and approved by the University of British Columbia's Animal Care Committee. The studies met current guidelines of the Canadian Council of Animal Care.

2.3.6 Permeability study

Liposomes were prepared as described above with ^{14}C-labeled sucrose was added to the hydration buffer. ^{14}C-labeled sucrose has been used as a marker of lipid membrane permeability (Van Veldhoven, Just et al. 1987; Franke, Galla et al. 1999). After separating un-encapsulated sucrose from the liposomes (SHE equilibrated spin columns), the liposomes were diluted in PBS to achieve a final lipid concentration of 25 µmol/mL and then incubated at 70°C. At specified

time points 100 μL aliquots were removed and placed onto 1 mL spin columns equilibrated with PBS. Liposomes were collected in the void volume after spinning the columns at 680 x g for 3 min. The amount of ^{14}C-labeled sucrose retained by the liposomes was quantified by scintillation counting.

2.3.7 Statistical analysis

One way ANOVA was performed in order to compare the results of drug loading studies, *in vitro* and *in vivo* drug release studies against appropriate controls. Significant differences between groups were identified using Students-Newman-Keuls multiple comparison post hoc test (GraphPad Instat software -San Diego, CA, USA). Differences between the groups were considered significant if $p<0.05$.

2.4 RESULTS AND DISCUSSION

2.4.1 Characterization of irinotecan loading

Previous studies have reported efficient encapsulation of camptothecins such as irinotecan and topotecan into preformed liposomes containing divalent metals either in the presence or absence of an initial pH gradient (Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006; Tardi, Gallagher et al. 2007). Drug loading using encapsulated manganese required the presence of the A23187 ionophore whereas ionophore was not required if the encapsulated metal was copper (Messerer, Ramsay et al. 2004; Taggar, Alnajim et al. 2006). These results were confirmed in Figure 2.1, where increases in drug-to-lipid ratio (mol/mol) were a measure of encapsulation of drug following addition to preformed liposomes prepared in 300 mM copper sulfate (Figure 2.1A) or 300 mM manganese sulfate (Figure 2.1B). Drug loading was done at 50^{o}C and in the presence (filled symbols) or absence (unfilled symbols) of A23187. This data highlighted two important points. First, as stated above, efficient drug loading using 300 mM

manganese as the internal buffer may only be accomplished through utilization of A23187, while liposomes prepared in 300 mM copper encapsulated drug in the presence and absence of the ionophore. Second, when the ionophore was used to facilitate drug loading, the loading rate was significantly faster for liposomes containing copper in the internal buffer. For example, liposomes prepared using 300 mM copper sulfate were able to encapsulate >98% of the added irinotecan within the first time point (10 min) while similar levels of loading were only achieved after 60 min for the liposomes prepared in 300 mM manganese sulfate. For the copper containing liposomes, loading rate (at 50^{o}C) was not influenced by the presence of ionophore A23187. Irinotecan loading was also determined at 40^{o}C and these results have been summarized in Figure 2.2. It is clear that drug loading rates were lower at 40^{o}C compared to 50^{o}C, but even at 40^{o}C irinotecan encapsulation was significantly faster when copper containing liposomes were used. It is argued that the copper containing formulations facilitate drug loading due to the pH gradient created following addition of A23187 as well as metal-ligand interactions and further, that copper forms complexes with irinotecan via a coordination complex with the oxygen atoms present on the E-ring of camptothecin (Kuwahara, Suzuki et al. 1986; Taggar, Alnajim et al. 2006). This mechanism does not necessarily explain why drug loading is more rapid in the copper containing liposomes. It is known that copper can interact with the phosphate moiety of phospholipids (Khomutov, Yakovenko et al. 1997; Suwalsky, Ungerer et al. 1998; Kamidate, Hashimoto et al. 2002; Lebedev, Volodina et al. 2005) and it may be this copper membrane interaction that is facilitating drug loading.

2.4.2 Influence of entrapped copper concentration on irinotecan loading

A range of liposomal preparations prepared with varying concentrations of copper were studied to determine the optimum concentration of copper required for effective irinotecan

loading. Liposomal preparations used for the initial studies were prepared using 300 mM metal sulfate solutions. This concentration was chosen to address potential issues arising from osmotic gradients that influence liposome shape and stability when mixed with solutions that are hypotonic (Dos Santos, Waterhouse et al. 2005). Hence, in preparations assessing the role of reduced copper concentration the total metal concentration was maintained at or around 300 mM where reductions in entrapped copper concentration were compensated by addition of manganese. A range of liposomal preparations with copper concentration ranging 1 mM to 300 mM were prepared and irinotecan encapsulation efficiency was determined 10 minutes after drug addition in the presence of A23187. The results are summarized in Figure 2.3. As evident in Figure 2.3A, reducing the encapsulated copper concentration from 300 mM to 50 mM did not have significant impact on irinotecan loading efficiency (95% vs 90%). However, further reductions in encapsulated copper concentration resulted in significant decreases in drug loading efficiency. Reducing the copper concentration from 50 mM to 25 mM reduced encapsulation efficiency from 90% to 42% ($p<0.05$) and this was further reduced as the copper concentration decreased to 10 mM. As noted in Figure 2.3B, when 50 mM copper was used the loading rate was not significantly different ($P>0.05$ at the 5, 10, and 20 minute time points) from that observed when using 300 mM copper. For comparison purposes irinotecan loading in manganese containing liposomes was included in Figure 2.3B (filled circles). The results from studies summarized in Figure 2.3 were completed in the presence of A23187. To determine whether A23187 influenced loading efficiency under these conditions, irinotecan loading into liposomes prepared with 50 mM copper in the absence of A23187 was assessed. The results, summarized in Figure 2.4, demonstrated that an encapsulation efficiency of only 60% can be achieved in the absence of A23187. This result highlighted the importance of both the pH gradient and

encapsulated copper in governing loading efficiency. It should be noted that the rate of drug loading in the absence of A23187 was essentially the same as that observed in the presence of A23187.

To further investigate the role of internal copper concentration, the amount of copper retained in liposomes following irinotecan addition was determined and these data are reported in Figure 2.5. For liposomes prepared using 300 mM copper, the amount of copper inside liposomes was reduced to 12% of the initial value within 10 minutes (the time point where maximum loading was noted). For the liposomal formulation prepared in 50 mM copper the retained copper concentration was reduced to 2% of the initial (before drug addition) value. No significant changes in entrapped copper concentrations were observed after the 10 minute time point (at least up to 60 min). This data demonstrated that drug loading occured while copper was exchanged from the liposome via A23187. A23187 facilitates the exchange of copper (inside) with protons present in the external medium and this, in turn, is important to help establish and maintain the transmembrane pH gradient.

2.4.3 Influence of entrapped copper concentration on drug retention

The results thus far established that the presence of entrapped copper facilitated the rate of irinotecan loading and that in the presence of A23187 drug loading efficiencies for formulations containing 50 mM or 300 mM copper were identical. Importantly, the amount of retained copper was different in these formulations e.g. as low as 1 mM when the starting formulation of copper was 50 mM and as high as 40 mM when the starting formulation was 300 mM. This data created two points of discussion. First, these results raised a potential concern for a drug loading model emphasizing formation of a coordination complex between copper and irinotecan. Irinotecan was loaded at a drug-to-lipid ratio of 0.2 (mol:mol) and it can be estimated

that the entrapped irinotecan concentration was at least 100 mM assuming the aqueous trap volume of these liposomes is 2 μL/μmole lipid (Elorza, Elorza et al. 1993; Maurer, Wong et al. 1998). Thus when the starting formulation used contains 300 mM copper the irinotecan to copper ratio after loading can be estimated to be 2.5 (mol:mol) while for the 50 mM copper formulation this ratio would be 100 (mol:mol). This analysis again highlights the fact that both encapsulated copper and the transmembrane pH gradient influence drug loading. However previous studies demonstrated that drug retention was unexpectedly better in formulations prepared with copper, thus differences in these formulations (the formulation prepared with 50 mM copper vs. the one prepared with 300 mM copper) could be observed if drug retention was measured.

Drug release from liposomal irinotecan formulations prepared using 300 mM and 50 mM copper was determined *in vivo* because previously it has been demonstrated that *in vitro* release rates do not reflect those observed *in vivo*. In these studies mice were injected via the tail vein with irinotecan loaded liposomes at a drug dose of 40 mg/kg. At various time points blood was collected and processed to produce plasma and the concentration of irinotecan and liposomal lipid in the plasma was determined as described in the Methods section. This data was then used to calculate an irinotecan to lipid ratio which was subsequently used to estimate drug release rates based on the assumption that 100% of the measured drug was associated with the liposomes in the plasma compartment. The results from this study have been summarized in Figure 2.6. Four liposomal irinotecan formulations were evaluated in these studies including ones prepared with liposomes that contained: i) 300 mM copper sulfate with A23187 (filled triangle), ii) 50 mM copper sulfate plus 250 mM manganese sulfate with A23187 (open circle), iii) 300 mM manganese sulfate in presence of A23187 (filled circle) and iv) 300 mM copper in the absence of A23187 (open triangle). Consistent with the previous results (Ramsay, Alnajim et al. 2006), the

plasma elimination rate of liposomal lipid from different liposomal formulations was not significantly affected by the loading conditions used (data not shown). Thus the differences in drug-to-lipid ratios noted in Figure 2.6 are primarily a consequence of changes in the plasma elimination rate of irinotecan that had been released from the liposomal carriers. Optimal drug retention and irinotecan circulation half-life ($t_{1/2}$= 9.5 h) was observed when the liposomal irinotecan formulation was prepared with liposomes containing 300 mM copper sulfate and A23187. When the formulation was prepared with 50 mM copper sulfate and 250 mM manganese sulfate and A23187, the changes in drug-to-lipid ratio observed over time ($t_{1/2}$= 3.7 h) were comparable to that seen following injection of liposomes prepared with 300 mM copper sulfate in the absence of A23187 ($t_{1/2}$= 3.6 h). Manganese containing liposomes prepared with A23187 released irinotecan more rapidly ($t_{1/2}$= 6.5 h) than copper containing liposomes.

In summary, the results indicated that 50 mM copper was sufficient to achieve irinotecan loading in the DSPC/CH liposomes, but these formulations released irinotecan more rapidly *in vivo* than formulations prepared with 300 mM copper. If copper is influencing drug release rates then it is likely that the amount of copper retained by the 50 mM copper containing liposomes following irinotecan loading (~1 mM) is not sufficient to optimize drug retention. Previous studies established that faster drug release from irinotecan containing liposomal formulations results in significant reductions in therapeutic activity (Ramsay, Alnajim et al. 2008). This data suggested that the 300 mM formulation prepared with A23187 exhibits the best drug retention characteristic of the formulations tested here, but provided no insight into why retained copper (~40 mM) improved drug retention. As indicated already this may be due to formation of a complex between irinotecan and copper, but the stoichiometry between encapsulated irinotecan and retained copper (2.5 to 1) was not entirely consistent with complex formation. Although

interaction of copper with camptothecins have been previously documented (Kuwahara, Suzuki et al. 1985; Kuwahara, Suzuki et al. 1986) a recent report completed using copper gluconate demonstrated no direct interaction between copper and irinotecan (Dicko, Tardi et al. 2007); although it should be noted that this formulation maintained an internal pH that is >6.5. As noted above, it has been reported that copper can bind to phospholipids (Suwalsky, Ungerer et al. 1998; Kamidate, Hashimoto et al. 2002) and it could be argued that the copper lipid interaction may affect membrane permeability in a more generic way. For this reason the influence that copper has on membrane permeability was assessed as determined using sucrose as a membrane permeable marker.

2.4.4 Effect of copper on membrane permeability

The results in Figure 2.1 and 2.2 clearly suggested that the presence of copper enhanced irinotecan loading rates. Faster drug loading rates are typically associated with more rapid drug release rates, but this is not the case for the liposomal irinotecan formulations described here. Radioactive (^{14}C labeled) sucrose was used to provide a measure of membrane permeability to help address this point. The amount of labeled sucrose retained by liposomes following incubation at 70^{o}C was determined at specified time points. The temperature used in these studies was selected based on preliminary work establishing that sucrose release from DSPC/CH liposomes (55:45 molar ratio) could be measured within a 24 h time course when the incubation temperature was 70^{o}C. This temperature was well above the transition temperature of DSPC and was expected to enhance sucrose permeability. The results, summarized in Figure 2.7, indicated that for liposomes prepared in saline, encapsulated [14C]-sucrose was lost gradually from the liposomes over time, with less than 20% of encapsulated sucrose being retained after the 24 h incubation period. Somewhat surprisingly, when the liposomes were prepared with 300 mM

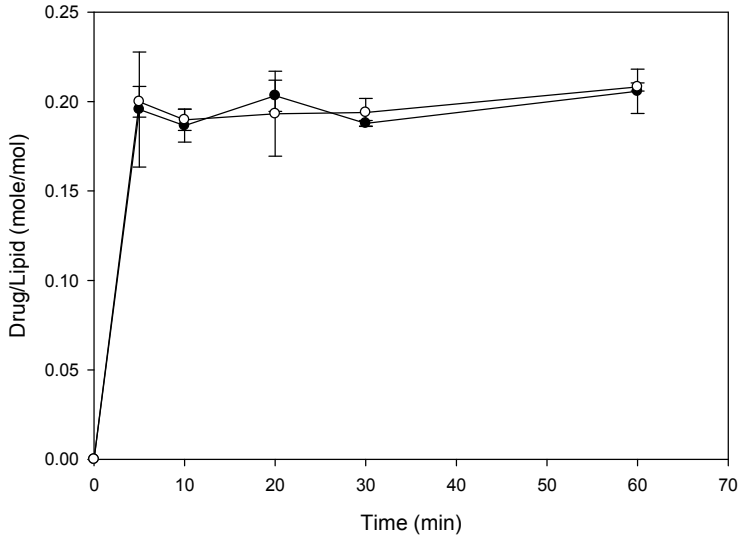
copper, sucrose permeability was increased dramatically. Liposomes prepared using saline retained greater than 90% of the initially encapsulated sucrose for at least 4 hours while liposomes prepared using 300 mM copper retained less than 20% of initially encapsulated sucrose after 4 hours. This data clearly suggested that the presence of copper significantly enhanced membrane permeability. This result was consistent with the influence of copper on irinotecan drug loading rates, but did not explain why the copper formulation exhibited improved drug retention. Therefore, once again these results would suggest that formation of an irinotecan-copper complex may explain why the copper containing irinotecan loaded formulations exhibit improved drug retention. Additional work will be required to fully characterize these purported copper-camptothecin interactions.

2.5 CONCLUSION

A summary of the results obtained here would suggest that the use of copper as an internal aqueous solution has a distinct advantage over manganese with respect to the active loading of irinotecan into DSPC/CH liposomes. The minimum concentration of copper required for efficient encapsulation of irinotecan was 50 mM. However optimal retention of irinotecan following intravenous administration was achieved when using formulations prepared with liposomes containing 300 mM copper prior to irinotecan addition. Studies assessing the influence of copper on membrane permeability helped to explain increased drug loading rates for those formulations prepared with copper, however it remains unclear why retained copper is important to achieve improved drug retention. It can be suggested from these studies that the formulation of liposomal irinotecan being developed for clinical use (Irinophore C^{TM}) may need to consider defining product release specifications that include retained copper.

Further, systematic characterization of drug loading methodology carried out in these experiments can be useful in defining formulation parameters while developing LNP formulation for topotecan.

(A)



(B)

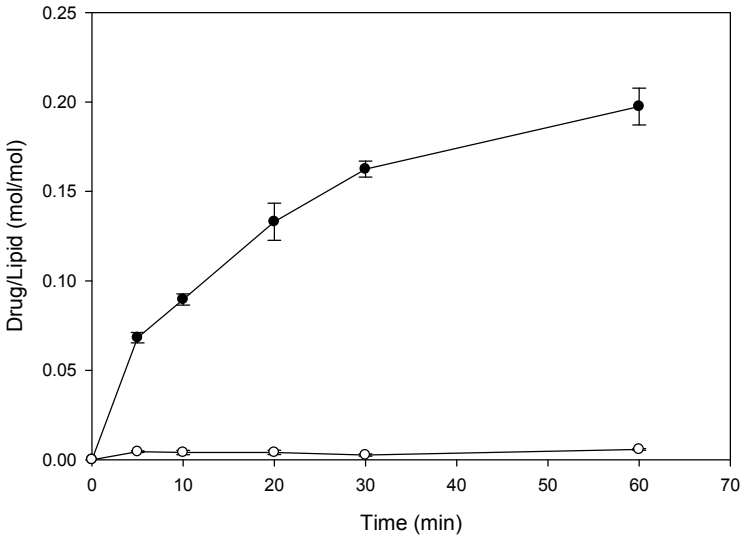


Figure 2.1: $CuSO_4$ mediated irinotecan encapsulation into DSPC/CH (55:45) liposomes. (A) Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) plus the transmembrane ionophore A23187 (●), and $CuSO_4$ (pH 3.5) alone (○) and (B) Liposomes were prepared with entrapped $MnSO_4$ (pH 3.5) plus the transmembrane ionophore A23187 (●), and MnSO4 (pH 3.5) alone (○). Cu^{+2} and Mn^{+2} gradients were created by exchanging the exterior liposome solution with PBS pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50ºC for 60 minutes. At 5, 10, 30 and 60 minutes aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD (n=3)

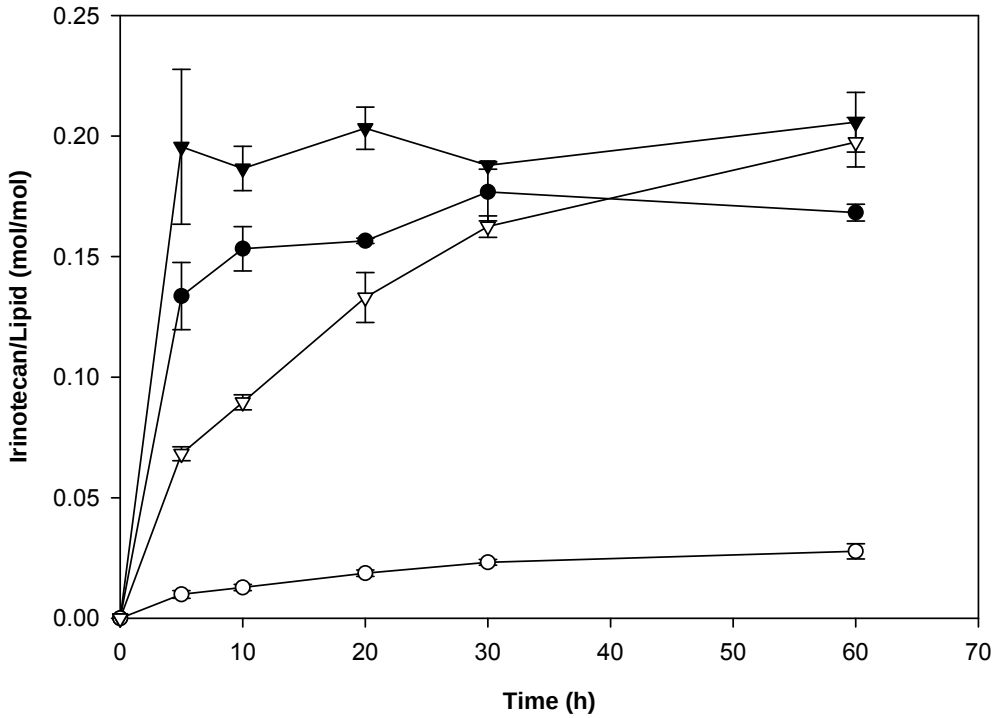
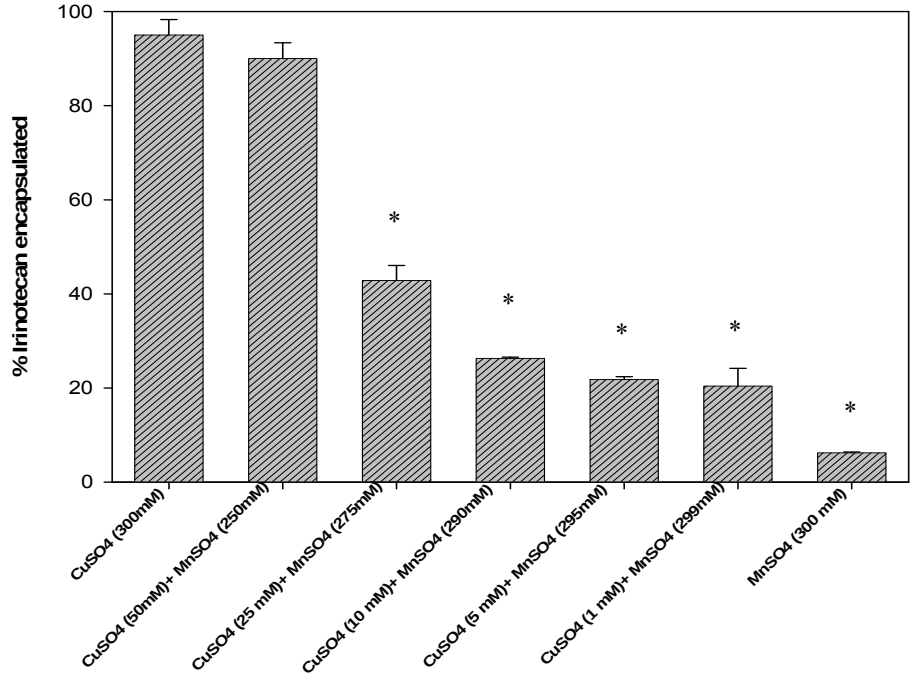


Figure 2.2: $MnSO_4$ and $CuSO_4$ mediated irinotecan encapsulation into DSPC/CH (55:45) liposomes at 50°C (triangles) and 40°C (circles). Liposomes were prepared with entrapped 300 mM $MnSO_4$ (open symbols) or 300 mM $CuSO_4$ (filled symbols) (pH 3.5) plus the transmembrane ionophore A23187. Mn^{+2}/Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at either 50°C or 40°C for 60 minutes. At 5, 10, 30 and 60 minutes aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD (n=3)

A



B

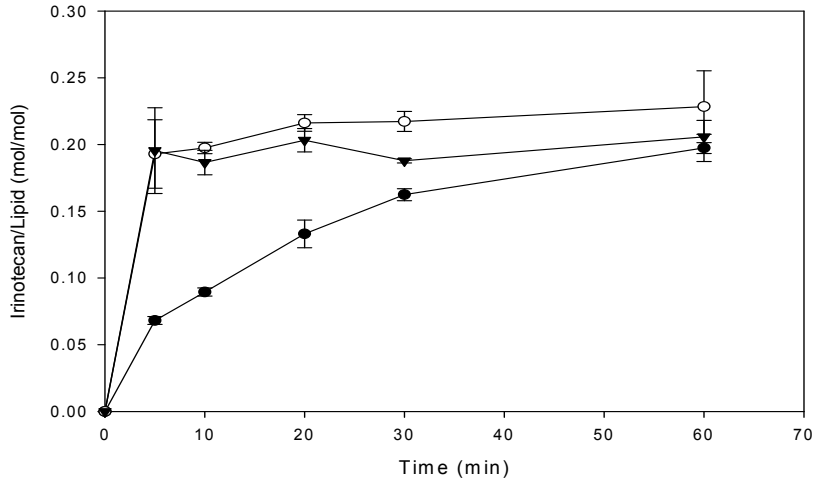


Figure 2.3: Influence of entrapped copper concentration on irinotecan encapsulation into DSPC/CH (55:45) liposomes. **A**- Liposomes were prepared using progressively reduced copper concentration. Mn^{+2} and Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 10 minutes. After 10 minutes, aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared with PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD (n=3) **B**- Liposomes were prepared with i.) entrapped $MnSO_4$ (300 mM) plus ionophore A23187 (●); ii.) $MnSO_4$ (250 mM) plus $CuSO_4$ (50 mM) plus ionophore A23187 (○) or iii) $CuSO_4$ (300 mM) plus ionophore A23187(▼). Mn^{+2} and Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 60 minutes. At 5, 10, 20, 30 and 60 minutes aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared in PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD (n=3). *$P<0.05$ vs. control.

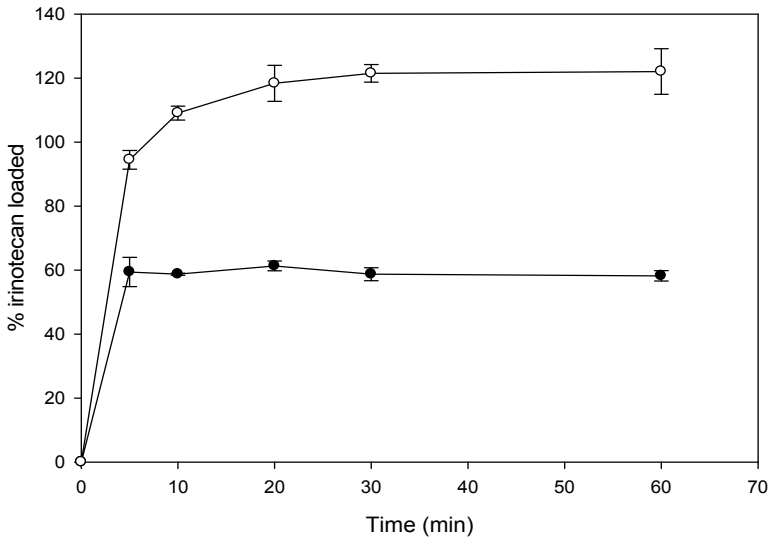


Figure 2.4: Irinotecan encapsulation into DSPC/CH (55:45) liposomes. Liposomes were prepared using $CuSO_4$ (50 mM) and $MnSO_4$ (250 mM) and irinotecan loading was carried out i.) in the presence of A23187 (○) and ii.) in the absence of A23187 (●). Mn^{+2} and Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 60 minutes. At 5, 10, 20, 30 and 60 minutes aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared in PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD (n=3).

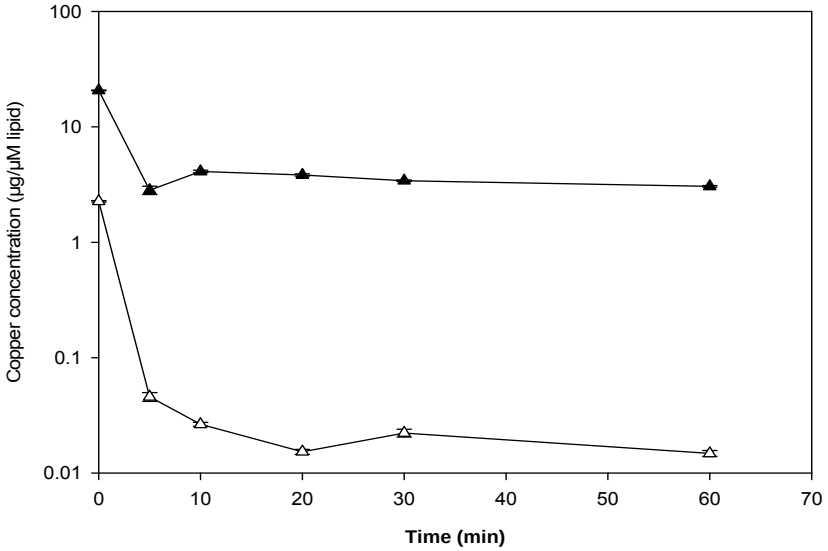


Figure 2.5: Release of copper from liposomes during irinotecan loading. Liposomes were prepared using i.) $CuSO_4$ (300 mM) (-▲-) and ii.) $CuSO_4$ (50 mM) + MnSO4 (250 mM) (-Δ-), incubated initially with ionophore A23187 at 30°C for 30 minutes and then with irinotecan at 50°C for 60 min. Aliquots were withdrawn at time intervals of 5, 10, 20, 30, 60 min and passed through spin columns prepared in PBS (pH 7.5) and analyzed for copper concentration. Data represents mean ± SD of triplicate measurements.

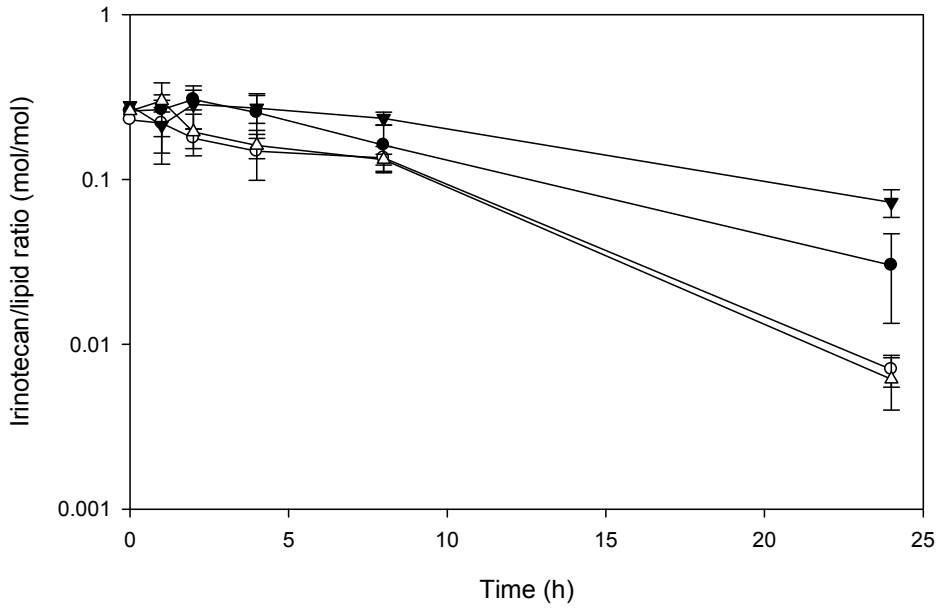


Figure 2.6: Plasma elimination profiles of different liposomal Irinotecan formulations: Female Balb/C mice were injected intravenously with a single dose (40 mg/kg irinotecan) of DSPC/CH (55:45 mol%) liposomal Irinotecan formulations: 300 mM $CuSO_4$ + A23187 (▼);300 mM $MnSO_4$ + A23187 (●); 300 mM $CuSO_4$ alone (Δ); 50 mM $CuSO_4$ + 250 mM unbuffered $MnSO_4$ + A23187 (○). At the indicated time points plasma samples were obtained (see Methods) and the concentration of liposomal lipid and irinotecan was determined as described in the Methods. These data were then used to calculate the irinotecan-to-lipid ratio (mol:mol) in the plasma as a function of time. Data points represent the mean ± SD (n=4).

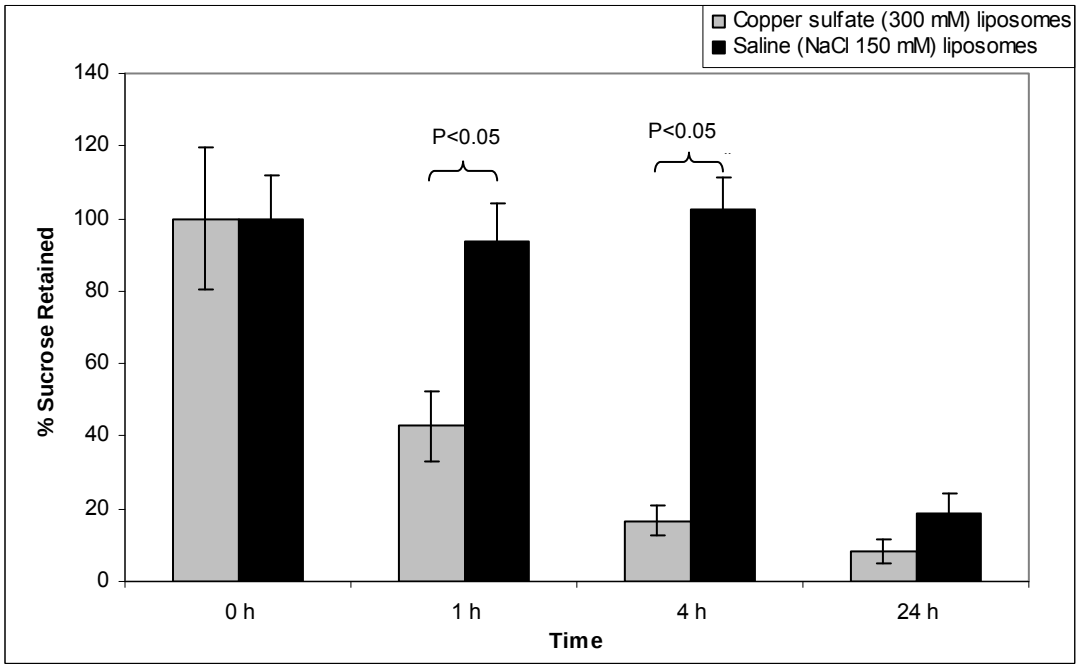


Figure 2.7: Membrane permeability study of DSPC/CH liposomes: Liposomes prepared using either copper sulfate (300 mM) or saline (NaCl 150 mM) were kept in 70ºC water bath. Amount of ^{14}C-labeled sucrose retained inside the liposomes was measured by passing the aliquots through a sephadex G-50 spin column equilibrated with PBS. Data points represent the mean ± SD (n=3).

2.6 REFERENCES

Abraham, S. A., Edwards, K., Karlsson, G., Hudon, N., Mayer, L. D. and Bally, M. B. (2004). "An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes." J Control Release **96**(3): 449-61.

Baker, J. H., Lam, J., Kyle, A. H., Sy, J., Oliver, T., Co, S. J., Dragowska, W. H., Ramsay, E., Anantha, M., Ruth, T. J., Adam, M. J., Yung, A., Kozlowski, P., Minchinton, A. I., Ng, S. S., Bally, M. B. and Yapp, D. T. (2008). "Irinophore C, a novel nanoformulation of irinotecan, alters tumor vascular function and enhances the distribution of 5-fluorouracil and doxorubicin." Clin Cancer Res **14**(22): 7260-71.

Bangham, A. D., Hill, M. W. and Miller, N. G. A., Eds. (1974). Membranes in Membrane Biology. Ney York, Plenum.

Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965). "Diffusion of univalent ions across the lamellae of swollen phospholipids." J Mol Biol **13**(1): 238-52.

Batist, G., Ramakrishnan, G., Rao, C. S., Chandrasekharan, A., Gutheil, J., Guthrie, T., Shah, P., Khojasteh, A., Nair, M. K., Hoelzer, K., Tkaczuk, K., Park, Y. C. and Lee, L. W. (2001). "Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer." J Clin Oncol **19**(5): 1444-54.

Bruggemann, E. P. and Melchior, D. L. (1983). "Alterations in the organization of phosphatidylcholine/cholesterol bilayers by tetrahydrocannabinol." J Biol Chem **258**(13): 8298-303.

Burkitt, M. J. (2001). "A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin." Arch Biochem Biophys **394**(1): 117-35.

Cheung, B. C., Sun, T. H., Leenhouts, J. M. and Cullis, P. R. (1998). "Loading of doxorubicin into liposomes by forming Mn2+-drug complexes." Biochim Biophys Acta **1414**(1-2): 205-16.

Cullis, P. R., Hope, M. J., Bally, M. B., Madden, T. D., Mayer, L. D. and Fenske, D. B. (1997). "Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles." Biochim Biophys Acta **1331**(2): 187-211.

Davis, C. D. (2003). "Low dietary copper increases fecal free radical production, fecal water alkaline phosphatase activity and cytotoxicity in healthy men." J Nutr **133**(2): 522-7.

Dicko, A., Tardi, P., Xie, X. and Mayer, L. (2007). "Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes." Int J Pharm **337**(1-2): 219-28.

Dos Santos, N., Waterhouse, D., Masin, D., Tardi, P. G., Karlsson, G., Edwards, K. and Bally, M. B. (2005). "Substantial increases in idarubicin plasma concentration by liposome encapsulation mediates improved antitumor activity." J Control Release **105**(1-2): 89-105.

Elorza, B., Elorza, M. A., Sainz, M. C. and Chantres, J. R. (1993). "Analysis of the particle size distribution and internal volume of liposomal preparations." J Pharm Sci **82**(11): 1160-3.

Emerson, D. L. (2000). "Liposomal delivery of camptothecins." Pharm Sci Technolo Today **3**(6): 205-209.

Franke, H., Galla, H. J. and Beuckmann, C. T. (1999). "An improved low-permeability in vitro-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol." Brain Res **818**(1): 65-71.

Gordon, A. N., Fleagle, J. T., Guthrie, D., Parkin, D. E., Gore, M. E. and Lacave, A. J. (2001). "Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan." J Clin Oncol **19**(14): 3312-22.

Gregoriadis, G., Ed. (1993). Liposome Technology. Liposomes Preparation and Related Techniques. Boca Raton, Florida, CRC Press.

Haran, G., Cohen, R., Bar, L. K. and Barenholz, Y. (1993). "Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases." Biochim Biophys Acta **1151**(2): 201-15.

Kamidate, T., Hashimoto, Y., Tani, H. and Ishida, A. (2002). "Uptake of transition metal ions using liposomes containing dicetylphosphate as a ligand." Anal Sci **18**(3): 273-6.

Khomutov, G. B., Yakovenko, S. A., Soldatov, E. S., Khanin, V. V., Nedelcheva, M. D. and Yurova, T. V. (1997). "Interaction of copper ions with stearic acid Langmuir monolayers and formation of cluster structures in monolayers and Langmuir-Blodgett films." Membr Cell Biol **10**(6): 665-81.

Kim, J. C., Bae, S. K. and Kim, J. D. (1997). "Temperature-sensitivity of liposomal lipid bilayers mixed with poly(N-isopropylacrylamide-co-acrylic acid)." J Biochem **121**(1): 15-9.

Kuwahara, J., Suzuki, T., Funakoshi, K. and Sugiura, Y. (1986). "Photosensitive DNA cleavage and phage inactivation by copper(II)-camptothecin." Biochemistry **25**(6): 1216-21.

Kuwahara, J., Suzuki, T. and Sugiura, Y. (1985). "Studies on antitumor drugs targeting DNA: photosensitive DNA cleavage of copper-camptothecin." Nucleic Acids Symp Ser(16): 201-4.

Lebedev, V. S., Volodina, L. A., Deinega, E. and Fedorov Iu, I. (2005). "[Structural modifications of the surface of Escherichia coli bacteria and copper-induced permeability of plasma membrane]." Biofizika **50**(1): 107-13.

Lichtenberg, D. and Barenholz, Y. (1988). "Liposomes: preparation, characterization, and preservation." Methods Biochem Anal **33**: 337-462.

Maurer, N., Wong, K. F., Hope, M. J. and Cullis, P. R. (1998). "Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR study." Biochim Biophys Acta **1374**(1-2): 9-20.

Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986). "Vesicles of variable sizes produced by a rapid extrusion procedure." Biochim Biophys Acta **858**(1): 161-8.

Messerer, C. L., Ramsay, E. C., Waterhouse, D., Ng, R., Simms, E. M., Harasym, N., Tardi, P., Mayer, L. D. and Bally, M. B. (2004). "Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer." Clin Cancer Res **10**(19): 6638-49.

Mozafari, M. R. (2005). "Liposomes: an overview of manufacturing techniques." Cell Mol Biol Lett **10**(4): 711-9.

Ramsay, E., Alnajim, J., Anantha, M., Taggar, A., Thomas, A., Edwards, K., Karlsson, G., Webb, M. and Bally, M. (2006). "Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation." Pharm Res **23**(12): 2799-808.

Ramsay, E., Alnajim, J., Anantha, M., Zastre, J., Yan, H., Webb, M., Waterhouse, D. and Bally, M. (2008). "A novel liposomal irinotecan formulation with significant anti-tumour activity: use of the divalent cation ionophore A23187 and copper-containing liposomes to improve drug retention." Eur J Pharm Biopharm **68**(3): 607-17.

Ramsay, E. C., Anantha, M., Zastre, J., Meijs, M., Zonderhuis, J., Strutt, D., Webb, M. S., Waterhouse, D. and Bally, M. B. (2008). "Irinophore C: a liposome formulation of irinotecan with substantially improved therapeutic efficacy against a panel of human xenograft tumors." Clin Cancer Res **14**(4): 1208-17.

Strausak, D., Mercer, J. F., Dieter, H. H., Stremmel, W. and Multhaup, G. (2001). "Copper in disorders with neurological symptoms: Alzheimer's, Menkes, and Wilson diseases." Brain Res Bull **55**(2): 175-85.

Suwalsky, M., Ungerer, B., Quevedo, L., Aguilar, F. and Sotomayor, C. P. (1998). "Cu2+ ions interact with cell membranes." J Inorg Biochem **70**(3-4): 233-8.

T. G. Burke, T. X. X., B. anderson, L. Latus Recent Advances in Camptothecin Drug Design and Delivery Strategies. Totowa, NJ, Humana Press Inc.

Taggar, A. S., Alnajim, J., Anantha, M., Thomas, A., Webb, M., Ramsay, E. and Bally, M. B. (2006). "Copper-topotecan complexation mediates drug accumulation into liposomes." J Control Release **114**(1): 78-88.

Tardi, P. G., Gallagher, R. C., Johnstone, S., Harasym, N., Webb, M., Bally, M. B. and Mayer, L. D. (2007). "Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo." Biochim Biophys Acta **1768**(3): 678-87.

Valko, M., Morris, H. and Cronin, M. T. (2005). "Metals, toxicity and oxidative stress." Curr Med Chem **12**(10): 1161-208.

Van Veldhoven, P. P., Just, W. W. and Mannaerts, G. P. (1987). "Permeability of the peroxisomal membrane to cofactors of beta-oxidation. Evidence for the presence of a pore-forming protein." J Biol Chem **262**(9): 4310-8.

3. TOPOPHORE C^{TM}: A LIPOSOMAL NANOPARTICLE FORMULATION OF TOPOTECAN FOR TREATMENT OF OVARIAN CANCER

[2] A version of this chapter has been submitted for publication as:

Patankar, N., Strutt, D., Waterhouse, D. and Bally, M. (2010). "TopophoreC^{TM}: A liposomal nanoparticle formulation of topotecan for treatment of ovarian cancer."

3.1 SUMMARY

The lipid nanoparticle (LNP) formulation of the camptothecin derivative irinotecan (referred to as Irinophore C™) relies on use of transition metal complexation with irinotecan and a transmembrane pH gradient to encapsulate the drug into preformed liposomes. Irinophore C™ exhibited surprising improvements in drug retention attributes and this was associated with remarkable improvements in therapeutic activity. The loading methodology developed for irinotecan has the potential to be applied to other cancer drugs, in particular other camptothecin analogues. In this report the methodology was used to develop a LNP topotecan formulation. Topotecan was encapsulated in preformed liposomes containing 300 mM copper sulfate and the divalent metal ionophore A23187. Optimization studies included assessments of maximum loading capacity, influence of temperature on drug loading rates and *in vitro* stability of the resulting formulations. One formulation (referred to herein as TopophoreC™) was selected for *in vivo* assessments. Following intravenous administration in mice, drug and liposome pharmacokinetics were measured, drug levels within the peritoneal cavity were determined and efficacy studies in two ovarian cancer models (ES-2 and SKOV-3) were completed. Topotecan loading into liposomes was optimized to achieve encapsulation efficiency of >95% based on a final drug-to-lipid (D/L) mole ratio of 0.1. Higher D/L ratios could be achieved, but the resulting formulations were less stable as judged by *in vitro* drug release studies. Pharmacokinetic data indicated that following i.v. administration of Topophore C™ the topotecan plasma half-life ($t_{1/2}$) and AUC were increased compared to free topotecan (Hycamtin®) by 10 and 22-fold, respectively. Topophore C™ was 2- to 3-fold more toxic than free topotecan; however this product candidate showed significantly better anti-tumor activity when compared to free topotecan administered at equivalent or at equitoxic doses. In the ES2 model, for example,

Topophore C™ at a dose of 1.25 mg/kg (Q7D x 3) achieved improved therapeutic effects when compared to free topotecan given at a dose of 5 mg/kg. Topophore C™ is a therapeutically interesting product candidate and studies described in chapter 4 were carried out in the interest of developing its use in combination with Doxil (doxorubicin HCl liposome injection) for treatment of platinum refractory ovarian cancer.

3.2 INTRODUCTION

Camptothecin (CPT) was first discovered in 1958 as a potent antitumor antibiotic from the bark of the Chinese tree *Camptotheca acuminata* (Wall 1993). Although this compound was interesting from a preclinical perspective its poor aqueous solubility compromised clinical development (Gottlieb and Luce 1972; Moertel, Schutt et al. 1972). Semi-synthetic and more water soluble analogues of camptothecin have been developed to address this issue and two of these derivatives have now been approved by regulatory authorities for use in patients. Camptosar® (Irinotecan hydrochloride) is used for the treatment of patients with colorectal cancer (Saltz, Cox et al. 2000) and is showing some promise in other indications including lung cancer (Noda, Nishiwaki et al. 2002). Hycamtin® (Topotecan) is approved for use in ovarian cancer patients that have relapsed following treatment with a taxane/platinum combination (Giovanella, Stehlin et al. 1989; Ozols 2000; Saltz, Cox et al. 2000). Acceptance of camptothecins as an additional class of cytotoxic agents was driven in part because of their unique mechanism of activity. Camptothecins exert their cytotoxic effect during the S-phase of the cell cycle by stabilizing the cleavable complex formed between the enzyme topoisomerase I (TOP-I) and DNA. This process prevents re-ligation of DNA strand and ultimately leads to apoptosis due to the accumulation of DNA-TOP-I complexes (Hsiang and Liu 1988; Hsiang, Wu et al. 1988; Hsiang, Lihou et al. 1989). As topoisomerase I inhibitors, the camptothecins act

additively or synergistically with other drug classes (Wall and Wani 1995; Saltz, Cox et al. 2000; Noda, Nishiwaki et al. 2002; Baek, Kim et al. 2006; Ghesquieres, Faivre et al. 2006; Harasym, Tardi et al. 2006; Main, Bojke et al. 2006; Verhaar-Langereis, Karakus et al. 2006; Tardi, Gallagher et al. 2007), and in particular with selected topoisomerase II (TOP-II) inhibitors such as doxorubicin (Dupont, Aghajanian et al. 2006; Ghesquieres, Faivre et al. 2006; Main, Bojke et al. 2006; Verhaar-Langereis, Karakus et al. 2006). More recently, there has been a great deal of excitement about the use of combinations of poly(ADP-ribose) polymerase (PARP) inhibitors (e.g. olaparib) and TOP-I inhibitors such as topotecan and irinotecan (Comen and Robson et al. ; Mahany, Lewis et al. 2009), particularly in the context of patients with BRCA1 mutations (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005). Further, as exposure to irinotecan can increase expression of EGFR receptor (Correale, Marra et al. 2010) camptothecins are also providing unexpected benefits in the context of chemotherapeutic regimes that include therapeutic antibodies targeting the EGFR receptor. It is therefore anticipated that the use of these camptothecins will expand over the next decade even with the introduction of more personalized therapies targeting dysregulated signaling pathways in cancer cells.

Importantly, it has been recognized that the therapeutic activity of camptothecins is compromised due to a chemical characteristics. The therapeutic action of camptothecins is dependent on the integrity of the drug's α-hydroxy-lactone ring (Burke 1992, Burke 1996; Giovanella, Harris et al. 2000). This lactone ring is prone to undergo reversible hydrolysis at physiological pH, producing an inactive carboxylate derivative (Giovanella, Harris et al. 2000; Sai, Kaniwa et al. 2002). Strategies proposed to improve the therapeutic activity of camptothecins have therefore primarily focused on preventing hydrolysis of the lactone ring. These strategies have included: i) structural modification of the compound (Burke and Mi 1994;

Emerson, Besterman et al. 1995; Larsen, Gilbert et al. 2001), ii) derivatization of the lactone form (Emerson, Besterman et al. 1995) or iii) encapsulation into nanoparticulate carriers such as liposomes (Burke 1992; Burke, Mishra et al. 1993; Burke and Gao 1994; Cullis, Hope et al. 1997; Emerson 2000; Hatefi and Amsden 2002; Kehrer, Bos et al. 2002; Abraham, Edwards et al. 2004; Ramsay, Alnajim et al. 2006), polymer micelles (Kawano, Watanabe et al. 2006; Watanabe, Kawano et al. 2006) or microemulsions (Nastruzzi 1997). Several liposomal formulations of camptothecins have been reported previously (Sadzuka, Hirotsu et al. 1999; Liu, Hong et al. 2002; Chou, Chen et al. 2003; Abraham, Edwards et al. 2004; Lei, Chien et al. 2004; Messerer, Ramsay et al. 2004; Seiden, Muggia et al. 2004; Hao, Deng et al. 2005; Hao, Deng et al. 2005; Pal, Khan et al. 2005; Drummond, Noble et al. 2006; Ramsay, Alnajim et al. 2006; Taggar, Alnajim et al. 2006; Ramsay, Anantha et al. 2008) with varying degree of therapeutic promise. Irinotecan and topotecan are weakly basic drugs and can be loaded into preformed liposomes through use of a trans-membrane pH gradient (acidic inside) (Cullis, Hope et al. 1997). The lactone ring is stabilized in its ring closed configuration when it is retained within the acidic core of the liposome. Several approaches can be used to generate a transmembrane pH gradient including: i) preparing liposomes using buffered acidic solutions (Mayer, Hope et al. 1986), ii) preparing liposomes using an ammonium sulfate solution capable of generating/maintaining a pH gradient (Haran, Cohen et al. 1993) or iii) preparing liposomes using solutions of monovalent or divalent metal ions coupled with the addition of an appropriate ionophore (Fenske, Wong et al. 1998). Recently a loading method was described that involves formation of a complex between copper and irinotecan (Ramsay, Alnajim et al. 2006; Ramsay, Anantha et al. 2008); a method that is not necessarily dependent on formation of a pH gradient. Importantly, when using encapsulated copper in combination with a transmembrane pH gradient

there were unexpected improvements in drug retention. It is not clear whether the improvements in drug retention are due to complexation of the drug to copper or, alternatively, involve a copper membrane interaction that engender decreases in the liposomal membrane's permeability to irinotecan.

Regardless of mechanism, the improvements in drug retention were associated with improved antitumor activity. This irinotecan formulation (referred to as Irinophore C^{TM}) maintains the drug in its therapeutically active lactone conformation (Ramsay, Alnajim et al. 2006). Since topotecan is a structurally comparable camptothecin analog it was reasonable to consider whether the formulation approach developed for Irinophore C^{TM} could be used to prepare a LNP topotecan formulation. The objectives of this study were: i) to characterize and optimize the use of the copper/pH gradient loading methodology for topotecan; ii) to assess how a selected LNP formulation of topotecan influenced drug pharmacokinetics and distribution to the peritoneal cavity following intravenous administration; and iii) to measure the therapeutic activity of the selected formulation in pseudo-orthotopic models of ovarian cancer. An optimized preparation of topotecan loaded liposomes referred to as Topophore C^{TM} was identified as a suitable candidate for further development. This product candidate maintained topotecan in the lactone ring closed configuration following i.v. administration, achieved significant increases in plasma circulation half-life and AUC, and was therapeutically active in two models of ovarian cancer.

3.3 MATERIALS AND METHODS

3.3.1 Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) from PerkinElmer Life Sciences (Boston, MA). Hycamtin® injection (GlaxoSmithKline, Mississauga, ON, Canada; active ingredient: Topotecan-HCl; inactive ingredients: mannitol, and tartaric acid; pH of reconstituted solution: 2.5–3.5) was purchased from the pharmacy of the BC Cancer Agency (Vancouver, BC, Canada). ^{14}C-sucrose and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Multi-use floating dialysis bags (DispoDialyzer®) were purchased from Spectrum Labs (USA). All other chemicals used were analytical or HPLC grade. The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH) and all other chemicals (Reagent grade) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Liposome preparation

Large unilamellar vesicles (LUVs) were prepared using DSPC and cholesterol (CH) by extrusion. For detailed methodology please refer to chapter 2, section 2.3.2.

3.3.3 Preparation of ion gradient and optimization of topotecan loading

Liposomes with encapsulated copper sulfate (unbuffered solution, pH 3.5) were suspended in SHE buffer (pH 7.5). Subsequently, A23187 (0.5 μg per 1 mg lipid) was added to the liposomal suspension which was then incubated at 30°C for 30 min. This mixture and a reconstituted solution of topotecan were warmed separately at 60°C for 5 minutes using a temperature bath. Just prior to mixing of topotecan with the liposomes a sufficient quatity of 1N NaOH was added to liposomes to raise the pH such that the final pH of the drug loading mixture was between 7.0

to 7.5 (see Results). The final drug/liposome mixture was incubated in a water bath at 60°C. At specified time points, 100 μL of this mixture was placed onto 1 mL Sephadex G-50 spin columns pre-equilibrated with phosphate buffer saline (PBS, pH 7.5) to separate un-encapsulated drug from liposomes. Liposomes collected in the void volumes after spinning the columns at 680x g for 3 min were analyzed for topotecan and liposomal lipid concentration. Lipid concentrations were measured using scintillation counting to determine ^{3}H-CHE. Topotecan concentrations were determined by measuring the absorbance at 370 nm on a spectrophotometer (Agilent/Hewlett Packard, model: 8453, Agilent Technologies. Mississauga, ON, Canada). Briefly, an aliquot of the sample collected from the spin columns was adjusted to 100 μL followed by addition of 900 μL Triton X-100 (1% v/v). This sample was heated in a 90°C water bath until the cloud point of the detergent was observed. Subsequently, the sample was cooled to room temperature and the absorbance was determined and compared against topotecan standard curve in which known concentrations were treated identically to that of samples.

3.3.4 *In vitro* drug release

The rate of topotecan release from liposomes *in vitro* was determined by a dialysis method. Briefly, 300 μL of topotecan loaded liposomal suspension was mixed with 1.5 mL phosphate buffer saline (PBS) (pH 7.5) and this mixture was placed inside disposable dialysis bag (DispoDialyzer®, SPECTRUM Laboratories, USA, MW cut-off of 10,000). The dialysis bag was then suspended in one liter PBS (pH 7.5) maintained at 40°C ± 1°C or 50°C ± 1°C. At specified time points, 100 μL samples were withdrawn from the dialysis bag and placed onto 1 mL Sephadex G-50 spin columns to separate liposomes from unencapsulated drug. The concentration of liposomal lipid (^{3}H-CHE) in the void volume was analyzed by scintillation counting and the topotecan concentration was determined by high performance liquid

chromatography (HPLC). The HPLC analysis was perfomed using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump, a controller and a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by dilution with ice cold methanol. 10 µL of the diluted sample was injected onto a Waters Symmetry Shield RP C18 cartridge column (100 Å, particle size 3.5 µm; 75 x 4.6 mm, Waters). The mobile phase consisted of mobile phase 'A' (1% Triethyleneamine in water, pH 6.4 adjusted with glacial acetic acid) and mobile phase 'B' (100 % acetonitrile). The sample temperature was maintained at 4°C and the column temperature was adjusted to 55°C. Each sample was run for 14 min at a flow rate of 1.0 mL/min using a gradient method, where the amount of organic phase was increased from 12% to 40% over 8 min. This method was able to detect ring opened carboxylate (eluted at 3 min) and ring closed lactone form (eluted at 8 min) of topotecan in a single run.

3.3.5 Measurement of copper concentration

Concentration of copper present inside the liposomes was determined using atomic absorption spectrometer (AA) (AANALYST 600 PerkinElmer Instruments, Woodbridge, ON). This instrument is equipped with THGA furnace with AS-800 Autosampler. Hollow cathode lamp (Cu-LUMINA.HCL) was used as a light source for copper detection. Liposomes were prepared and topotecan was loaded into pre-formed liposomes using the copper ion gradient method described above. For detailed procedure on copper measurement please refer to chapter 2, section 2.3.4.

3.3.6 Storage stability study

After selecting a LNP topotecan formulation for biological studies the stability of the product at 4^{o}C was determined over a time frame of 2 months. This formulation, referred to as Topophore C^{TM}, was monitored for a number of parameters including: i) color; ii) appearance; iii) particle size distribution; iv) drug concentration; v) liposome concentration as well as vi) drug-to-lipid ratio.

3.3.7 *In vivo* plasma elimination of topotecan

A single dose (5 mg/kg) of topotecan (Hycamtin) or Topophore C^{TM} was administered intravenously (iv) into female Balb/c mice (Taconic, Hudson, NY; 20-25 g). Four mice were used per time point and blood samples were collected via cardiac puncture after the mice were terminated by CO_2 asphyxiation. Blood was immediately placed into EDTA-containing microtainers (Becton Dickinson, NJ) and stored on ice until they could be centrifuged at 2500 rpm for 15 min to separate plasma from blood cells. The concentration of liposomal lipid (^{3}H-CHE) in the plasma was determined by scintillation counting and concentration of topotecan was determined by HPLC methods as described above. The drug-to-lipid ratio was estimated from these data and the plasma AUC and half-life of topotecan was determined from this data using non-compartmental pharmacokinetic model with the help of WinNonlin^{TM} (PharSight$^{®}$ Corp., Mountain View, CA) software. These animal studies were completed under an animal care protocol reviewed and approved by the University of British Columbia's Animal Care Committee. The studies met current guidelines of the Canadian Council of Animal Care.

3.3.8 Accumulation of topotecan in the peritoneal cavity following i.v. injection

Since the pseudo-orthotopic ovarian cancer models used (see below) involve injection of the indicated tumor cell lines into the peritoneal cavity of mice, studies were completed to

determine the amount of topotecan that accesses this site after i.v. administration of free topotecan (Hycamtin) or TopophoreC^{TM}. ES2 tumor bearing mice (see below) were injected i.v. with a 5 mg/kg dose of topotecan. At specified time points the mice were terminated by asphyxiation with CO_2 and subsequently 5 mL of ice cold hank's balanced salt solution (HBSS) was injected along the midline into the peritoneal cavity with a 27 G needle. The peritoneal cavity was vigorously massaged to achieve good distribution of the lavage fluid which was then collected with a syringe equipped with a 20 G needle. The lavage fluid was transferred to a 15-mL polypropylene tube maintained on ice. No further processing of the samples was done, thus the measured concentration of topotecan include both cell associated and free material. The topotecan concentration was determined by HPLC as described above.

3.3.9 *In vivo* antitumor activity

The antitumor activity of Topophore C^{TM} was evaluated in female mice using two different pseudo-orthotopic models of ovarian cancer. ES-2 cells are model of a chemorefractory clear cell carcinoma (Lau, Lewis et al. 1991) while SKOV-3 cells are representative of a serous adenocarcinoma (Cao, Lu et al. 2006). Both ES-2 and SKOV-3 cell lines were purchased from American Type Culture Collection, ATCC (Rockville, MD). ES-2 cells (1 x 10^5/500 µl) were inoculated intraperitoneally (i.p.) into female NCr-Fox1^{nu} mice (Taconic, Hudson, NY). Seven days after tumor cell inoculation free topotecan (Hycamtin) or Topophore C^{TM} was administered i.v. (Q7D x 3) at the indicated drug doses. Control mice groups were injected with saline. The health status of all animals inoculated with tumor cells is monitored carefully to assess animal health as judged by sign of ill health or suffering as well as tumor growth and associated morbidity. If such signs were noted, mice were terminated by CO_2 asphyxiation. Suffering is assessed by trained animal care technicians who score and document animal health status daily

as specified by a prepared standard operating procedure. Euthanasia is based on a balance between measurable signs (weight loss and stool softness) and behavioral changes (activity) as well as physical appearance (coat and eye appearance). Morbidity measured in efficacy studies is understood to be a reflection of both treatment related side effects and tumor progression. Improvements in "survival" of mice inoculated with tumor cells was recorded for all groups, as follows: the overall health status of the animal was used to assess morbidity and a scoring system utilized to indicate when an animal should be terminated due to that morbidity. The day of death was typically reported as 1 day following termination due to morbidity. Necropsies were performed on terminated animals to assess gross signs of toxicity and tumor progression. As indicated above these animal studies were conducted according to the protocol approved by Institutional Animal Care Committee (IACC) of the University of British Columbia.

For the SKOV-3 tumor model, 5 x 10^6 SKOV-3 luc-D3 (luciferase transfected) cells/500 µL (obtained from Caliper Life Sciences, USA) were inoculated intraperitoneally into female mice (Ncr-nude, 20-25 g). Tumor growth was monitored weekly by non-invasive bioluminescent imaging with an IVIS^{TM}200 imager (Xenogen, Alameda, CA, USA). The commercially available Living Image^{TM} software (Xenogen, Alameda, CA, USA) was used to obtain and analyze images. Briefly, prior to imaging mice were injected i.p. with 500 µl luciferin solution (15 mg/mL), anaesthetized with isoflurane and then imaged. Regions of interest covering the entire peritoneal cavity were selected for the determination of total photon counts emitted per second.

3.3.10 Statistical analysis

Results were analyzed using ANOVA. Significant differences between groups were identified using Students-Newman-Keul's multiple comparison post hoc test (GraphPad Instat

software -San Diego, CA, USA). Differences between the groups were considered significant if $p<0.05$.

3.4 RESULTS AND DISCUSSION

3.4.1 Topotecan loading into copper containing liposomes

Topotecan loading into pre-formed liposomes was achieved using a modification of methods developed for irinotecan. In brief, addition of topotecan (Hycamtin) to the liposomes prepared in 300 mM copper sulfate and suspended in SHE (pH 7.5) buffer resulted in a dramatic decrease in the solution pH. If the pH of the solution was below 7.0, then the topotecan loading efficiency was very poor. To address this issue a pre-determined volume of 1N NaOH was added to the liposomes prior to topotecan addition. The volume added was such that when topotecan was mixed with the liposomes the pH of the resulting solution was 7.0-7.5. It was critical to have an external pH of 7.0-7.5 after topotecan addition in order to achieve optimum drug loading because similar to that observed at low pH, poor loading efficiency was observed at pH greater than 8. This is summarized in Table 3.1 for formulations prepared to achieve a final drug-to-lipid ratio of 0.1 (mol/mol). Depending on the loading conditions (lipid concentration, drug concentration, temperature) this meant that the liposomes were exposed to a pH as high as 12 for a brief period (<10 seconds). When the incubation temperature was 60^{o}C topotecan encapsulation efficiencies of >98% could be achieved for starting drug-to-lipid ratios of 0.1 (mol/mol). As summarized in Figure 3.1A, topotecan loading was rapid (>80% loading within 5 minutes) however to achieve loading efficiencies of >98% samples required incubation for 60 minutes. Particle size distribution as determined by PALS was between 95 – 110 nm prior to drug loading and the mean particle size or distribution around the mean did not change following drug loading (data not shown). Unlike irinotecan which was encapsulated in DSCP/CH

liposomes (55:45 mol ratio) efficiently at 50°C, topotecan loading required an incubation temperature of 60°C. The effect of temperature on drug loading rate is illustrated by the data summarized in Figure 3.1B. Topotecan loading efficiency was reduced significantly when the incubation temperature was decreased to 50°C and little drug loading was noted when the samples were incubated at 40°C.

Based on an understanding that the pH of the sample and temperature are critical to achieve rapid and efficient drug loading, additional studies were conducted to establish what the maximum drug loading capacity is for liposomes prepared with 300 mM copper. This work was initiated, in part, because other LNP topotecan formulations have been described in the literature with varying drug-to-lipid ratios (Drummond, Noble et al. ; Abraham, Edwards et al. 2004; Taggar, Alnajim et al. 2006; Dadashzadeh, Vali et al. 2008). Since the drug-to-lipid ratio influences lipid dose which in turn influences pharmacokinetics of injected formulations (Proffitt, Williams et al. 1983; Zhigaltsev, Maurer et al. 2005) it was important to establish the range of formulations which could be prepared. As summarized in Figure 3.2, the loading efficiency decreased as the initial drug-to-lipid ratio increased. When the initial drug-to-lipid ratio was 0.5 (mol/mol) the loading efficiency was 68%, compared to >95% for drug-to-lipid ratios of 0.2 or 0.1 (mol/mol). To better characterize the resulting formulations, drug release was measured under the dialysis conditions described in the Methods (excess volume of PBS). These data, summarized in Figure 3.3, were obtained at 40°C (Figure 3.3A) and at 50°C (Figure 3.3B) for LNP topotecan formulations prepared at 0.1, 0.2, 0.3 and 0.4 drug-to-lipid ratios (mol/mol). The results demonstrate rapid release of topotecan from the liposomes at both 40°C and 50°C when the initial drug-to-lipid ratio was 0.4 (~70% drug loading). This instability was also reflected in samples prepared using an initial drug-to-lipid ratio of 0.3 (mol/mol) when the

incubation temperature was 50°C. Both the 0.1 and 0.2 drug-to-lipid ratio formulations exhibited excellent drug retention under the conditions used, where less than 30% drug loss occurred over 8 h when incubated at 40°C. Although the differences between drug release from the 0.1 and 0.2 drug-to-lipid formulations were small, the improvements in drug retention noted for the 0.1 drug-to-lipid ratio were slightly better than that for 0.2. Therefore, this formulation was selected for further studies and has been referred to herein as TopophoreC™.

3.4.2 Retention of copper following topotecan loading

Although the mechanisms through which encapsulated copper improves drug retention for the Irinophore C™ formulation are not understood, it has been shown that the amount of retained copper after drug loading is important to achieve optimal drug retention attributes (chapter 2). For Irinophore C™ a retained copper concentration of at least 40 mM is required for optimal drug retention. Assuming similar mechanisms govern topotecan retention in Topophore C™, it was important to determine the amount of copper retained following topotecan loading. Aliquots obtained at various time points following topotecan addition to the liposomes at 60°C were passed through a size exclusion column (see Methods) in order to separate the drug loaded liposomes from any copper that may have been released during drug loading. Subsequently the samples were analyzed for copper as described in the Methods and the results of this study have been summarized in Figure 3.4. As topotecan (open circles) was encapsulated copper (filled circles) was released. In this study 90% of the added topotecan was encapsulated within 10 min. At this time point 60% of the encapsulated copper was released. After 60 min at 60°C, topotecan loading was >98% and approximately 75% of the initial liposome associated copper was released. Based on these data, the estimated copper concentration remaining in the liposomes would be approximately 75 mM.

3.4.3 Storage stability

Prior to initiating biological studies it was important to determine whether Topophore C^{TM} could be stored at 4^{o}C for extended time periods. In the context of the studies described here, it was known that treatment schedules to be used were based on weekly injections for at least 3 weeks, thus stability of the formulation for at least 1 month was a required. A batch of Topophore C^{TM} was prepared, filter sterilized and placed into sealed 5 mL vials prior to placing the samples at 4ºC. At selected time points vials were removed and the samples were analyzed for a number of parameters as specified in the Methods. These data have been summarized in Table 3.2. No significant changes in the formulation were noted in terms of appearance, color, particle size distribution, or drug-to-lipid ratio following fractionation on a size exclusion column. Topophore C^{TM} was able to retain > 98% of the initially encapsulated drug for at least 2 months.

3.4.4 *In vivo* pharmacokinetics following i.v. administration of TopophoreC^{TM}

Topotecan pharmacokinetics was evaluated in mice following administration of single i.v. bolus injection of free topotecan (Hycamtin) or Topophore C^{TM} administered at a dose of 5 mg/kg drug. At selected time points blood was obtained from injected animals and the concentration of liposomal lipid and topotecan were measured in the plasma as described in the Methods. The results, summarized in Figure 3.5, demonstrate that following injection of free topotecan the plasma concentration of drug decreased rapidly, with less than 0.08% of the injected dose in the plasma compartment after 2 h. The concentration of topotecan was below detection limits at time points beyond 4 h. In contrast, following administration of an equivalent drug dose of Topophore C^{TM} ~ 70% of the injected drug dose was in the plasma compartment at 2 h. Topotecan levels were still measureable 24h following administration of TopophoreC^{TM} at

levels comparable to those seen in plasma 2 h following administration of free drug. The plasma elimination half life was 15 min for free topotecan and 2.5 h for TopophoreC^{TM}. The plasma $AUC_{0\text{-}24h}$ following administration of free topotecan was 14.31 μg.h.mL^{-1} compared to 317.9 μg.h.mL^{-1} following administration of TopophoreC^{TM}. HPLC results (see representative chromatograms in Figure 3.6) demonstrated that 1 h after i.v. administration of Topophore C^{TM} >98% of the drug measured in the plasma was in the active lactone ring closed conformation (Figure 3.6 B) and no detectable carboxy form was measured for as long as 18 h (data not shown). This suggests that the drug in the plasma compartment was primarily present in the encapsulated form. In contrast following administration of free topotecan ~40% of the drug measured in the plasma compartment 1 h after drug administration was in the ring opened carboxy form (Figure 3. 6C). It should be noted that at the drug dose used in these studies, the associated liposomal lipid dose was 60 mg total lipid/kg and 24 h after administration >90% of the injected lipid dose was eliminated (Figure 3.5B). Liposome circulation longevity could be enhanced by incorporation of PEG-modified lipids (Blume and Cevc 1990; Klibanov, Maruyama et al. 1990; Allen, Hansen et al. 1991; Senior, Delgado et al. 1991) but addition of PEG lipids is often associated with increases in drug leakage rates and compromised intracellular delivery (Silvander, Johnsson et al. 1998; Dadashzadeh, Vali et al. 2008; Atyabi, Farkhondehfai et al. 2009).

3.4.5 Topotecan accumulation within the peritoneal cavity following i.v. administration

In advanced serous ovarian carcinoma, there is often significant disease burden restricted to the peritoneal cavity. Angiogenesis associated with ovarian cancer development and progression promotes peritoneal carcinomatosis and malignant ascites formation; two attributes linked to disease engendered morbidity and mortality (Vermorken 2000; Fujiwara, Armstrong et

al. 2007). For these reasons, preclinical models of ovarian cancer often involve inoculation of tumor cells within the peritoneal cavity. Given this site of tumor progression, it was reasonable to assess the extent to which topotecan distributed to the peritoneal cavity following i.v. administration of free topotecan (Hycamtin) or TopophoreC^{TM}. Further, studies have already demonstrated that following i.v. administration of LNP anticancer drug formulations there is significant accumulation of drug within the peritoneal cavity in the presence or absence of tumor (Harasym, Cullis et al. 1997). To determine if Topophore C^{TM} was able to enhance topotecan delivery to the peritoneal cavity of mice the concentration of topotecan in peritoneal fluid obtained from ES2 tumor bearing mice was measured following i.v. administration of either free topotecan (Hycamtin) or Topophore C^{TM} (see Methods). Results of this study are summarized in Figure 3.7. Approximately 400 ng of topotecan was recovered from the peritoneal cavity 1h following free topotecan administration; equivalent to about 0.4% of the injected dose. In comparison delivery of drug to the peritoneal cavity was significantly lower 1 h after administration of TopophoreC^{TM}. This is consistent with previous results suggesting that extravasation of circulating LNPs from the blood compartment to sites of tumor growth is a slow process (Noguchi, Wu et al. 1998; Maeda, Sawa et al. 2001; Laginha, Verwoert et al. 2005). After the 1 h time point, the amount of topotecan decreased in the peritoneal cavity of mice receiving free topotecan, but increased in those animals given TopophoreC^{TM}. At 8 h, for example, the level of topotecan in the peritoneal cavity was not detectable following administration of free topotecan while the level obtained following administration of Topophore C^{TM} was 250 ng. These data clearly demonstrated that following i.v. administration of Topophore C^{TM} significantly higher topotecan levels were observed for prolonged duration in the peritoneal cavity of ES-2 tumor bearing mice when compared to animals treated with free

topotecan (Hycamtin). Based on this data alone, one would anticipate that Topophore C^{TM} would exhibit improved therapeutic activity when used to treat animals with ovarian cancers developing within the peritoneal cavity.

3.4.6 *In vivo* efficacy studies in models of ovarian cancer

Clear cell carcinoma is a rare subtype of ovarian cancer that is clinically distinct from serous adenocarcinoma of the ovary, and is resistant to chemotherapy (Sugiyama, Kamura et al. 2000; Pather and Quinn 2005; Pectasides, Pectasides et al. 2006). In these studies human ovarian cancer cell lines were used that have been classified as clear cell carcinoma (ES2 cells) and serous adenocarcinoma (SKOV3 cells). As indicated in the Methods section, these cells were inoculated i.p. and tumor progression (SKOV3) or tumor related morbidity (ES2 cells) were monitored as a function of time following treatment. Treatments were given i.v. using a Q7D x 3 schedule. Dose response curves were generated and it should be noted that free topotecan (Hycamtin) was tolerated better than Topophore C^{TM} under this dosing schedule. Non-tumor related toxicity data summarized in Table 3.3 indicate that free topotecan can be administered at doses as high as 15 mg/kg without inducing morbidities requiring euthanasia of mice. Topophore C^{TM} administered at doses of 10 mg/kg topotecan caused significant weight loss in mice. For both free topotecan and TopophoreC^{TM} the toxicity observed were comparable.. Additional studies evaluating toxicity are currently ongoing. Results obtained using the ES2 ovarian cancer model are summarized in Figure 3.8. Although free topotecan (Hycamtin) could be administered at 15 mg/kg, the antitumor effects observed were not better than that observed following administration of 5 mg/kg (data not shown). Thus the 5 mg/kg dose was defined in this model as the maximum therapeutic dose of free topotecan. 100% of control animals (saline treated) were terminated due to disease progression within 29 days. In contrast 100% of the free topotecan

treated animals were terminated due to disease progression by day 36. The median survival time for control animals was 19 days and for animals treated with free topotecan (5 mg/kg) was 29 days, representing a 53% increase in median life span. The therapeutic activity of Topophore C^{TM} given at 1.25, 2.5 and 5.0 mg/kg topotecan was significantly better than that observed using the maximum efficacious dose of free topotecan. For example, when animals were treated at a dose of 1.25 mg/kg the median survival time for the treated animals was 48 days, representing a 152% increase in median survival time relative to controls. Similar to results obtained with free topotecan (Hycamtin), Topophore C^{TM} did not exhibit a significant dose response curve, i.e. the median survival time for mice treated with 5 mg/kg of Topophore C^{TM} (44 days) was not significantly different from the results obtained with 1.25 mg/kg Topophore C^{TM}.

The ES2 ovarian cancer model is considered to be a treatment refractory, aggressive model. In contrast, disease progression in the SKOV-3 ovarian cancer model is slow. To better assess tumor progression in the SKOV3 model, SKOV3 cells transfected with luciferease were obtained (see Methods) and this allowed use of bioluminescent imaging to non-invasively assess tumor progression as a function of time (see Methods). The tumor burden can be estimated by the amount of luminescent light emitted (photons/second) by the luciferase-modified cells. This data has been summarized in Figure 3.9. It should be noted that animals were imaged every week over a 6-week time frame, but for simplicity only the results obtained 1, 28 and 42 days after cell inoculation are presented. As noted in the representative images provided in Figure 3.9A, the imaging method used was sensitive enough to detect cells one day after cell inoculation. By day 7 when treatment was initiated, the bioluminescence was not greater than that observed on day 1, highlighting the very slow growth of this tumor model. By day 42, animals treated with saline exhibited a 218% increase in tumor cell burden as measured by

bioluminescence (Figure 3.9B). When animals were treated i.v. with free topotecan (Hycamtin; 15 mg/kg, Q7D x 3 starting on day 7) the increase in tumor burden was 176%. Animals treated with Topophore C^{TM} at a dose of 5 mg/kg exhibited a 95% increase in tumor burden. The differences in efficacy were not significant between the free topotecan or Topophore C^{TM} treatment groups ($p>0.05$). When comparisons were made to the saline treated control group, only those animals treated with Topophore C^{TM} exhibited a significant difference ($P<0.001$) in tumor progression. Clearly more research needs to be completed using other ovarian cancer models, but the results provided in Figures 3.7 and 3.8 provided sufficient proof of concept data to warrant further assessments of the Topophore C^{TM} formulation described here.

3.5 CONCLUSION

Topotecan has been recently approved by FDA to be used as a single agent in the second line treatment of recurrent ovarian cancer. It has proven to be a potent anticancer agent and has shown very good therapeutic efficacy in terms of reductions in measurable disease and improving the progression free survival time for patients with ovarian cancer (Creemers, Bolis et al. 1996). There is a growing recognition among drug delivery research community that application of suitable nanoscale drug delivery technology will enhance the therapeutic effects of topotecan. Enhanced activity would be due to: i) maintaining the drug in its lactone ring closed configuration following administration, ii) enhanced delivery of therapeutically active drug to the site of tumor growth and iii) increased efficacy at lower drug doses which may be better tolerated and more suitable for use in a drug combination setting. As indicated other groups have pursed development of LNP topotecan formulations. In some examples low topotecan loading efficiency and rapid drug loss from the formulation limited their further development (Burke and Gao 1994; Subramanian and Muller 1995; Dadashzadeh, Vali et al. 2008). Others have reported on

formulations prepared using novel methods relying on transmembrane gradients of triethylammonium salts of polyphosphate or sucroseoctasulfate to form topotecan precipitates within the liposomes. These formulations exhibit extended circulation lifetimes, increased topotecan AUC, and improved therapeutic activity (Drummond, Noble et al. ; Drummond, Noble et al. 2006). At this time it is too early to assess the benefits/limitations of these formulation methods when compared to the one described in this report. The formulation described here relies on use of encapsulated copper and a transmembrane pH gradient to achieve improvements in topotecan retention. The formulation developed (TopophoreC^{TM}) was stable for at least 2 months at 4^{o}C, exhibited a topotecan encapsulation efficiency of >98%, maintained the drug in its active ring closed lactone form, resulted in a significant decrease in topotecan elimination rate and changed topotecan distribution in a manner that resulted in improved therapeutic activity as judged in two different models of ovarian cancer. It will be clearly important to assess the toxicity and efficacy of Topophore C^{TM} against other aggressive models of recurrent ovarian cancer. In addition there needs to be a comparison made between different LNP topotecan formulations currently under development. At this point in time there does not appear to be any compelling reason to develop multiple LNP topotecan formulations, thus in the interest of patients in need of better treatment options it would be ideal if comparator studies could be completed to identify one formulation that has the best chance of providing benefits for patients. Further evaluation of Topophore C^{TM} was carried out in combination with Doxil$^{®}$, a liposomal formulation of doxorubicin that is approved for use in patients with relapsed, platinum refractory ovarian cancer. These studies are described and discussed in detail in chapter 4 of this thesis.

Table 3.1: Optimization of drug loading conditions for the LNP topotecan formulation.

Initial pH of liposomes	pH after NaOH addition	Vol. of NaOH (1N) required (μL)	pH after topotecan addition	Incubation time/temp.	% topotecan loading
7.5	9.0	14	5.0	30 min/60°C	80%
7.5	10.5	30	6.0	30 min/60°C	89%
7.5	12.0	50	7.25 ± 0.25	30 min/60°C	>98%

Table 3.2: Storage stability of Topophore C^{TM} at 4ºC.

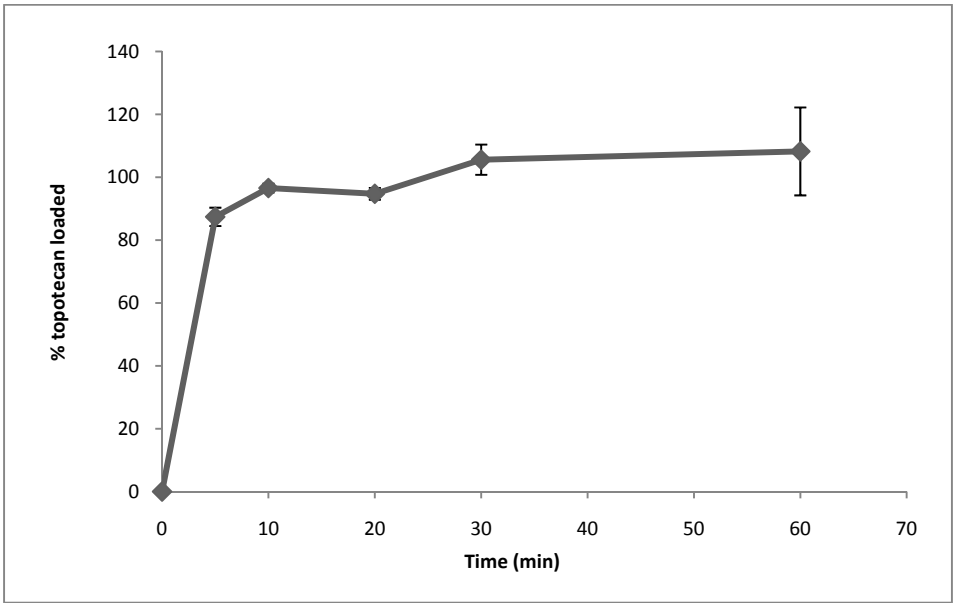
Time	Appearance	Size (nm)	D/L	% Drug Retained
Initial	Translucent yellow	99.4	0.08	>98
1 week	No change	105.8	0.08	>98
2 week	No change	104.8	0.08	>98
3 week	No change	108.1	0.08	>98
1 month	No change	108.1	0.07	>98
2 month	No change	102.9	0.08	>98

Table 3.3: Non-tumor related toxicity signs observed in mice following increasing doses of Hycamtin (free topotecan) or Topophore C™

Formulation	Dose (Q7D x 3) (mg/kg)	Major signs of toxicity (BCS)[a]	Morbidity due to toxicity[b]
Hycamtin	1.25	1-5% weight loss (1)	None
	2.5	6-10% weight loss (2)	None
	5	1-5% weight loss (1)	None
	7.5	Dry skin, sunken eyes (2)	None
	15	Dry skin, decreased motor activity, hunched (3)	None
Topophore C™	1.25	None (0)	None
	2.5	None (0)	None
	5	Hunched, 20% weight loss in two mice (3)	2(14)
	10	Hunched, >20% weight loss (4)	None

a. Body condition score (BCS): based on overall health status mice were euthanized at a score of ≥4.
b. Number of mice needed to be euthanized due to poor BCS.

(A)



(B)

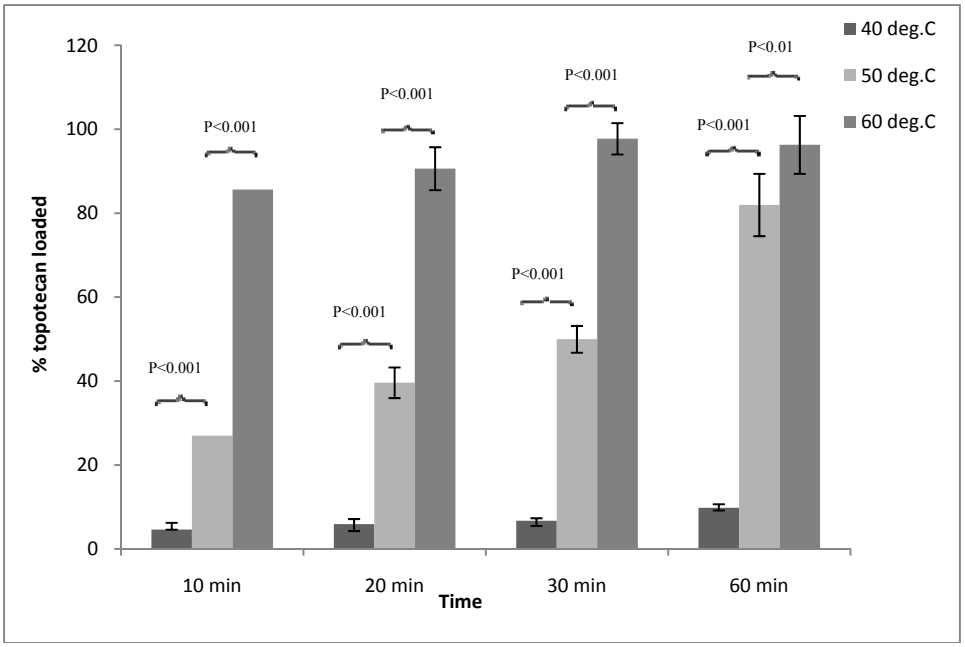


Figure 3.1: $CuSO_4$ mediated topotecan encapsulation into DSPC/CH (55:45) liposomes. (A) Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) and incubated with A23187. Topotecan was mixed with liposomal suspension to achieve a drug-to-lipid ratio of 0.1 (mol/mol) at 60ºC for 60 minutes. (B) Effect of incubation temperature on the loading efficiency of topotecan into liposomes prepared in the similar manner as described for figure (A). Aliquots were removed intermittently and fractionated onto 1 mL Sephadex G-50 size exclusion column to separate unencapsulated drug. Data points represent mean ± SD of values obtained from at least three experiments.

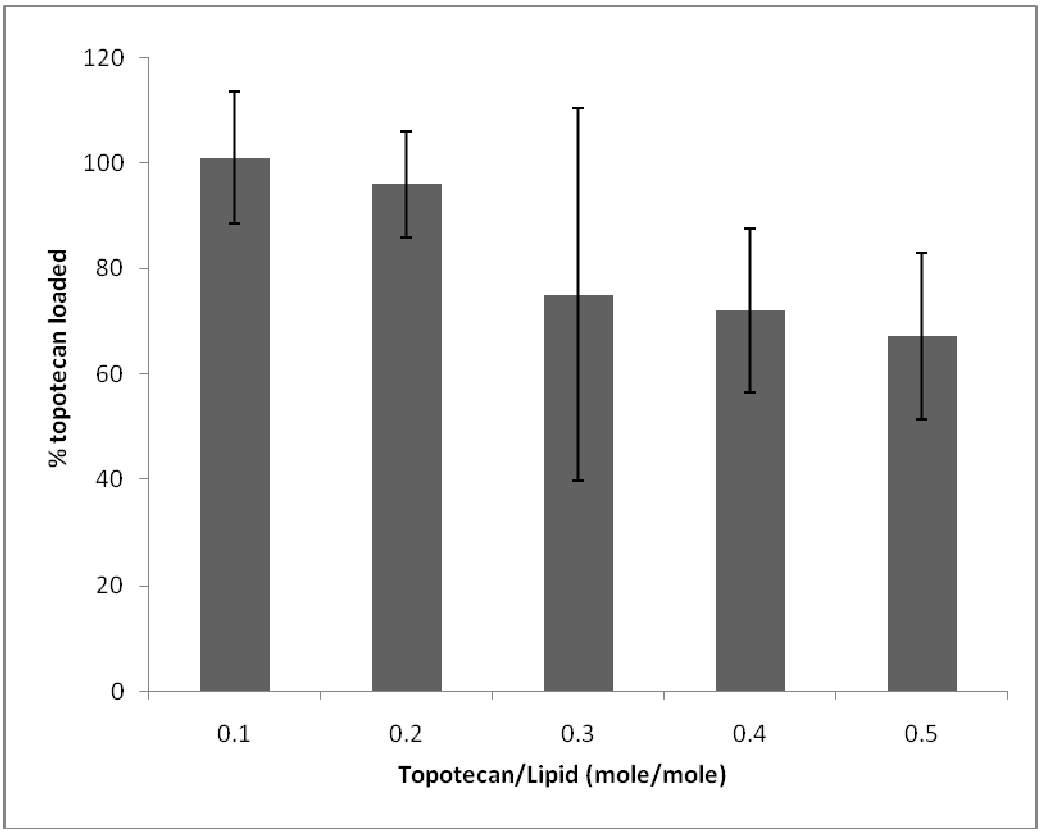
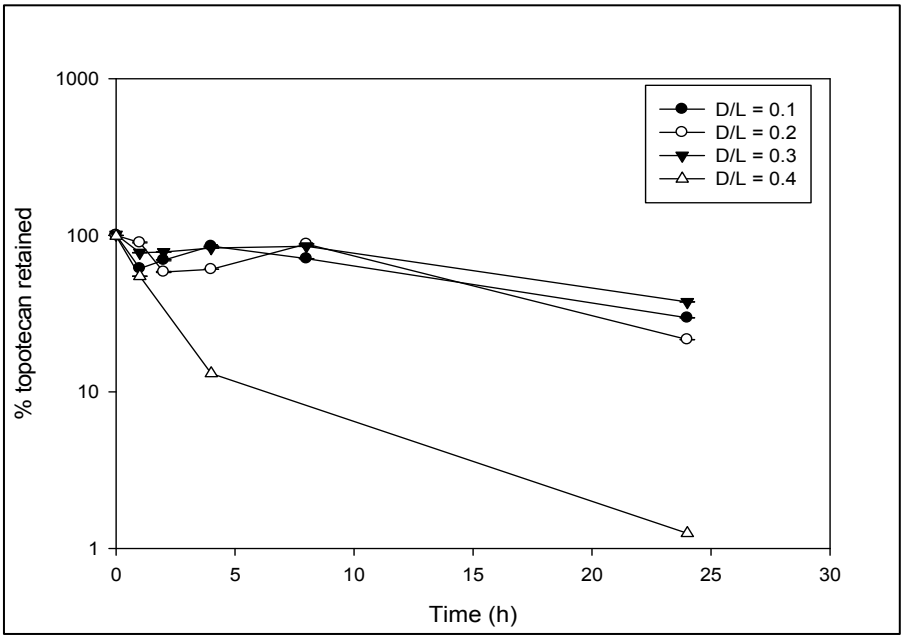


Figure 3.2: Encapsulation efficiency of liposomal topotecan formulations as a function of increasing target D/L (mole/mole). Topotecan was added to liposomes prepared in 300 mM copper sulfate and A23187. The liposomes and drug were incubated separately at 60^{o}C prior to mixing. Immediately before drug addition to the liposomes, the liposome solution pH was adjusted with 1N NaOH such that when topotecan was mixed with the liposomes the final pH was 7.0-7.5. Data points represent mean ± SD of values obtained from at least three separate experiments.

(A)



(B)

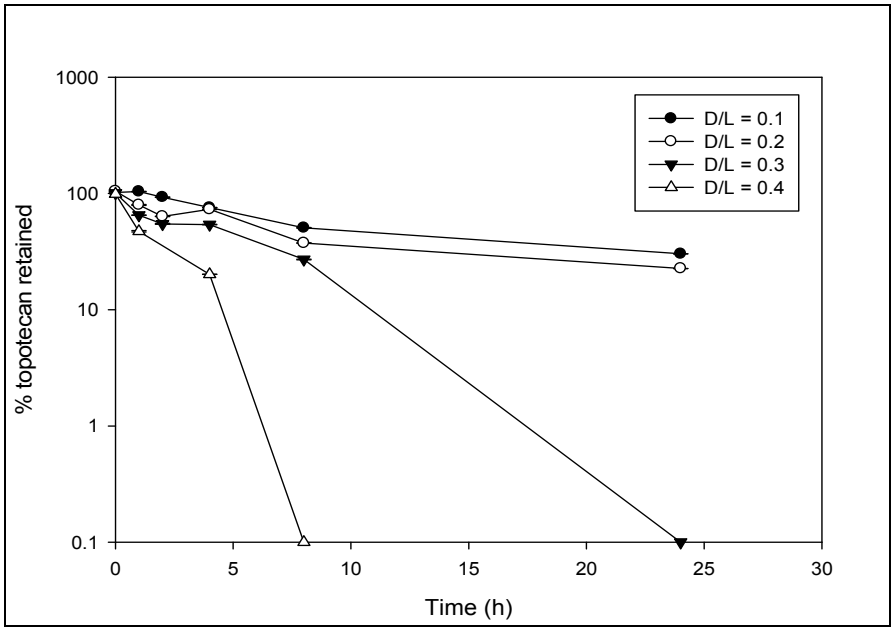


Figure 3.3: *In vitro* drug release profile of liposomal topotecan following incubation in PBS (pH 7.4) at (A) 40ºC and (B) 50ºC. Briefly, liposomal suspensions were diluted appropriately with PBS buffer and placed in a dialysis bag (MWC 10000). Aliquots were removed from the dialysis bag intermittently and liposomes were separated from the free drug using spin columns prepared with Sephadex G50. Topotecan and liposomal lipids were measured using HPLC and scintillation counting respectively. Values indicate mean ± SD of three individual measurements.

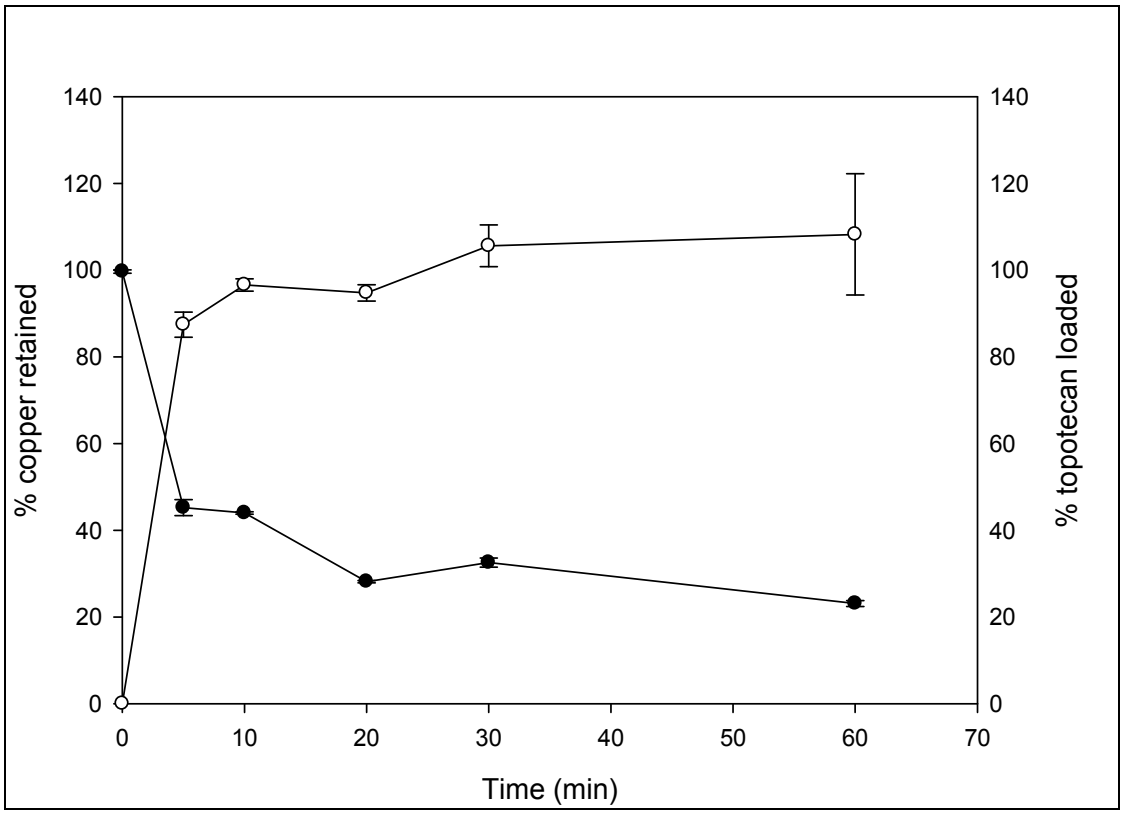
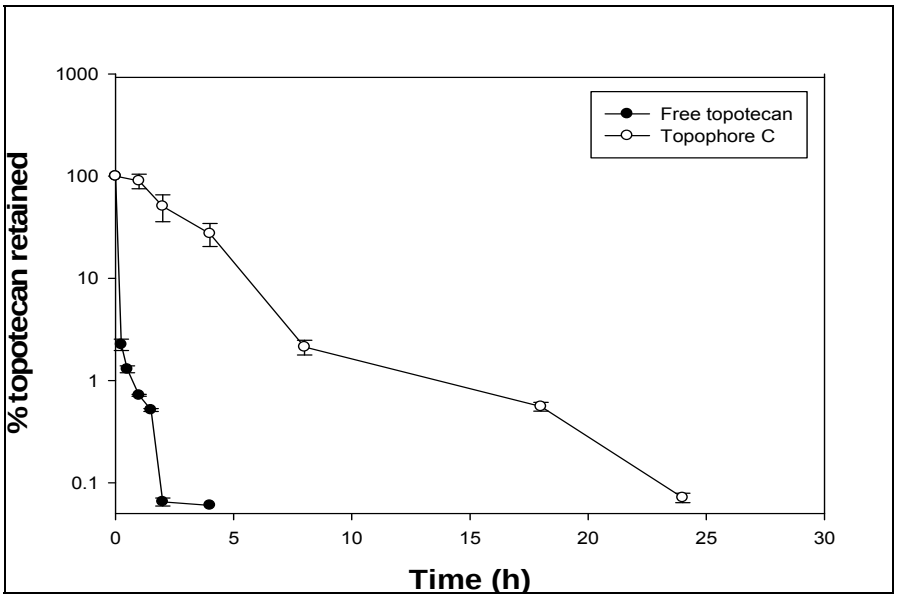


Figure 3.4: Relationship between topotecan loading (○) and copper release (●) from the liposomes. Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) and incubated with ionophore A23187. Topotecan was mixed with liposomal suspension to achieve a drug-to-lipid ratio of 0.1 (mol/mol), at 60^{o}C. Aliquots were removed intermittently and fractionated onto 1 mL Sephadex G-50 size exclusion column to separate unencapsulated topotecan and released copper which were measured using UV-spectrometer and atomic absorption spectroscopy respectively.

(A)



(B)

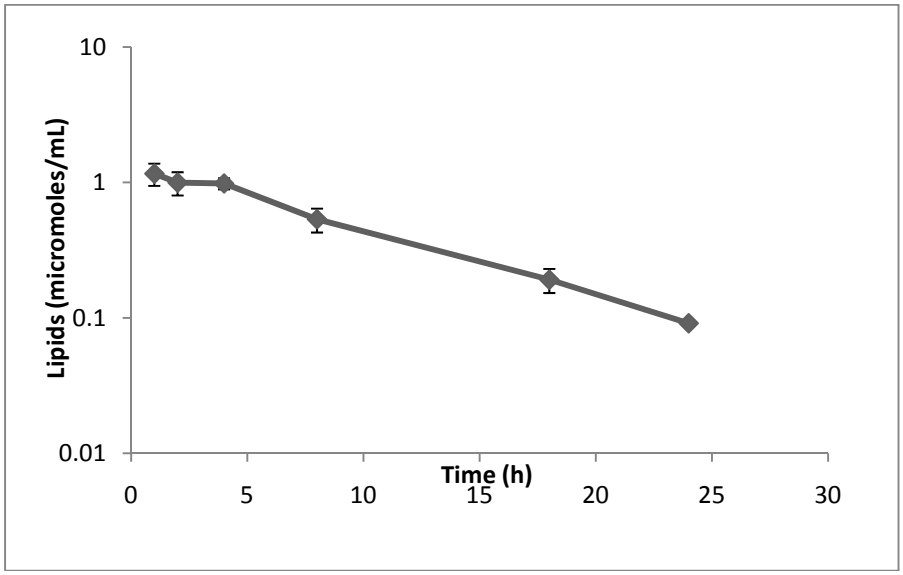
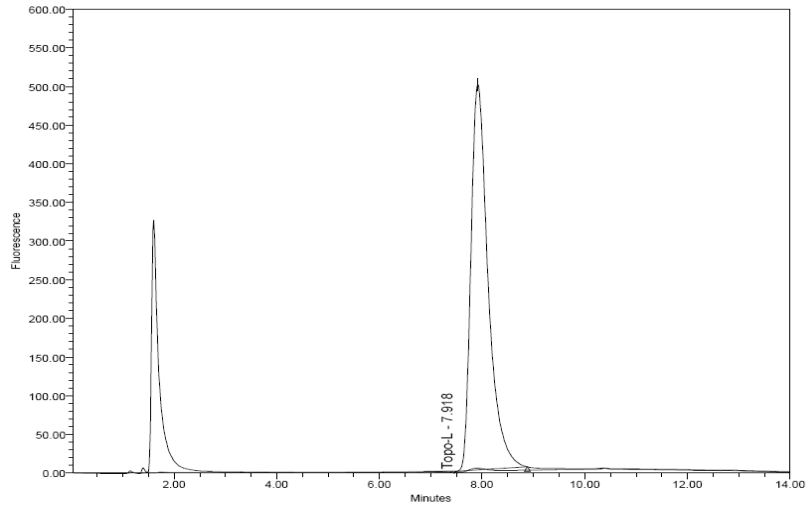
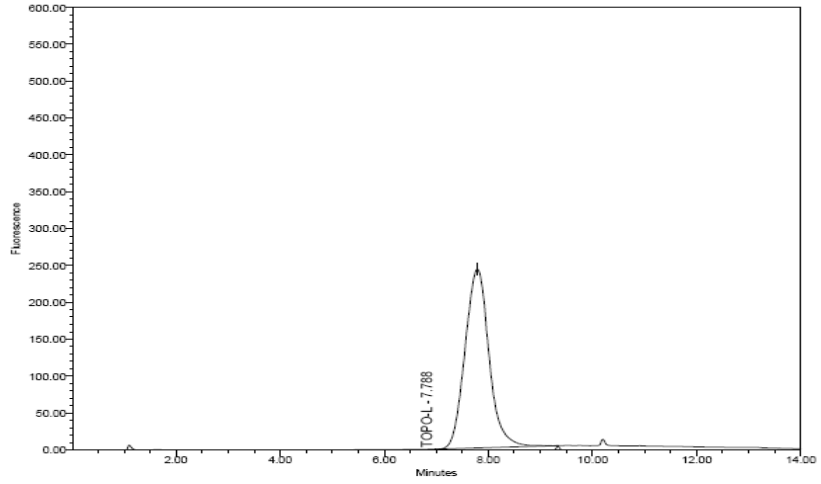


Figure 3.5: Plasma elimination profiles of Topophore C compared against free topotecan (Hycamtin): Female Balb/C mice were injected intravenously with a single dose (5 mg/kg topotecan) of DSPC/Chol (55:45 mol%) liposomal topotecan formulations: (A) Concentration of topotecan remaining in plasma as a function of time. (B) Amount of liposomal lipid (3H-CHE) remaining in plasma as a function of time. Data points represent the mean ± SD (n=4).

(A)



(B)



(C)

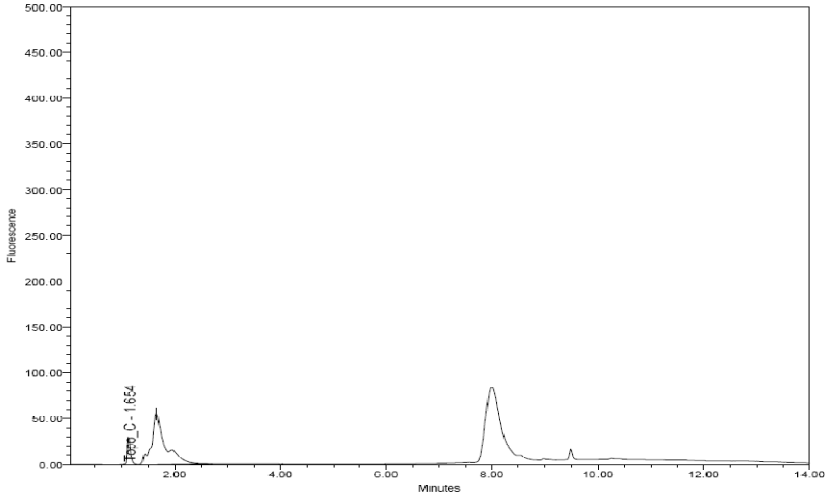


Figure 3.6: HPLC chromatograms of topotecan representing (A) overlay spectra for separately injected carboxy and lactone standards in mouse plasma. (B) 1 h mouse plasma sample following i.v. administration of Topophore C (5 mg/kg) and (C) 1 h mouse plasma sample following i.v. administration of Free topotecan (5 mg/kg).

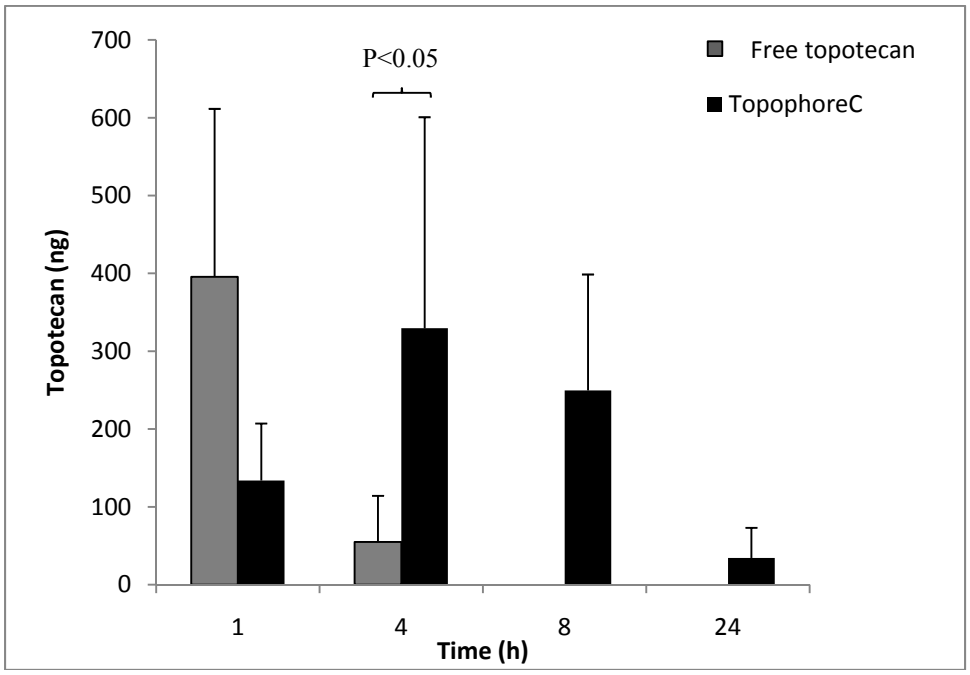


Figure 3.7: Amount of topotecan recovered from the peritoneal fluid of ES-2 ovarian tumor bearing mice following i.v. administration of single dose (5 mg/kg) of free topotecan (Hycamtin) or TopophoreC^{TM}. Data points indicate mean ± SEM, n = 4, P<0.01.

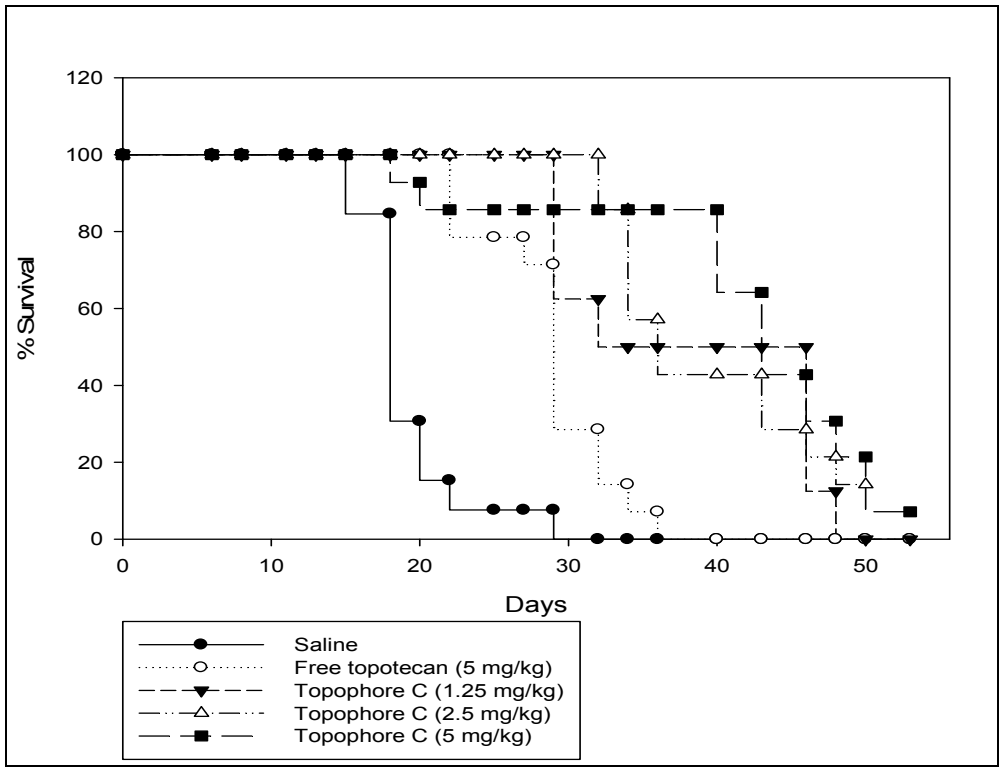
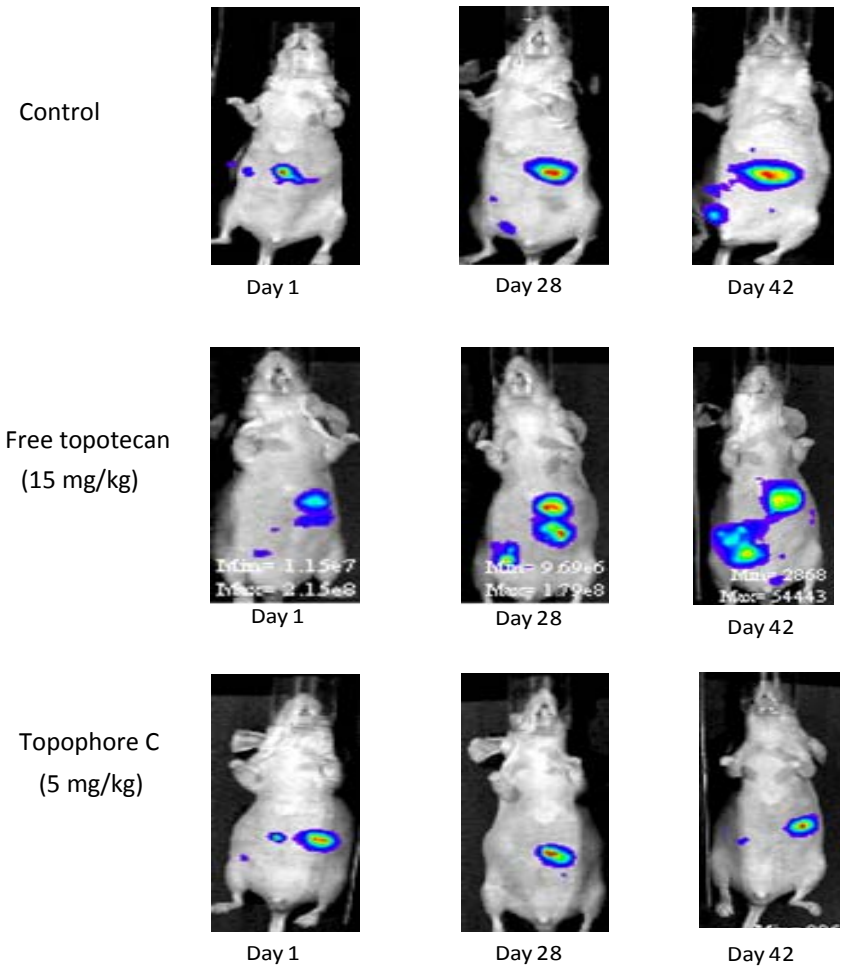


Figure 3.8: Kaplan-Meier survival plot for ES-2 ovarian tumor bearing mice following i.v. administration (q7d x3) of free topotecan (5 mg/kg) or Topophore C^{TM} (1.25 mg/kg, 2.5 mg/kg and 5 mg/kg). Control mice were administered equivalent volume of saline. Data points represent mean ± SD ($n \geq 8$).

(A)



(B)

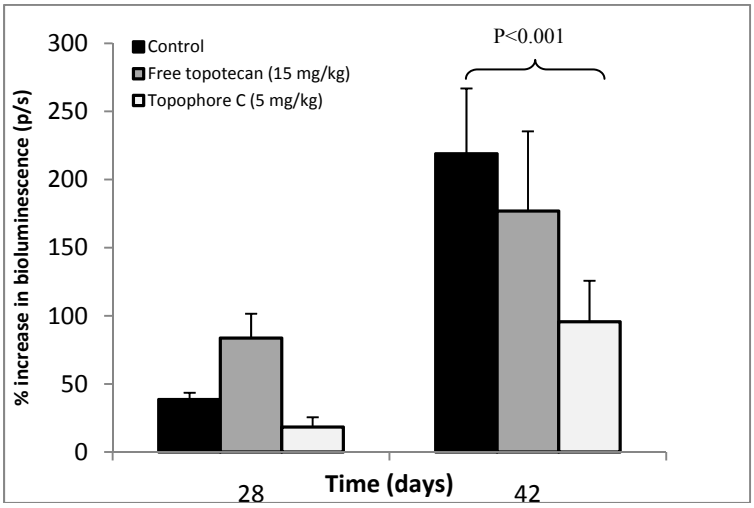


Figure 3.9: Tumor progression in i.p. inoculated luciferase modified SKOV-3 mice xenografts. Treatment groups were administered intravenously (q7d x3) with either free topotecan (15 mg/kg) or Topophore C^{TM} (5 mg/kg,) and control mice were administered equivalent volume of saline. Tumor measurements were conducted using IVIS system. Data points represent mean ± SD (n = 6).

3.6 REFERENCES

Abraham, S. A., Edwards, K., Karlsson, G., Hudon, N., Mayer, L. D. and Bally, M. B. (2004). "An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes." J Control Release **96**(3): 449-61.

Allen, T. M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991). "Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo." Biochim Biophys Acta **1066**(1): 29-36.

Atyabi, F., Farkhondehfai, A., Esmaeili, F. and Dinarvand, R. (2009). "Preparation of pegylated nano-liposomal formulation containing SN-38: In vitro characterization and in vivo biodistribution in mice." Acta Pharm **59**(2): 133-44.

Baek, J. H., Kim, J. G., Jeon, S. B., Chae, Y. S., Kim, D. H., Sohn, S. K., Lee, K. B., Choi, Y. J., Shin, H. J., Chung, J. S., Cho, G. J., Jung, H. Y. and Yu, W. (2006). "Phase II study of capecitabine and irinotecan combination chemotherapy in patients with advanced gastric cancer." Br J Cancer **94**(10): 1407-11.

Blume, G. and Cevc, G. (1990). "Liposomes for the sustained drug release in vivo." Biochim Biophys Acta **1029**(1): 91-7.

Burke, T. G. (1996). "Chemistry of the camptothecins in the bloodstream. Drug stabilization and optimization of activity." Ann N Y Acad Sci **803**: 29-31.

Burke, T. G. and Gao, X. (1994). "Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine." J Pharm Sci **83**(7): 967-9.

Burke, T. G. and Mi, Z. (1994). "The structural basis of camptothecin interactions with human serum albumin: impact on drug stability." J Med Chem **37**(1): 40-6.

Burke, T. G., Mishra, A. K., Wani, M. C. and Wall, M. E. (1993). "Lipid bilayer partitioning and stability of camptothecin drugs." Biochemistry **32**(20): 5352-64.

Burke, T. G. e. a. (1992). "Liposomal stabilization of camptothecin's lactone ring." J. Am. Chem. Soc. **114**: 8318-8319.

Cao, Q., Lu, X. and Feng, Y. J. (2006). "Glycogen synthase kinase-3beta positively regulates the proliferation of human ovarian cancer cells." Cell Res **16**(7): 671-7.

Chou, T. H., Chen, S. C. and Chu, I. M. (2003). "Effect of composition on the stability of liposomal irinotecan prepared by a pH gradient method." J Biosci Bioeng **95**(4): 405-8.

Comen, E. A. and Robson, M. "Inhibition of poly(ADP)-ribose polymerase as a therapeutic strategy for breast cancer." Oncology (Williston Park) **24**(1): 55-62.

Correale, P., Marra, M., Remondo, C., Migali, C., Misso, G., Arcuri, F. P., Del Vecchio, M. T., Carducci, A., Loiacono, L., Tassone, P., Abbruzzese, A., Tagliaferri, P. and Caraglia, M. "Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-targeted antibody-dependent cell-mediated-cytotoxicity (ADCC)." Eur J Cancer.

Creemers, G. J., Bolis, G., Gore, M., Scarfone, G., Lacave, A. J., Guastalla, J. P., Despax, R., Favalli, G., Kreinberg, R., Van Belle, S., Hudson, I., Verweij, J. and Ten Bokkel Huinink, W. W. (1996). "Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study." J Clin Oncol **14**(12): 3056-61.

Cullis, P. R., Hope, M. J., Bally, M. B., Madden, T. D., Mayer, L. D. and Fenske, D. B. (1997). "Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles." Biochim Biophys Acta **1331**(2): 187-211.

Dadashzadeh, S., Vali, A. M. and Rezaie, M. (2008). "The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats." Int J Pharm **353**(1-2): 251-9.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Connolly-Ingram, C., Gabriel, B. S., Hann, B., Liu, B., Park, J. W., Hong, K., Benz, C. C., Marks, J. D. and Kirpotin, D. B. "Development of a highly stable and targetable nanoliposomal formulation of topotecan." J Control Release **141**(1): 13-21.

Drummond, D. C., Noble, C. O., Guo, Z., Hong, K., Park, J. W. and Kirpotin, D. B. (2006). "Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy." Cancer Res **66**(6): 3271-7.

Dupont, J., Aghajanian, C., Andrea, G., Lovegren, M., Chuai, S., Venkatraman, E., Hensley, M., Anderson, S., Spriggs, D. and Sabbatini, P. (2006). "Topotecan and liposomal doxorubicin in recurrent ovarian cancer: is sequence important?" Int J Gynecol Cancer **16 Suppl 1**: 68-73.

Emerson, D. L. (2000). "Liposomal delivery of camptothecins." Pharm Sci Technolo Today **3**(6): 205-209.

Emerson, D. L., Besterman, J. M., Brown, H. R., Evans, M. G., Leitner, P. P., Luzzio, M. J., Shaffer, J. E., Sternbach, D. D., Uehling, D. and Vuong, A. (1995). "In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues." Cancer Res **55**(3): 603-9.

Fenske, D. B., Wong, K. F., Maurer, E., Maurer, N., Leenhouts, J. M., Boman, N., Amankwa, L. and Cullis, P. R. (1998). "Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients." Biochim Biophys Acta **1414**(1-2): 188-204.

Fujiwara, K., Armstrong, D., Morgan, M. and Markman, M. (2007). "Principles and practice of intraperitoneal chemotherapy for ovarian cancer." Int J Gynecol Cancer **17**(1): 1-20.

Ghesquieres, H., Faivre, S., Djafari, L., Pautier, P., Lhomme, C., Lozahic, S., Djazouli, K., Armand, J. P. and Raymond, E. (2006). "Phase I dose escalation study of pegylated liposomal doxorubicin (Caelyx) in combination with topotecan in patients with advanced malignancies." Invest New Drugs **24**(5): 413-21.

Giovanella, B. C., Harris, N., Mendoza, J., Cao, Z., Liehr, J. and Stehlin, J. S. (2000). "Dependence of anticancer activity of camptothecins on maintaining their lactone function." Ann N Y Acad Sci **922**: 27-35.

Giovanella, B. C., Stehlin, J. S., Wall, M. E., Wani, M. C., Nicholas, A. W., Liu, L. F., Silber, R. and Potmesil, M. (1989). "DNA topoisomerase I--targeted chemotherapy of human colon cancer in xenografts." Science **246**(4933): 1046-8.

Gottlieb, J. A. and Luce, J. K. (1972). "Treatment of malignant melanoma with camptothecin (NSC-100880)." Cancer Chemother Rep **56**(1): 103-5.

Hao, Y. L., Deng, Y. J., Chen, Y., Wang, K. Z., Hao, A. J. and Zhang, Y. (2005). "In-vitro cytotoxicity, in-vivo biodistribution and anti-tumour effect of PEGylated liposomal topotecan." J Pharm Pharmacol **57**(10): 1279-87.

Hao, Y. L., Deng, Y. J., Chen, Y., Wang, X. M., Zhong, H. J. and Suo, X. B. (2005). "In vitro and in vivo studies of different liposomes containing topotecan." Arch Pharm Res **28**(5): 626-35.

Haran, G., Cohen, R., Bar, L. K. and Barenholz, Y. (1993). "Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases." Biochim Biophys Acta **1151**(2): 201-15.

Harasym, T. O., Cullis, P. R. and Bally, M. B. (1997). "Intratumor distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes." Cancer Chemother Pharmacol **40**(4): 309-17.

Harasym, T. O., Tardi, P. G., Bally, M. and Janoff, A. (2006). Fixed drug ratio liposome formulations of combination cancer therapeutics. New York, informa healthcare.

Hatefi, A. and Amsden, B. (2002). "Camptothecin delivery methods." Pharm Res **19**(10): 1389-99.

Hsiang, Y. H., Lihou, M. G. and Liu, L. F. (1989). "Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin." Cancer Res **49**(18): 5077-82.

Hsiang, Y. H. and Liu, L. F. (1988). "Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin." Cancer Res **48**(7): 1722-6.

Hsiang, Y. H., Wu, H. Y. and Liu, L. F. (1988). "Topoisomerases: novel therapeutic targets in cancer chemotherapy." Biochem Pharmacol **37**(9): 1801-2.

Kawano, K., Watanabe, M., Yamamoto, T., Yokoyama, M., Opanasopit, P., Okano, T. and Maitani, Y. (2006). "Enhanced antitumor effect of camptothecin loaded in long-circulating polymeric micelles." J Control Release **112**(3): 329-32.

Kehrer, D. F., Bos, A. M., Verweij, J., Groen, H. J., Loos, W. J., Sparreboom, A., de Jonge, M. J., Hamilton, M., Cameron, T. and de Vries, E. G. (2002). "Phase I and pharmacologic study of liposomal lurtotecan, NX 211: urinary excretion predicts hematologic toxicity." J Clin Oncol **20**(5): 1222-31.

Klibanov, A. L., Maruyama, K., Torchilin, V. P. and Huang, L. (1990). "Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes." FEBS Lett **268**(1): 235-7.

Laginha, K. M., Verwoert, S., Charrois, G. J. and Allen, T. M. (2005). "Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors." Clin Cancer Res **11**(19 Pt 1): 6944-9.

Larsen, A. K., Gilbert, C., Chyzak, G., Plisov, S. Y., Naguibneva, I., Lavergne, O., Lesueur-Ginot, L. and Bigg, D. C. (2001). "Unusual potency of BN 80915, a novel fluorinated E-ring modified camptothecin, toward human colon carcinoma cells." Cancer Res **61**(7): 2961-7.

Lau, D. H., Lewis, A. D., Ehsan, M. N. and Sikic, B. I. (1991). "Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin." Cancer Res **51**(19): 5181-7.

Lei, S., Chien, P. Y., Sheikh, S., Zhang, A., Ali, S. and Ahmad, I. (2004). "Enhanced therapeutic efficacy of a novel liposome-based formulation of SN-38 against human tumor models in SCID mice." Anticancer Drugs **15**(8): 773-8.

Liu, J. J., Hong, R. L., Cheng, W. F., Hong, K., Chang, F. H. and Tseng, Y. L. (2002). "Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation." Anticancer Drugs **13**(7): 709-17.

Maeda, H., Sawa, T. and Konno, T. (2001). "Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS." J Control Release **74**(1-3): 47-61.

Mahany, J. J., Lewis, N. and Heath, E. I. e. a. (2009). A Phase IB study evaluating BSI-201 in combination with chemotherapy in subjects with advanced solid tumors. Proc. Am. Soc. Clin. Oncol.

Main, C., Bojke, L., Griffin, S., Norman, G., Barbieri, M., Mather, L., Stark, D., Palmer, S. and Riemsma, R. (2006). "Topotecan, pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line or subsequent treatment of advanced ovarian cancer: a systematic review and economic evaluation." Health Technol Assess **10**(9): 1-132 iii-iv.

Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986). "Vesicles of variable sizes produced by a rapid extrusion procedure." Biochim Biophys Acta **858**(1): 161-8.

Messerer, C. L., Ramsay, E. C., Waterhouse, D., Ng, R., Simms, E. M., Harasym, N., Tardi, P., Mayer, L. D. and Bally, M. B. (2004). "Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer." Clin Cancer Res **10**(19): 6638-49.

Moertel, C. G., Schutt, A. J., Reitemeier, R. J. and Hahn, R. G. (1972). "Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer." Cancer Chemother Rep **56**(1): 95-101.

Nastruzzi, R. C. E. E. A. M. E. M. C. (1997). "Formulation study for the antitumor drug camptothecin: Liposomes, micellar solution and a microemulsion." International Journal of Pharmaceutics **159**: 95-103.

Noda, K., Nishiwaki, Y., Kawahara, M., Negoro, S., Sugiura, T., Yokoyama, A., Fukuoka, M., Mori, K., Watanabe, K., Tamura, T., Yamamoto, S. and Saijo, N. (2002). "Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer." N Engl J Med **346**(2): 85-91.

Noguchi, Y., Wu, J., Duncan, R., Strohalm, J., Ulbrich, K., Akaike, T. and Maeda, H. (1998). "Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues." Jpn J Cancer Res **89**(3): 307-14.

Ozols, R. F. (2000). "Optimum chemotherapy for ovarian cancer." Int J Gynecol Cancer **10**(S1): 33-37.

Pal, A., Khan, S., Wang, Y. F., Kamath, N., Sarkar, A. K., Ahmad, A., Sheikh, S., Ali, S., Carbonaro, D., Zhang, A. and Ahmad, I. (2005). "Preclinical safety, pharmacokinetics and antitumor efficacy profile of liposome-entrapped SN-38 formulation." Anticancer Res **25**(1A): 331-41.

Patankar, N., Ramsay, E., Anantha, M., Waterhouse, D. and Bally, M. (2010). "The role of the transition metal copper and the ionophore A23187 in the development of Irinophore C^{TM} (Submitted)" Pharm Res.

Pather, S. and Quinn, M. A. (2005). "Clear-cell cancer of the ovary-is it chemosensitive?" Int J Gynecol Cancer **15**(3): 432-7.

Pectasides, D., Pectasides, E., Psyrri, A. and Economopoulos, T. (2006). "Treatment issues in clear cell carcinoma of the ovary: a different entity?" Oncologist **11**(10): 1089-94.

Proffitt, R. T., Williams, L. E., Presant, C. A., Tin, G. W., Uliana, J. A., Gamble, R. C. and Baldeschwieler, J. D. (1983). "Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles." Science **220**(4596): 502-5.

Ramsay, E., Alnajim, J., Anantha, M., Taggar, A., Thomas, A., Edwards, K., Karlsson, G., Webb, M. and Bally, M. (2006). "Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation." Pharm Res **23**(12): 2799-808.

Ramsay, E. C., Anantha, M., Zastre, J., Meijs, M., Zonderhuis, J., Strutt, D., Webb, M. S., Waterhouse, D. and Bally, M. B. (2008). "Irinophore C: a liposome formulation of irinotecan with substantially improved therapeutic efficacy against a panel of human xenograft tumors." Clin Cancer Res **14**(4): 1208-17.

Sadzuka, Y., Hirotsu, S. and Hirota, S. (1999). "Effective irinotecan (CPT-11)-containing liposomes: intraliposomal conversion to the active metabolite SN-38." Jpn J Cancer Res **90**(2): 226-32.

Sai, K., Kaniwa, N., Ozawa, S. and Sawada, J. (2002). "An analytical method for irinotecan (CPT-11) and its metabolites using a high-performance liquid chromatography: parallel detection with fluorescence and mass spectrometry." Biomed Chromatogr **16**(3): 209-18.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., Maroun, J. A., Ackland, S. P., Locker, P. K., Pirotta, N., Elfring, G. L. and Miller, L. L. (2000). "Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group." N Engl J Med **343**(13): 905-14.

Sandhu, S. K., Yap, T. A. and de Bono, J. S. "Poly(ADP-ribose) polymerase inhibitors in cancer treatment: a clinical perspective." Eur J Cancer **46**(1): 9-20.

Seiden, M. V., Muggia, F., Astrow, A., Matulonis, U., Campos, S., Roche, M., Sivret, J., Rusk, J. and Barrett, E. (2004). "A phase II study of liposomal lurtotecan (OSI-211) in patients with topotecan resistant ovarian cancer." Gynecol Oncol **93**(1): 229-32.

Senior, J., Delgado, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991). "Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles." Biochim Biophys Acta **1062**(1): 77-82.

Silvander, M., Johnsson, M. and Edwards, K. (1998). "Effects of PEG-lipids on permeability of phosphatidylcholine/cholesterol liposomes in buffer and in human serum." Chem Phys Lipids **97**(1): 15-26.

Subramanian, D. and Muller, M. T. (1995). "Liposomal encapsulation increases the activity of the topoisomerase I inhibitor topotecan." Oncol Res **7**(9): 461-9.

Sugiyama, T., Kamura, T., Kigawa, J., Terakawa, N., Kikuchi, Y., Kita, T., Suzuki, M., Sato, I. and Taguchi, K. (2000). "Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy." Cancer **88**(11): 2584-9.

Taggar, A. S., Alnajim, J., Anantha, M., Thomas, A., Webb, M., Ramsay, E. and Bally, M. B. (2006). "Copper-topotecan complexation mediates drug accumulation into liposomes." J Control Release **114**(1): 78-88.

Tardi, P. G., Gallagher, R. C., Johnstone, S., Harasym, N., Webb, M., Bally, M. B. and Mayer, L. D. (2007). "Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo." Biochim Biophys Acta **1768**(3): 678-87.

Verhaar-Langereis, M., Karakus, A., van Eijkeren, M., Voest, E. and Witteveen, E. (2006). "Phase II study of the combination of pegylated liposomal doxorubicin and topotecan in platinum-resistant ovarian cancer." Int J Gynecol Cancer **16**(1): 65-70.

Vermorken, J. B. (2000). "The role of intraperitoneal chemotherapy in epithelial ovarian cancer." Int J Gynecol Cancer **10**(S1): 26-32.

Wall, M. E. (1993). Chronicles of Drug Discovery. D. Lednicer. Washington D.C., American Chemical Society. **3:** 327.

Wall, M. E. and Wani, M. C. (1995). "Camptothecin and taxol: discovery to clinic--thirteenth Bruce F. Cain Memorial Award Lecture." Cancer Res **55**(4): 753-60.

Watanabe, M., Kawano, K., Yokoyama, M., Opanasopit, P., Okano, T. and Maitani, Y. (2006). "Preparation of camptothecin-loaded polymeric micelles and evaluation of their incorporation and circulation stability." Int J Pharm **308**(1-2): 183-9.

Zhigaltsev, I. V., Maurer, N., Akhong, Q. F., Leone, R., Leng, E., Wang, J., Semple, S. C. and Cullis, P. R. (2005). "Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention." J Control Release **104**(1): 103-11.

4. TOPOTECAN AND DOXORUBICIN COMBINATIONS FOR TREATMENT OF REFRACTORY OVARIAN CANCER: THE INFLUENCE OF DRUG EXPOSURE TIME ON DRUG COMBINATION EFFECTS AND THE ROLE FOR DELIVERY SYSTEMS TO ACHIEVE OPTIMIZED THERAPEUTIC ACTIVITY *IN VIVO*.

[3] A version of this chapter will be submitted for publication as:

Nilesh Patankar, Julia Pritchard, Mariska van Grinsven, Maryam Osooly, Marcel Bally. "Topotecan and doxorubicin combinations for treatment of refractory ovarian cancer: The influence of drug exposure time on drug combination effects and the role for delivery systems to achieve optimized therapeutic activity in vivo."

4.1 SUMMARY

Ovarian cancer is a lethal gynecological cancer and is associated with poor prognosis and high relapse rate. Doxil® and topotecan are used as second line chemotherapy in treating recurrent ovarian cancer. Recently developed therapeutically active lipid nanoparticle (LNP) formulation of topotecan (referred to as Topophore C™) was discussed in chapter 3 and the studies presented here were designed to provide proof-of-concept data to support the use of Doxil® and Topophore C™ in combination for use in the treatment of platinum refractory ovarian cancer.

Cytotoxic activities of topotecan and doxorubicin against ES-2, OVCAR-3 and SKOV-3 ovarian cancer cell lines were tested with the help of cell based screening assays measuring metabolic activity (MTT assay). Fixed ratio combinations of doxorubicin, the active pharmaceutical ingredient in Doxil®, and topotecan were generated and tested for activity against these cells. The effect of extended drug exposure on the cancer cells was studied by exposing the cells to the drugs alone and in combination for 1, 4, 8, 24, 48 or 72 hours. *In vivo* studies evaluated plasma elimination of the drugs when administered alone and in combination. Efficacy assessments using single agents or combinations were completed in two pseudo-orthotopic models of ovarian cancer (clear cell carcinoma (ES-2) and serous adenocarcinoma (SKOV-3)).

Based on drug doses capable of achieving 50% reduction in cell viability (MTT assay) over a 72 hour time course; combination index analysis indicated that combinations of doxorubicin and topotecan were additive when tested in the SKOV3 cells but highly synergistic when used against the ES-2 and OVCAR-3 cells. Favorable drug-drug interactions increased when drug exposure time increased. The pharmacokinetic behavior of Topophore C™ was not affected when co-administered with Doxil®, a small decrease in plasma elimination of free

topotecan was noted when the drug was co-administered with Doxil®, and the plasma levels of doxorubicin were significantly lower when Doxil® was co-administered with free topotecan or Topophore C™. Doxil® administered at its maximum tolerated dose (MTD, 7.5 mg/kg Q7D x 3) provided no therapeutic benefit when used to treat mice bearing established ES-2 tumors and when used in combination with free topotecan (MTD of 15 mg/kg Q7D x3). Doxil® did not enhance survival time in this model over that which could be achieved with topotecan alone. In contrast, median survival time (MST) was increased to 52 days when the ES-2 bearing mice were treated with combinations of Topophore C™ (MTD 2.5 mg/kg) and Doxil® (7.5 mg/kg) (Q7D x 3) when compared to untreated animals (MST of 18 days) or those treated with Topophore C™ alone (MTD 5 mg/kg, Q7D x 3) (MST of 40 days). In the SKOV3 model, where bioluminescence was used to monitor tumor progression, Doxil® exhibited significant therapeutic effects when used alone and all combination treatments showed better therapeutic efficacy than that achievable when using the individual drugs, however the combination of Doxil® and Topophore C™ was therapeutically superior at doses exhibiting equivalent toxicity.

Thus, topotecan and doxorubicin combinations produced additive or synergistic effects against three ovarian cancer cells and these were best be achieved under conditions when the tumor cells were exposed to both drugs over extended time periods. LNP formulations of these drugs can achieve extended exposure times *in vivo* and combinations of LNP formulations of doxorubicin and topotecan are therapeutically superior as judged in two models of ovarian cancer. Drug:drug interactions affecting the pharmacokinetic behavior of Doxil® need to be explored further.

4.2 INTRODUCTION

Ovarian cancer is among the most common gynecological cancers and is the leading cause of cancer-related deaths among female cancer patients (Kurman and Shih Ie ; Jemal, Siegel et al. 2008). Platinum-refractory ovarian cancer is considered an incurable disease and the treatment options available at present are primarily palliative in nature (Ahmad and Gore 2004). There is a need to develop improved treatment options for these patients, and a great deal of hope has been placed on the identification and development of molecularly targeted drugs; drugs that can affect signaling pathways uniquely expressed in the ovarian cancer patients (Agarwal, Linch et al. 2006). While these more specific therapeutic agents are offering benefits in many cancers, they are not replacing the use of existing cytotoxic drugs. In fact the targeted drugs often exhibit poor therapeutic effects when used alone and their therapeutic value is achieved primarily in the combination setting. Thus there remains a strong rationale for exploring the use of strategies that can enhance the effects of existing cytotoxic drugs when given alone and in combination. Combination chemotherapy is an effective strategy that has shown promise in the treatment of ovarian cancer. Strategies used for the selection of drugs to be used in combination have been developed in light of well established criteria: i) the drugs must exhibit complementary mechanism of action, ii) they must be effective when used as a single agent, iii) they should exhibit different mechanisms of resistance and iv) they should exhibit non-overlapping toxicities (Frei 1991; Jonsson, Fridborg et al. 1998; Zimmermann, Lehar et al. 2007). More recently, the concept of developing drug combinations selected on the basis of synergistic drug-drug interactions has been promoted (Ramsay, Dos Santos et al. 2005; Waterhouse, Gelmon et al. 2006).

Synergy can be defined as an interaction that results in therapeutic effects that are greater than that which could be expected from single agent activities (Chou and Talalay 1984; Chou 1991; Chou 2006). An alternative perspective is that a synergistic drug combination can achieve therapeutic effects equal to that achievable with single agents, but at significantly lower, better tolerated, drug doses (Ramsay, Dos Santos, Dragowska, Laskin and Bally 2005). It is not well understood what factors influence synergy, however existing evidence suggests that drug dose, drug-drug ratio and drug sequencing all influence combination interactions (Zoli, Ricotti et al. 2001; Ramsay, Dos Santos, Dragowska, Laskin and Bally 2005; Zimmermann, Lehar and Keith 2007). In this study the role of drug exposure time on drug:drug interaction is being explored with the goal of defining an improved drug combination for use in patients with relapsed ovarian cancer. Currently the FDA has approved topotecan (Creemers, Bolis et al. 1996; Swisher, Mutch et al. 1997; ten Bokkel Huinink, Gore et al. 1997; Ozols 2000; Gordon, Fleagle et al. 2001; Horowitz, Hua et al. 2004) and a liposomal formulation of doxorubicin (Doxil®) as single agents for use in second line therapy in the treatment of ovarian cancer. Further, there are ongoing clinical trials exploring the use of topotecan in combination with Doxil® in this patient population (Dupont, Aghajanian et al. 2006; Ghesquieres, Faivre et al. 2006). However based on the current understanding of the factors that influence drug:drug interactions *in vivo,* the maximum therapeutic benefits of this combination may not be realized.

Topotecan, is camptothecin analogue that specifically targets DNA-topoisomerase I complex and induces DNA damage by stabilizing the complex and thereby acts as a topoisomerase I (TOP I) inhibitor (D'Arpa and Liu 1989). Doxorubicin, an anthracycline analogue, acts by stabilizing the DNA-topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping

the process of replication (Swift, Rephaeli et al. 2006). Combination of TOP I and II inhibitors have been evaluated and results suggest synergy between them when administered sequentially (Jonsson, Fridborg et al. 1998; Dupont, Aghajanian et al. 2006; Ghesquieres, Faivre et al. 2006; Main, Bojke et al. 2006). Interestingly, the combinations of TOP I and TOP II inhibitors currently being pursued for treatment of patients with relapsed ovarian cancer actually involve the use of a free TOP I inhibitor (topotecan) and a lipid nanoparticle (LNP) formulation of a TOP II inhibitor (Doxil™). However, considering the pharmacokinetic differences the LNP formulation is expected to exhibit remarkably different plasma elimination rates and biodistribution behavior when compared to free topotecan, therefore the drug levels and the drug-to-drug ratio at the sites of tumor growth will likely be variable over time. It has already been demonstrated that the pharmacokinetic and biodistribution behavior of drug combinations can be controlled better when using LNP formulations of drug combinations (Harasym, Tardi et al. 2006; Mayer, Harasym et al. 2006; Tardi, Johnstone et al. 2009). This concept envisioned development of novel combination products (two drugs formulated in a single LNP composition) or the combination of two different LNP formulations. A number of groups, have been pursuing the development of optimized LNP formulations for topotecan (Drummond, Noble et al. ; Liu, Hong et al. 2002; Dadashzadeh, Vali et al. 2008) and many of these exhibit promising therapeutic potential based on preclinical studies. A recently developed formulation described in chapter 3 of this thesis utilizes pH gradient encapsulation methods combined with drug complexing ability of encapsulated copper to prepare a formulation that exhibits improved drug retention *in vivo* and improved therapeutic activity when compared to the free drug. This formulation, referred to as Topophore C^{TM}, is now undergoing extensive preclinical evaluations. In an effort to guide the clinical development of Topophore C™ preclinical studies have placed

emphasis on its use in relapse ovarian cancer. In the studies described here, the use of Topophore C™ in combination with Doxil® was studied and this effort was initiated around the hypothesis that drug:drug interactions promoting synergy will be dependent on exposure time for the drugs used and, since LNP formulations of drugs provide a means to enhance exposure time of drugs, combinations of Topophore C™ and Doxil® should provide means to achieve optimal drug combination effects *in vivo*. The results indicated that the synergistic effects achieved when using topotecan and doxorubicin in combination can increase when exposure time increases and the therapeutic effects of Topophore C™ in combination with Doxil® were significantly better than those obtained when using combinations of topotecan and Doxil®.

4.3 MATERIALS AND METHODS

4.3.1 Materials and chemicals

Doxorubicin (Adriamycin®), topotecan (Hycamtin®) and Doxil® were purchased from the British Columbia Cancer Agency pharmacy (Vancouver, BC). ES-2, SKOV-3 and OVCAR-3 human ovarian carcinoma cells lines were obtained from the American Tissue Culture Collection, ATCC (Rockville, MD). SKOV-Luc-D3 cell line was obtained from Caliper Life Sciences (USA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) from PerkinElmer Life Sciences (Boston, MA). ^{3}H-cholesterylhexadecyl ether ([3H]-CHE), ^{14}C-sucrose and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH) and all other chemicals (Reagent grade) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

4.3.2 Cell culture

ES-2 and SKOV-3 cells were grown in McKoy's 5A medium containing 1.5 mM L-glutamine and 10% FBS. OVCAR-3 cells were grown in RPMI-1640 medium supplemented with 20% FBS, 0.01 mg/mL bovine insulin, 2.5 mM L-Glutamine. Cells were sub-cultured when 80-90% confluent by rinsing with phosphate buffer saline and detached from flask with 0.25% trypsin. Once detached, cells were counted using a hemocytometer and diluted in media to the appropriate concentration (see below) prior to addition to 96-well microtitre Falcon plates. Cells were maintained in culture for up to 20 passages. After 20 passages, new cells were expanded from frozen stock vials, stored in liquid nitrogen.

4.3.3 Cell viability assay

The MTT assay was used as a measure of cell viability. In brief, 100 μL of cell suspension containing required cell number (6×10^3 for ES-2 and SKOV3 and 1×10^4 for OVCAR-3) was added to the wells of 96-well plates and incubated at 37°C in humidified air with 5% CO_2. After 24 hours, 100 μL of cell culture medium containing appropriate concentration of either topotecan hydrochloride or doxorubicin hydrochloride (0 to 100000 nM) was added to these cells. Following 72 hours of this treatment, 50 μL of MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to the wells and plates were incubated at 37°C for 3.5 hours. Supernatant was aspirated before dissolving the formazan crystals in 150 μL DMSO. Plates were agitated for 10 min and the optical density of each well was read at 570 nm using a microplate reader (Thermo Multiskan Spectrum). Cell viability was determined by comparing absorbance from treated wells against that from control wells (cells treated with culture medium instead of drug). Concentrations of topotecan and doxorubicin needed to cause 50 % loss in viability as judged by the MTT assay (IC_{50}) were determined. To determine the

effect of prolong exposure of drugs onto the cells, cells were treated with increasing concentrations of topotecan or doxorubicin and incubated for different time periods (1, 4, 8, 24, 48 and 72 h). Following each time point media-containing drug was aspirated off the cells and 200 µl fresh media was added and the cells were incubated for an additional time frame such that the total time in culture was 72 hours. Prior to addition of MTT at 72 hours, media was aspirated and fresh media was added. Drug concentrations were used that encompassed the IC_{10}, IC_{50} and IC_{90} values for the respective drug (50-32000 nM for doxorubicin and 5-3200 nM for topotecan).

To determine the activity of the topotecan-doxorubicin combinations, fixed ratio of the two agents were generated based on a ratio defined by the IC_{50} of doxorubicin and topotecan in the indicated cell line. These ratios were then tested over a broad range of effective doses for activity against the three cell lines using the MTT assay as described above. To determine the effect of exposure time, fixed ratio combinations of the two agents were exposed to the cell lines for different lengths of time as described above.

All *in vitro* assays were conducted in triplicate and mean values obtained from three separate experiments were used for further analysis. The dose dependent effects of the drugs when used alone and in combination were analyzed using CompuSyn®, a computer program that analyzes dose response data according to the Chou and Talalay median effect principle (Chou and Talalay 1984; Chou 2006; Waterhouse, Gelmon et al. 2006) discussed in detail in chapter 1 (section 1.5.1). The program generates combination index (CI) values from the dose response curves and provide an indication as to whether the interaction between the two drugs results in synergistic (CI<1), additive (CI=1) or antagonistic (CI>1) effects.

4.3.4 Preparation of TopophoreC™

Large unilamellar vesicles (LUVs) were prepared using DSPC and cholesterol (CH) (55:45 molar ratio) by film hydration-extrusion as described previously (Please refer to chapter 2, section 2.3.2). The size of the LUVs generated using this method was determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). Liposomal lipid concentration was determined by measuring ^{3}H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer). To load topotecan, liposomes with encapsulated copper sulfate (unbuffered solution, pH 3.5) were suspended in SHE buffer (pH 7.5). Subsequently, A23187 (0.5 μg per 1 mg lipid) was added to the liposomes which were then incubated at 30°C for 30 min. This mixture and a reconstituted solution of topotecan were warmed separately at 60°C for 5 minutes using a temperature bath. Just prior addition of topotecan to the liposomes a sufficient volume of 1N NaOH was added to liposomal suspension such that the final pH of the drug loading mixture after addition of topotecan was 7.0 to 7.5. The final drug/liposome mixture was incubated in a water bath at 60°C for 60 min. Following loading the mixture was brought to the room temperature and un-encapsulated topotecan was separated from liposomes with the help of Sephadex G-50 column pre-equilibrated with phosphate buffer saline (PBS, pH 7.5). Lipid concentrations were measured by scintillation counting of the lipid marker ^{3}H-CHE. Topotecan concentrations were determined by measuring the absorbance at 370 nm on UV-vis spectrophotometer (Agilent/Hewlett Packard, model: 8453, Agilent Technologies. Mississauga, ON, Canada) as described previously (Chapter 3, section 3.3.3)..

4.3.5 Pharmacokinetic analysis of drug combinations

Doxil (7.5 mg/kg), a combination of free topotecan (5 mg/kg) and Doxil (7.5 mg/kg) or a combination of Doxil (7.5 mg/kg) and Topophore C (5 mg/kg) were administered intravenously

(i.v.) to female Ncr-Nude mice (Taconic, Hudson, NY; 20-25 g; 4 per time point). To administer a combination, the specified doses of the respective treatment agents were mixed just before the injection. Following administration, blood samples were collected at specified time intervals via cardiac puncture, after the mice were terminated by CO_2 asphyxiation. Blood was immediately placed into EDTA containing microtainers (Becton Dickinson, NJ) and stored on ice until they could be centrifuged at 2500 rpm for 15 min to separate plasma from blood cells. Plasma topotecan concentrations were determined by HPLC. HPLC assays were conducted using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump, a controller and a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by diluting with Acetonitrile:Methanol mixture (50:50 v/v) . 10 µL of diluted sample was injected onto a Waters Symmetry Shield RP C18 cartridge column (100 Å, particle size 3.5 µm; 75 x 4.6 mm, Waters). Mobile phase consisted of mobile phase 'A' (1% Triethyleneamine in water, pH 6.4 adjusted with glacial acetic acid) and mobile phase 'B' (100 % acetonitrile). The sample temperature was maintained at 4°C and the column temperature was adjusted to 55°C. Each sample was run for 14 min at a flow rate of 1.0 mL/min using a gradient method, where the amount of organic phase was increased from 12% to 40% over 8 min. Plasma AUC and half-life of topotecan were determined from this data using non-compartmental pharmacokinetic model with the help of PK Solutions software (Summit Research Services, Montrose, CO).

Doxorubicin concentrations in the plasma were determined by a previously established method (Bally, Nayar et al. 1990). Briefly, plasma samples were mixed with 10% sodium dodecyl sulfate (SDS) and 10 mM sulfuric acid (H_2SO_4) (1/1/1) and volume was adjusted to 1

mL with water. This was followed by organic extraction using isopropanol-chloroform solution (1/1) (organic phase to sample ratio of 2:1). The samples were frozen at -80°C for 48 h to facilitate the precipitation of proteins and then thawed at the room temperature. Doxorubicin containing organic phase was separated by centrifugation at 2500 x g for 10 min at room temperature. Doxorubicin equivalent fluorescence in the organic phase was determined using a luminescence spectrophotometer (Perkin Elmer LS50B) with an excitation wavelength of 470 nm (slit width = 2.5) and an emission wavelength of 550 nm (slit width = 10). The standard curve for doxorubicin was generated by extracting it into the organic phase using the procedure described above and fluorescence readings from the samples were compared against the freshly prepared standard curve.

4.3.6 Assessment of anti-tumor efficacy

In vivo assessments of anti-tumor efficacy were completed in two ovarian cancer models. For ES-2 model development, ES-2 cells (1×10^5/500 µl) were inoculated intraperitoneally (i.p.) into female NCr-Fox1^{nu} mice (Taconic, Hudson, NY). Seven days after tumor cell inoculation treatment groups were treated i.v. (Q7D x 3) at the indicated drug doses. Control mice groups were injected with saline. The drug doses were escalated to levels that were close to maximum tolerated doses (MTD) and the health status of all animals inoculated with tumor cells was monitored carefully, which included effects due to tumor growth and associated morbidity as well as drug induced morbidity. In the event that the health status was poor as judged using a scoring method defined in a standard operating procedure, mice were terminated by CO_2 asphyxiation. A balance between measurable signs (weight loss and stool softness) and behavioral changes (activity) as well as physical appearance (coat and eye appearance) was taken into consideration for euthanasia. Survival times of the mice were recorded for all groups and the

day of death was reported as 1 day after the mice were euthanized due to poor health status. Necropsies were performed on terminated animals to assess gross signs of toxicity and tumor progression. These animal studies were conducted according to the protocol approved by University of British Columbia Animal Care Committee.

For the SKOV-3 tumor model, 5 x 10^6 SKOV-3 luc-D3 (luciferase transfected) cells in 500 µL were inoculated intraperitoneally into female mice (Ncr-nude, 20-25 g). Tumor growth was monitored one time per week non-invasively by bioluminescent imaging with an IVIS^{TM}200 imager (Xenogen, Alameda, CA, USA). The commercially available Living Image^{TM} software (Xenogen, Alameda, CA, USA) was used to obtain and analyze images. Briefly, mice were injected i.p. with 500 µl luciferin solution (15 mg/mL), anaesthetized with isoflurane and were imaged 20 minutes (as accurately as possible) after luciferin injection. Regions of interest covering the entire peritoneal cavity were selected for the determination of total photon counts emitted per second. Following one week of tumor cell inoculation, mice were treated i.v. (Q7D x 3) at the drug doses specified. Control mice groups were injected with saline. Following administration of the first dose, treated and control mice were imaged once a week to monitor tumor progression. All animals were observed post inoculation at least two times a day, more if deemed necessary, for signs of morbidity. The health status of mice was monitored as described above.

4.3.7 Statistical analysis

The results were analyzed using ANOVA. Significant differences between groups were identified using Students-Newman-Keul's multiple comparison post hoc test (GraphPad Instat software - GraphPad, CA, USA). Survival curves generated using Kaplan-Meier plot were compared for statistical significance using Log-rank (Mantel-Cox) test (GraphPad Prism

software, GraphPad, CA, USA). Differences between the groups were considered significant if $P<0.05$.

4.4 RESULTS

4.4.1 Cell viability assays following treatment with topotecan and doxorubicin alone and in combination

Results from the MTT assays have been summarized in Table 4.1 and Figure 4.1 (topotecan) and Figure 4.2 (doxorubicin). Regardless of the cell line used, topotecan was more potent than doxorubicin. As noted in Table 4.1, which summarizes the drug IC_{50} following a 72 h exposure, the IC_{50} for topotecan ranged from a low of 18 nM in the SKOV-3 cell line to a high of 83 nM in the ES-2 cells. Doxorubicin IC_{50}s ranged from a low of 251 nM in the SKOV-3 cells to a high of 539 nM in the ES-2 cells. The ES-2 cells were least sensitive to these drugs, SKOV-3 cells were most sensitive. Exposure studies demonstrated for all three cell lines that drug concentration required to achieve an effect level of 50% (fa=0.5) decreased as the exposure time increased. The activity of topotecan (Figure 4.1) was much more exposure time dependent than doxorubicin (Figure 4.2). Using ES-2 cells (Figure 4.1A) as an example, the IC_{50} was about 780 nM if the exposure time was 1 h and this concentration decreased almost 10-fold to 83 nM when the exposure time was increased to 72 h. The exposure time dependency was even more dramatic for the OVCAR-3 cells (Figure 4.1C), where exposure times of 1 to 8 h was insufficient to achieve significant impacts on cell viability regardless of the drug concentration used. The effect of exposure time on the IC_{50} of topotecan has been summarized for all three cell lines in Figure 4.1D. There were decreases in the IC_{50} of doxorubicin as exposure time increased but the effect of exposure time was less than that noted for topotecan, albeit difference of 5 to 10 fold were obtained for the ES2 cells (Figure 4.2A) and the SKOV-3 cells (Figure 4.2B).

The activity of topotecan and doxorubicin used alone and in combination was also assessed and these data have been summarized in Figure 4.3 and Figure 4.4. Fixed ratio combinations of doxorubicin and topotecan were generated based on the IC_{50} values observed in the three ovarian cancer cell lines (see Table 4.1) which ranged from 15:1 to 6.5:1 (doxorubicin to topotecan). These combinations were tested over a broad range of effective doses and the dose response curves have been summarized in Figure 4.3A (ES2 cells), 4.3B (OVCAR-3 cells) and 4.3C (SKOV-3 cells). In all examples, these 72 h studies demonstrated that drug combination was as active (SKOV-3 cells) or more active than the agents when used alone. It is difficult to interpret drug:drug interactions on the basis of the sigmoidal dose response curves shown in Figure 4.3 and for this reason the results were analyzed using the median effect principle developed by Chou and Talaly (Chou and Talalay 1984; Berenbaum 1989; Zoli, Ricottiet al. 2001; Chou 2006; Waterhouse 2008) and functionalized in the CompuSyn® program described in the Methods. CompuSyn® analysis of the dose response curves (Figure 4.3D) indicated that topotecan-doxorubicin combination produced CI values of 0.22 and 0.15 against ES-2 and OVCAR-3 cell lines respectively and therefore appeared highly synergistic against these cells. The CompuSyn analysis of the drug combination data obtained for the SKOV-3 cells suggested that the interactions were additive (CI of 1.1) for this cell line.

The activities of topotecan and doxorubicin were dependent on exposure time and for this reason it was assumed that combinations of topotecan and doxorubicin would exhibit increased activity as the exposure time increased from 1 to 72 h. This is illustrated by the data (obtained for cells exposed to drug for 72h) summarized in Figure 4.4A (ES2 cells), 4.4B (OVCAR-3 cells) and 4.4C (SKOV-3 cells). To assess how exposure time influences the activity of the drugs when used in combination, the resultant dose response curves were then analyzed through use of the

CompuSyn program and the CI values measured at Fa values of 0.5 (IC_{50}) were plotted for different exposure times (Figure 4.4D). As observed in this figure, increased exposure time significantly improved the synergistic interactions of the combination against ES-2 and OVCAR-3 cells. This is exemplified by the ES-2 data where 4 h exposure suggested that the combination was additive (CI = 1.1) while a 72 h exposure suggested synergistic interactions (CI = 0.2). Similarly, for OVCAR-3 data, 4 h exposure to the drug combination suggested that the combination was antagonistic (CI = 1.4) while a 72 h exposure suggested synergistic interactions (CI = 0.2). The drug combination produced additive effects when tested against the SKOV3 cell line, regardless of the exposure time.

4.4.2 Pharmacokinetic (PK) analysis of the combination

The results thus far confirm that combinations of topotecan and doxorubicin can produce additive or synergistic interactions in selected ovarian cancer cell lines and that these interactions are maintained or increased with increasing exposure time. These data supported the further development of combinations of topotecan and doxorubicin *in vivo*, and the studies described here have explored the use of topotecan in combination with a LNP formulation of doxorubicin that has already been approved for use in the treatment of relapsed ovarian cancer. Recently developed liposomal formulation of topotecan (Topophore C^{TM}) as discussed in chapter 3 of this thesis, as well as some of the other LNP formulations of topotecan, extend the circulation lifetime of the associated drug and help to maintain the drug in its therapeutically active lactone form (Drummond, Noble et al.; Liu, Honget al. 2002). The extended circulation lifetime is associated with an increase in delivery of the drug to sites of tumor growth and this includes regions such as the peritoneal cavity (Harasym, Cullis et al. 1997). Topophore C™ was selected for the *in vivo* studies here, but prior to evaluating efficacy it was important to assess whether co-

administration of the various drug combinations affected the pharmacokinetics of the drugs compared to when the drugs were used alone.

The combinations to be tested included free topotecan and Doxil® as well as Topophore C™ and Doxil®. For this reason plasma elimination was assessed (see Methods) following a single i.v. dose in mice of Doxil® (7.5 mg/kg) alone, free topotecan (5 mg/kg) or Topophore C™ (5 mg/kg). These data were compared to studies where the drugs were administered in combination (Figure 4.5A, 4.5B and Figure 4.6). Results indicated that co-administration of Doxil® had a small impact on the plasma elimination profile of free topotecan (Figure 4.5A). The measured drug levels at 1 and 4 h were significantly ($p < 0.05$) greater than those achieved when topotecan was administered alone. This is reflected in a small increase in topotecan $AUC_{0\text{-}24h}$ from 15 µg.h/mL to 70 µg.h/mL. This could be explained by an association of free topotecan with the long circulating LNP Doxil®. The plasma elimination of Topophore C™ was not changed when co-administered with Doxil® (Figure 4.5B). Doxil® when administered as a single agent, the associated active ingredient doxorubicin showed bi-phasic plasma elimination with an initial $t_{(1/2)distribution}$ of 0.36 h corresponding rapid tissue distribution and second $t_{(1/2)elimination}$ of 36 h corresponding to an extended terminal elimination phase (Figure 4.6). These observations were consistent with the earlier reports (Gabizon, Shmeeda et al. 2003). Co-administration of Doxil® with either free Topotecan or Topophore C™ resulted in faster first phase elimination with 30% of initial doxorubicin concentration remaining in the plasma after 1 hour compared to that of 50% when administered as single agent (Figure 4.6). Doxil elimination rate after first hour was not affected when the drug was co-administered with the topotecan formulations. It was possible that the more rapid initial elimination of doxorubicin following administration of Doxil® is a result of topotecan mediated increased doxorubicin release from the LNP. To assess this, an *in*

vitro assay was conducted to measure drug loss from the Doxil® formulation upon incubation with free topotecan or Topophore C™ (in PBS buffer at 37°C for 1 hour). Free drug was separated from liposomes using Sephadex G-50 spin columns (standardized to minimize the elution variability) as described in methods for Topophore C™ preparation and liposomes were then analyzed for doxorubicin concentration as described before (see Methods). Results were compared to those obtained with Doxil® alone which was subjected to similar incubation and treatment. These data, (summarized in the insert to Figure 4.6) indicated that under the conditions used, there was no loss of LNP associated doxorubicin when Doxil® was mixed with free topotecan or liposomal topotecan.

4.4.3 *In vivo* efficacy in two models of ovarian cancer

Antitumor activity of single agents or combination treatments was determined *in vivo* using two different pseudo-orthotopic models of ovarian carcinoma. As indicated in the Methods, these ES-2 or SKOV-3 luc-D3 cells were inoculated i.p. and tumor progression (SKOV3) or tumor related morbidity (ES2 cells) were monitored as a function of time following treatment. Treatments were given i.v. using a Q7D x 3 schedule. Dose response curves were generated for treatment groups in ES-2 model where overall survival (OS) was used as an indicator of efficacy and tumor growth and associated ascites development were observed rapidly when animals were left untreated. The median survival time for control animals was 18 days from the day of tumor inoculation with more than 80% of mice terminated by day 21 due to tumor progression (Table 4.2). Animals treated with free topotecan showed improved OS over control group, but the dose effect observed was small. For example free topotecan administered at 5 mg/kg resulted in a 57% increase in median survival time (MST of 29 days) (Table 4.2A). This increased to 76% at 15 mg/kg (MST of 32 days, $P<0.005$) and a further increase in

topotecan dose to 20 mg/kg did not show any further improvement in OS and at this dose, some health status issues like decreased motor activity, dry skin were observed. Therefore, although the 20 mg/kg free topotecan dose was tolerated, 15 mg/kg was used for the drug combination studies with Doxil® because it was the most therapeutically efficacious dose. As noted in a chapter 2 Topophore C™ was more toxic than free topotecan and the maximum tolerated dose of Topophore C™ was 7.5 mg/kg, at least 2-fold lower that the dose that can be achieved with free drug. Treatment with Topophore C™, however, provided significantly better activity when compared to free topotecan when administered at equitoxic doses. The MST of mice treated with Topophore C™ at 2.5 mg/kg was 39 days, representing a 114% increase in median lifespan when compared to controls ($P<0.001$) and more than a 1.5-fold improvement in activity when compared to results obtained with free topotecan administered at 15 mg/kg. Similar to free topotecan, there was no significant dose response curve and when Topophore C™ was administered at 7.5 mg/kg (the MTD) the MST was 42 days, representing a 127% increase in median lifespan over controls. The maximum efficacious dose of Topophore C™ was 5 mg/kg and this dose was considered appropriate for the drug combination studies. It is notable that Doxil®, when used as a single agent at its maximum tolerated dose (MTD) of 7.5 mg/kg, exhibited no measurable therapeutic activity (Table 4.2A). Insensitivity to Doxil® was consistent with previous reports indicating that clear cell carcinoma (e.g. ES-2) is an aggressive and chemo-refractory subtype of ovarian cancers (Goff, Sainz de la Cuesta et al. 1996; Sugiyama, Kamura et al. 2000; Pather and Quinn 2005; Pectasides, Pectasides et al. 2006; Crotzer, Sun et al. 2007).

For the drug combination studies Doxil® was administered at its maximum tolerated dose (7.5 mg/kg) and it was co-administered with increasing doses of free topotecan or Topophore C™. The dose escalation studies were conducted to establish whether there was an increase in

toxicity when the drugs were used in combination. When Doxil® was combined with free topotecan given at the 5 mg/kg dose the median survival time was 31 days (Table 4.2B), demonstrating a small, but not significant increase in activity when compared to use of free topotecan alone. Increases in MST were observed when the dose of topotecan was escalated to 15 mg/kg, where the MST was 37 days when compared to 32 days achieved following administration of topotecan alone at the 15 mg/kg dose. There was some evidence of enhanced toxicity when these drugs were used in combination. For example, significant weight loss (10 to 15%) was noted following the third dose of the free topotecan-Doxil® combination (15 mg/kg and 7.5 mg/kg, respectively) but the mice recovered within one week. This was in dramatic contrast to studies completed with Doxil® and Topophore C™, where there was a significant increase in drug related morbidity. For example combinations of Topophore C™ and Doxil® were not tolerated when dosed at 5 mg/kg and 7.5 mg/kg, respectively and the combination produced a greater than expected (synergistic) toxicity that was reflected in significant weight loss (up to 25%) and the need to terminate animals due to poor health status. This is reflected in the data shown in Table 4.2B, where the median survival time of these animals was not significantly different from controls. The combination of Doxil® and Topophore C™, however exhibited significant therapeutic benefits when the animals were treated with well tolerated doses. When treated with 0.625 mg/kg Topophore C™ in combination with Doxil® (7.5 mg/kg) the MST was 28 days. Escalation of the Topophore C™ dose to 2.5 mg/kg resulted in significant improvements ($P<0.001$ vs. control) in treatment outcomes and MST of 52 days was noted. When Topophore C™ was used alone at the 2.5 mg/kg dose the MST noted was only 39 days. Given the lack of activity of Doxil® used as a single agent this provides strong evidence that the combination of the LNPs is synergistic. The efficacy results summarized in Table 4.2

have also been presented in the form of Kaplan Meier survival plot (Figure 4.7) to highlight the improvements in treatment outcomes achievable with this combination.

In contrast to the ES-2 model, disease progression in the SKOV-3 ovarian cancer model is slow and for this reason tumor progression in this model was assessed using bioluminescent imaging (see Methods). The tumor burden after inoculation of SKOV-3 luc-D3 cells was estimated by assessing increase in luminescent light emitted (photos/second) by the luciferase modified cells following luciferin injection (see Methods). Mice with established tumors were organized into treatment groups defined on the basis of data obtained using the ES-2 tumor model and these data have been summarized in Figure 4.8. Representative images for each treatment group used have been provided in this figure and these demonstrated that the SKOV-3 luc-D3 cells could be visualized even one day after tumor cell inoculation. Treatment was initiated 7 days after cell inoculation and imaging was completed every 7 days. The results obtained 28 days after tumor cell inoculation (the day when the last treatment was provided) and 42 days after tumor inoculation are provided. Control mice exhibited a steady increase in bioluminescence over the 42 day time course, where the increase in signal intensity from day 1 to day 42 was 580% (see histogram in Figure 4.8). When the mice were treated with free topotecan they exhibited, an average, a 131% increase in the bioluminescent signal, suggesting the topotecan was therapeutically active in this model. Topophore C™ (2.5 mg/kg) treated mice showed only 39% increase in signal on day 42. These data are consistent with the results obtained using the ES-2 model where Topophore C™ was therapeutically superior to free topotecan. In contrast to the results obtained with the ES-2 model, Doxil® (7.5 mg/kg) exhibited good therapeutic effects. In this treatment group there was a 92% increase in bioluminescence signal on day 42. When this effective dose of Doxil® was combined with free topotecan (15

mg/kg) there was a 139% increase in signal on day 42 which was comparable or slightly higher than that observed when animals were treated with free topotecan or Doxil® alone. Combination of Doxil® and Topophore C™ proved to be most effective, where only 6% increase in bioluminescence signal was noted on day 42. No significant weight loss or other treatment related toxicities were observed in any of the treatment groups.

4.5 DISCUSSION

Approaches to define effective combinations in the clinic have not taken into consideration a number of pharmacokinetic factors that could influence drug-drug interactions that result in improved treatment effects (Hanahan, Bergers et al. 2000; Kerbel and Folkman 2002; Harasym T. O. 2006). A variety of *in vitro* approaches have been used to determine the drug-drug interactions in cell culture systems (Carter and Wampler 1986; Berenbaum 1989; Zoli, Ricotti, Tesei, Barzanti and Amadori 2001); however the use of this type of *in vitro* data to predict synergy *in vivo* remains challenging, mainly because drug pharmacology and pharmacokinetics of different drugs cannot be adequately mimicked in the *in vitro* setting. In addition it is well understood that the *in vitro* conditions do not adequately represent the microenvironments seen within tumors. These concerns need to be addressed if the therapeutic potential of selected combinations are going to be realized in patients. *In vitro* assays, although limited, have already demonstrated that drug:drug synergy (or antagonism) is influenced by drug dose (as represented by measured effect level) and drug:drug ratio. These data have profound implications particularly since drugs are typically combined *in vivo* under conditions where drug dose and drug:drug ratio cannot be controlled. In recent studies it was observed that surprising improvements in therapeutic activity can be achieved by controlling the ratios of combinations of various drug combinations (irinotecan/floxuridine, daunorubicin/cytarabine or

cisplatin/daunorubicin) *in vivo* through use of well designed LNPs. In reference to the concept of surprising, efficacy of select combinations could be achieved at doses that are far lower than those required to achieve similar effects with the free drug (Mayer, Harasym et al. 2006).

The results obtained with carefully designed drug combination products are compelling, however these formulations rely on the use of drug delivery formulations which can control the drug:drug ratio within the plasma compartment and the tumor, but in addition these formulations extend the circulation lifetime of the associated drugs and benefits may arise simply as a result of these extended exposure times. This is an important point to consider, given that many *in vitro* assays rely on endpoints determined 3 to 5 days after drug addition, yet *in vivo* the drug exposure time may be considerably shorter depending on the plasma elimination rate of the administered drugs. The studies described in this report were designed to assess whether drug exposure time influenced treatment outcomes for a combination that appears to have some therapeutic potential for treatment of relapsed ovarian cancer. The cell viability assays completed with three ovarian cancer cell lines (ES-2, SKOV-3 and OVCAR-3) have demonstrated, not surprisingly, that prolonged exposure of topotecan and doxorubicin as single agents enhanced therapeutic effects. This effect can be attributed to the cell cycle specific nature of the drugs used. Topotecan and doxorubicin are known to produce greater effects during the *s*-phase of the cell cycle (Tewey, Rowe et al. 1984; Burden and Osheroff 1998; Bailly 2000), thus greater cytotoxicity observed on prolong exposure of these drugs to the cells would be a consequence of a greater proportion of cells entering in the *s*-phase. Topotecan is thought to be a more *s*-phase specific drug when compared to doxorubicin as doxorubicin is known to produce its activity by multiple mechanisms (Tewey, Rowe et al. 1984; Burden and Osheroff 1998; Minotti, Menna et al. 2004)

and this drug exhibited a greater dependency on exposure time than doxorubicin (see Figure 4.1 and Figure 4.2).

The enhanced treatment effects noted with increasing exposure time were also observed with the drugs used in combination (see Figure 4.4). These drug combination data were analyzed using the median effect principle (MEP) developed by Chou and Talalay (Chou 2006) to determine if synergistic interactions are increased or decreased as a function of exposure time. It should be noted that the MEP methodology is built around the concept that combination effects need to be studied at fixed drug ratios and that these effects must be determined over a broad range of effective doses (Chou 1991; Chou 2006; Waterhouse, Gelmon et al. 2006). Studies summarized here used fixed molar ratio (IC50 of doxorubicin/IC50 of topotecan, determined at 72 h, see Table 4.1) and as recommended this ratio was tested over a range of effective doses. However, it is obvious from the data plotted in Figure 4.1 and Figure 4.2 that this ratio would likely change depending on the exposure time used. This highlights the complexity of these studies, however the ratio used can be justified on the basis that the accuracy of the MEP in determining drug:drug interactions is greatest when the effect level measured is 0.5 (i.e. the IC_{50} dose). The results summarized here do suggest that drug:drug interactions as assessed by the MEP are influenced by exposure time, but this is exemplified primarily when comparing results obtained at 1 h or 4 h to those obtained at 72 h. Further the interactions noted were different for the cell lines studied. However; importantly, in all examples the combination appeared to produce additive or synergistic effects and the therapeutic activity measured using the MTT assay was always greatest when the exposure time was extended.

LNP formulations can be used as an effective means to achieve extended drug exposure *in vivo* and this is reflected in increased levels of drug in the blood compartment over time as

well as enhanced delivery to sites of tumor growth (Bally, Mayer et al. 1988; Bally, Nayar, et al. 1990; Sadzuka, Hirotsu et al. 1998; Emerson, Bendele et al. 2000; Batist, Ramakrishnan et al. 2001; Drummond, Noble et al. 2008). When evaluating drug exposure following administration of LNPs, two factors must be considered. First the drug measured in the plasma compartment is primarily present in a form that is associated with the circulating LNPs. For this reason drug exposure can be dependent on the elimination rate of the LNP. Second, in the absence of any surface features facilitating targeting to specific cell populations, the LNP associated drug must dissociate from the formulation in order to be active. Drug dissociation rates are controlled by the composition used when preparing the LNP as well as the mechanism through which the drug is associated with the LNPs. Thus LNP formulations provide a simple method of increasing drug exposure in tumors. Doxil® and Topophore C™ formulations have previously demonstrated these abilities and therefore were used in the studies here to increase the exposure of doxorubicin and topotecan to the tumor site. The approach used is reliant on a clinical development plan for Topophore C™ that would involve a phase II study comparing the effectiveness of topotecan vs Topophore C™ and subsequently a study evaluating combinations of Doxil®, which is already approved for use in the treatment of relapsed ovarian cancer, and Topophore C™. This approach is more conservative than one that would evaluate the combination incorporated in to one delivery system. This latter approach has an advantage with respect to the ease of administration or compliance. However, challenges associated with this approach are effective loading of two drugs and designing formulations capable of retaining multiple agents in one delivery vehicle. Differences in physico-chemical properties of the different drugs, limited flexibility in terms of dose adjustment, stringent regulatory norms to get regulatory approval for such products makes this approach more difficult, albeit efforts have resulted in several drug combination products

that show significant promise in the clinic (Gill, Wernz et al. 1996; Ozols, Bundy et al. 2003; Chan, Davidson et al. 2004; Penson, Seiden et al. 2005; Baek, Kim et al. 2006; Ghesquieres, Faivre, et al. 2006; Mahany, Lewis et al. 2009).

The drug combination effects observed when using combinations of topotecan and doxorubicin *in vitro* were reflected in the *in vivo* efficacy studies. Combination treatments involving LNPs demonstrated superior therapeutic efficacy over single agents in both ovarian cancer models evaluated. Doxil®/Topophore C™ combinations were therapeutically superior than the other combinations tested. However enhanced therapeutic effects of this combination were also reflected in an increase in toxicity likely due to synergism between the two drugs on normal proliferating cell populations in the GI tract and hematopoietic system. The studies presented provided preclinical evidence to support the use of combinations comprising a LNP formulation of topotecan and Doxil®, but the results also suggested that combinations of free topotecan and Doxil® may provide limited if any therapeutic benefit when compared to the agents used alone. This will eventually be determined in patients.

4.6 CONCLUSION

Cytotoxic activity of topotecan and doxorubicin against ovarian cancer cell lines was observed to be exposure time dependent. Synergistic interactions observed between topotecan and doxorubicin *in vitro* were translated very well *in vivo* and the results suggested that this synergy may best be achieved under conditions when the tumor cells are exposed to both drugs over extended time periods. Concurrent administration of Doxil® with Topophore C™ proved to be effective when used to treat ovarian cancer xenografts models. These results provided a proof of concept data to support the use of this combination for treatment of recurrent ovarian cancer.

Table 4.1: IC_{50} values of topotecan and doxorubicin as assessed in ovarian cancer cell lines ES-2, SKOV-3 and OVCAR-3.

	ES-2		**OVCAR-3**		**SKOV-3**	
	Topotecan	Doxorubicin	Topotecan	Doxorubicin	Topotecan	Doxorubicin
IC50	83 nM	539 nM	63 nM	497 nM	18 nM	251 nM

Table 4.2: Anti-tumor activity of Free topotecan, Topophore C™ and Doxil® in nude mice bearing ES-2 clear cell carcinoma xenografts following i.v. administration of free topotecan, Topophore C™ or Doxil® as (A) single agents, and (B) as combination treatments.

Treatment	Drug Dose (mg/kg)	Median Survival (days)	% ILS[a]
Saline (Control)	-	18.5	N/A
Free topotecan	5	29	57
Free topotecan	7.5	30.5	65
Free topotecan	15	32.5	76
Free topotecan	20	28	51
Topophore C	2.5	39.5	114
Topophore C	5	44	138
Topophore C	7.5	42	127
Doxil	7.5	19	3

Treatment	Drug Dose (mg/kg)	Median Survival (days)	% ILS[a]
Saline (Control)	-	20.3	N/A
Free topotecan + Doxil	5 + 7.5	31.5	55
Free topotecan + Doxil	10 + 7.5	37	82
Free topotecan + Doxil	15 + 7.5	37.3	84
Topophore C + Doxil	0.625 + 7.5	28	38
Topophore C + Doxil	1.25 + 7.5	43	112
Topophore C + Doxil	2.5 + 7.5	52	156
Topophore C + Doxil	5 + 7.5	20	-

[a] Percentage ILS (Increase in Life Span): values were determined from median survival times of treated and control (untreated) groups.

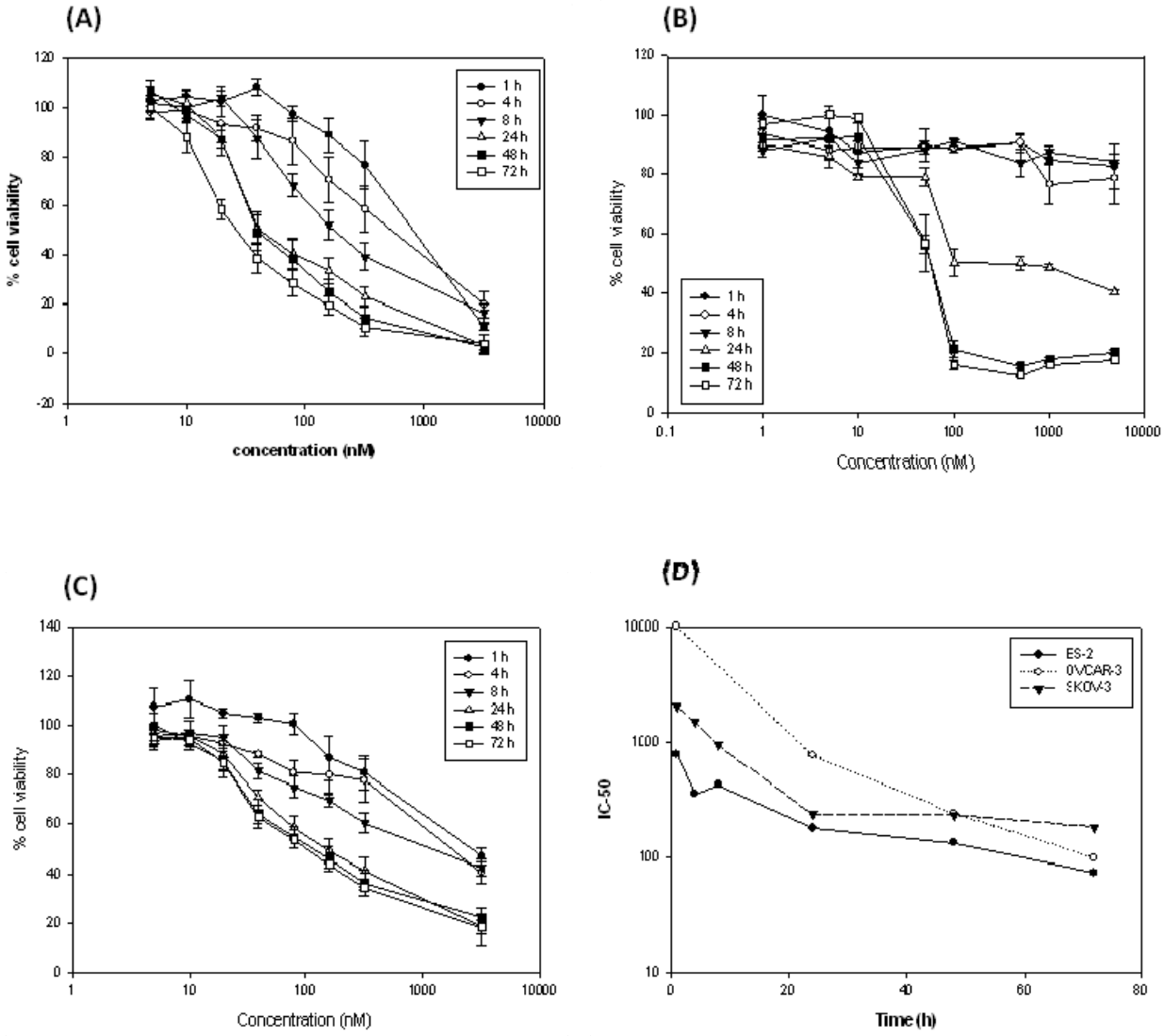


Figure 4.1: Effect of exposure time on the cytotoxicity (MTT assay endpoint) of topotecan against (A) ES-2, (B) OVCAR-3 and (C) SKOV-3 cell lines. Values indicate mean ± SE of at least three individual experiments. (D) Effect of exposure time on the IC_{50} of topotecan against ES-2, OVCAR-3 and SKOV-3 cells..

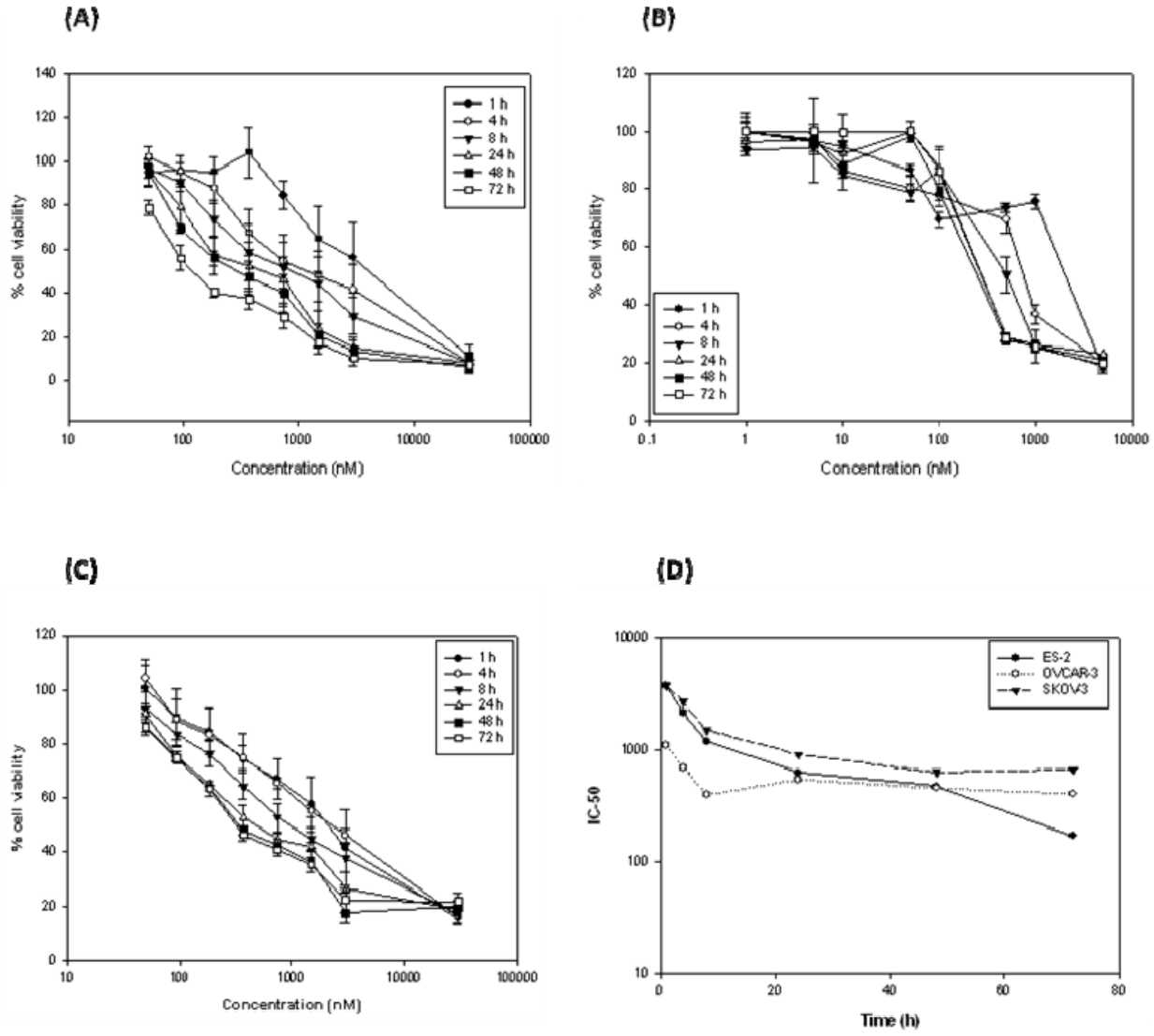


Figure 4.2: Effect of exposure time on the cytotoxicity (MTT assay) of doxorubicin against (A) ES-2, (B) OVCAR-3 and (C) SKOV-3 cell lines. Values indicate mean ± SE of at least three individual experiments. (D) Effect of exposure time on the IC_{50} of doxorubicin against ES-2, OVCAR-3 and SKOV-3 cells.

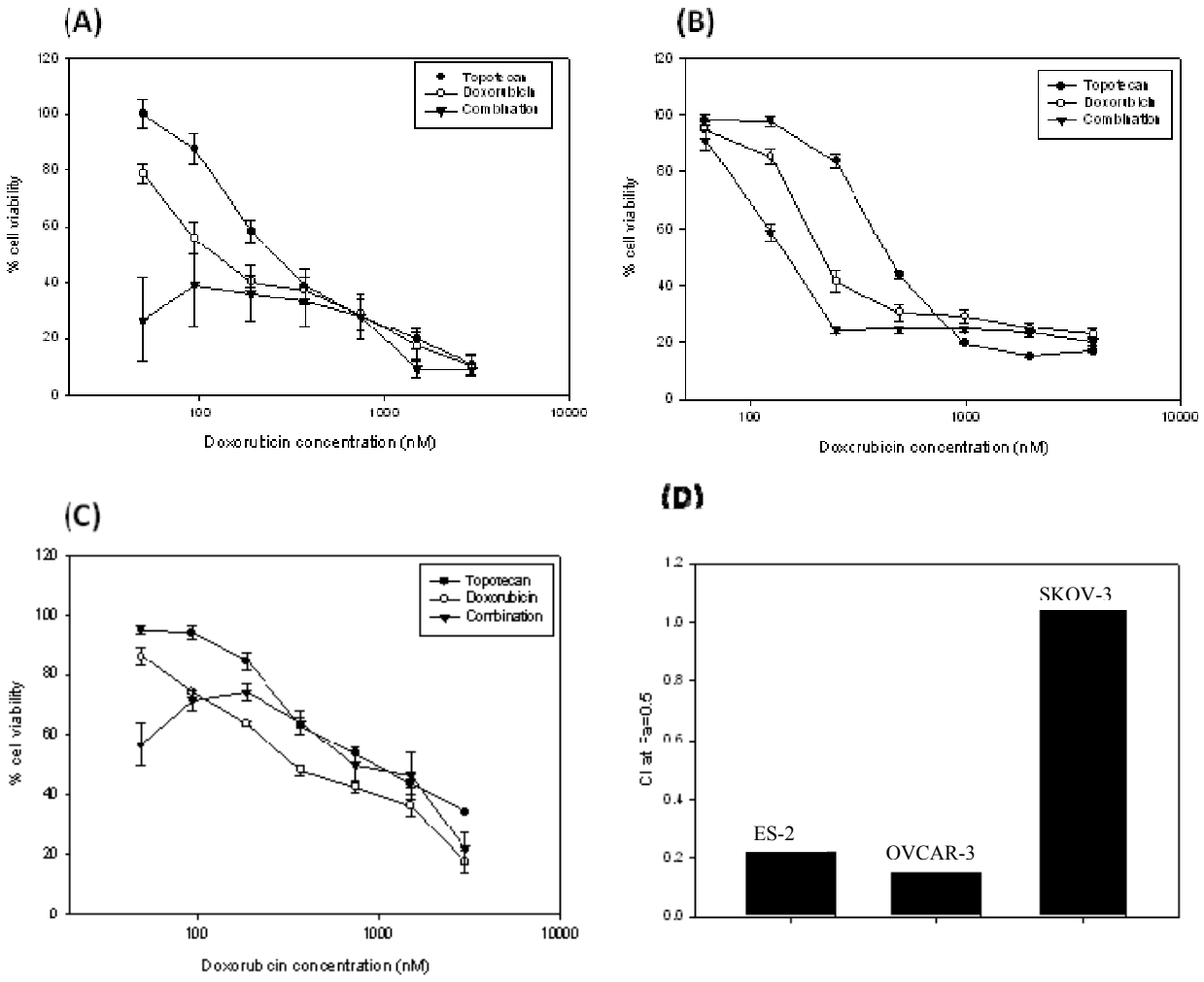


Figure 4.3: Dose response curves for topotecan and doxorubicin as single agents and as combination when tested on ovarian cancer cell lines; (A) ES-2, (B) OVCAR-3 and (C) SKOV-3. Values indicate mean ± SE of at least three individual experiments. (D) represent combination indices (CI) for topotecan-doxorubicin combination (IC_{50}:IC_{50}) at 50% effect level (Fa = 0.5) as calculated using CompuSyn program.

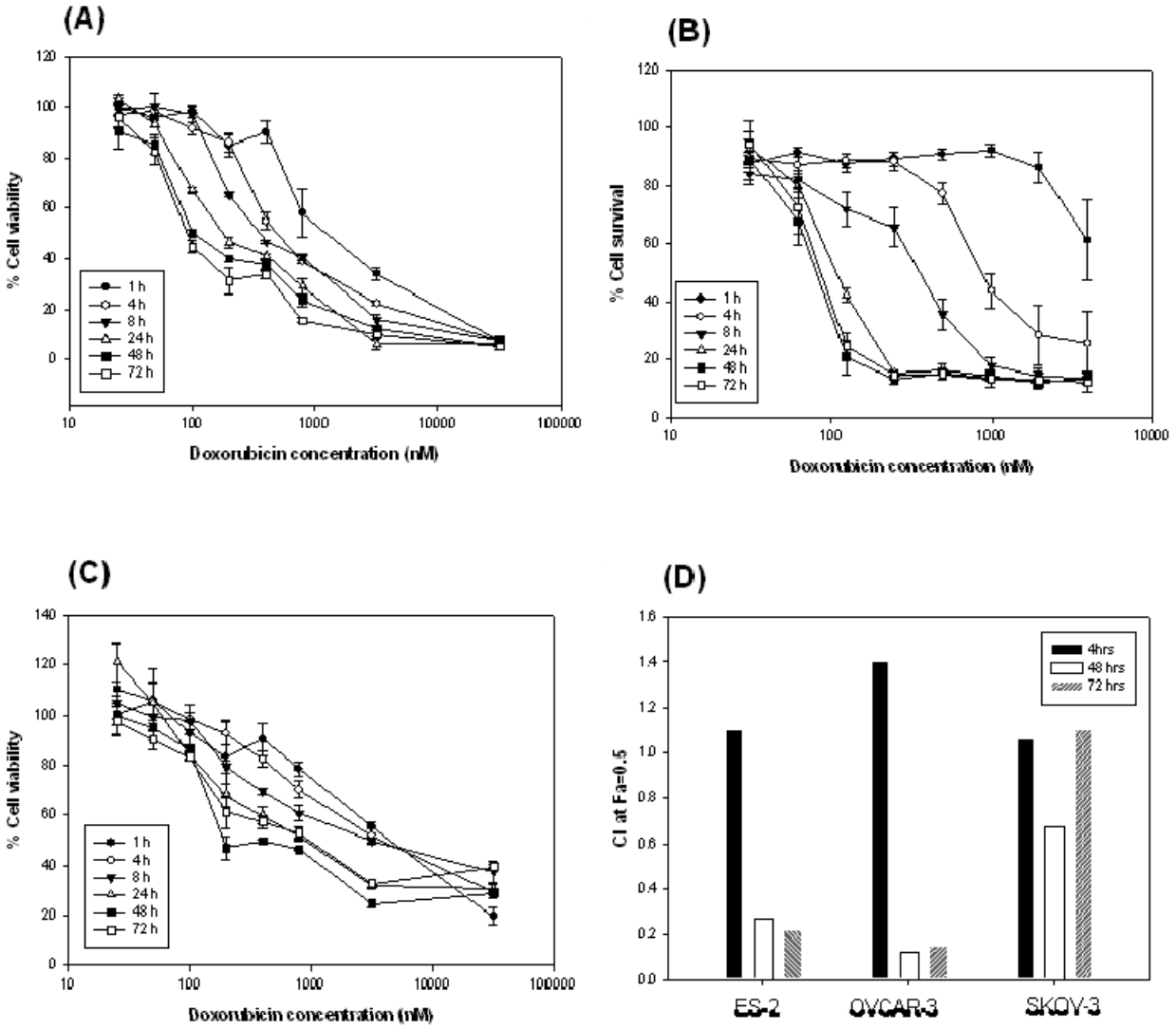
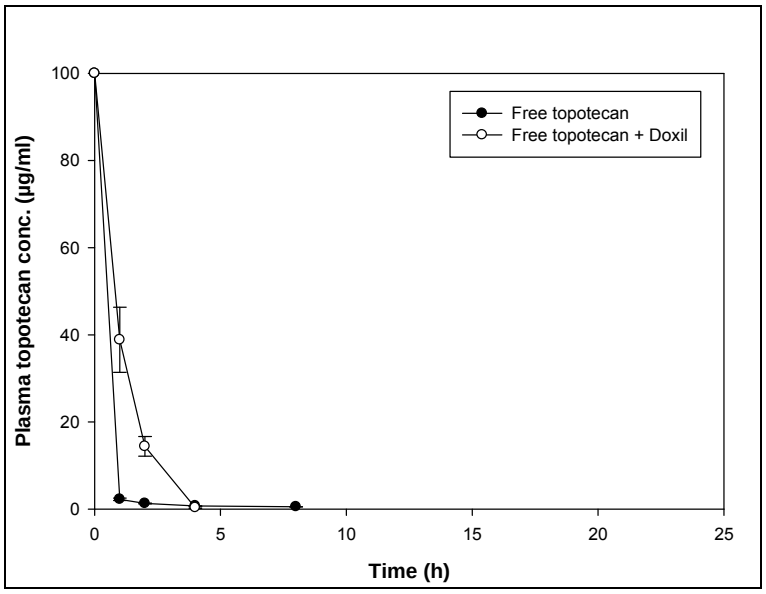


Figure 4.4: Effect of exposure time on the cytotoxicity of topotecan and doxorubicin when used in combination against (A) ES-2, (B) OVCAR-3 and (C) SKOV-3 cell lines. Values indicate mean ± SE of at least three individual experiments. (D) represent combination indices (CI) for topotecan-doxorubicin combination (IC_{50}:IC_{50}) at 50% effect level (Fa = 0.5) as calculated using CompuSyn program.

(A)



(B)

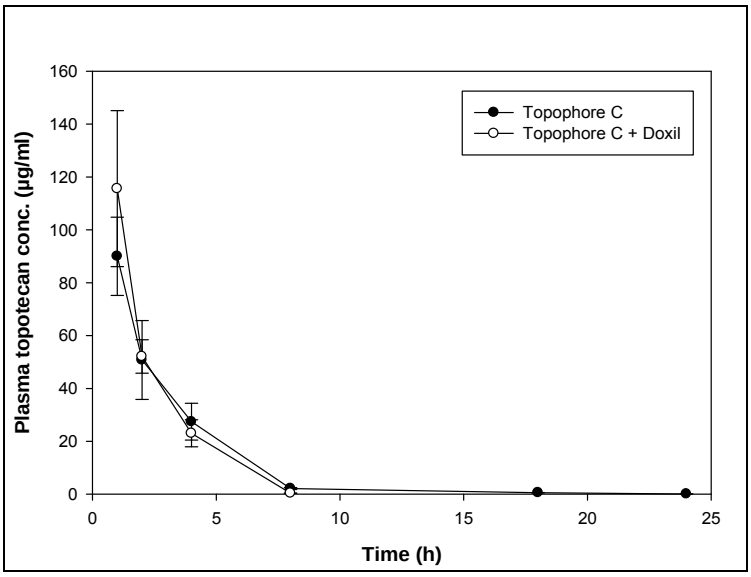


Figure 4.5: Plasma elimination profile of topotecan and doxorubicin following i.v. administration of single dose of (A) free topotecan (5 mg/kg) or free topotecan + Doxil (5 + 7.5) mg/kg and (B) Topophore C (5 mg/kg) or Topophore C + Doxil (5 + 7.5) mg/kg to female mice. Values indicate mean ± SD, n = 4.

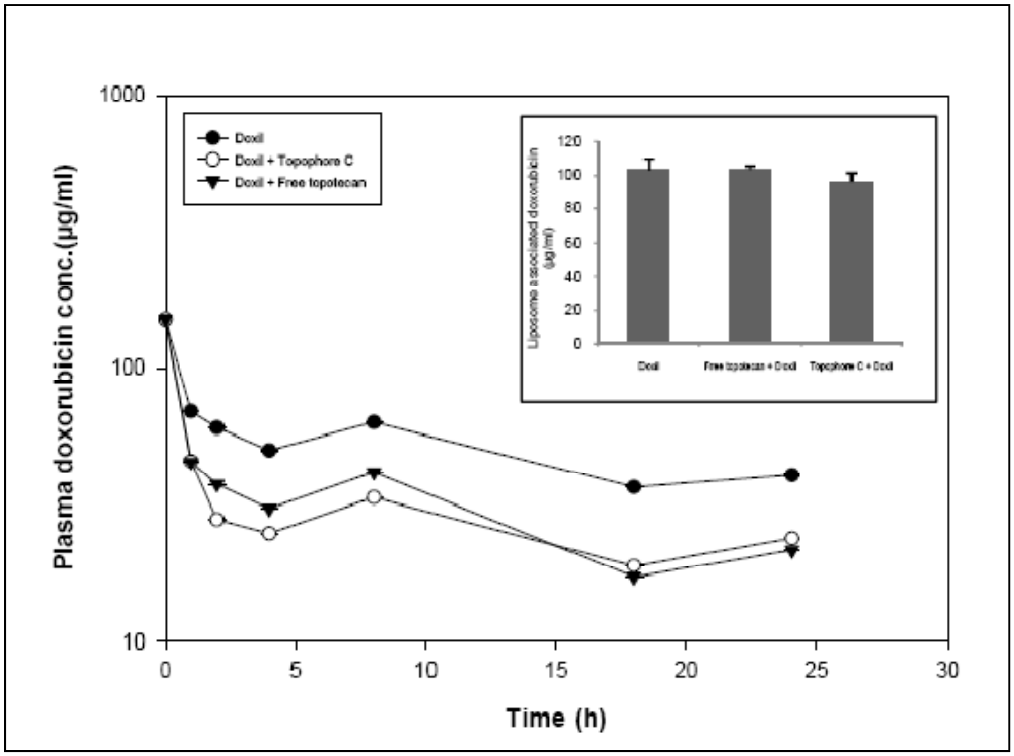


Figure 4.6: Plasma elimination profile of doxorubicin following i.v. administration of single dose of (A) Doxil (7.5 mg/kg) alone or in combination with either free topotecan (15 mg/kg) or Topophore C (5 mg/kg) to female mice. Values indicate mean ± SD, n = 4. Inserted figure indicate amount of doxorubicin retained by doxorubicin LNP (Doxil) following incubation with free topotecan or Topophore C in PBS at 37°C/1h. Values indicates mean ± SD of three separate measurements.

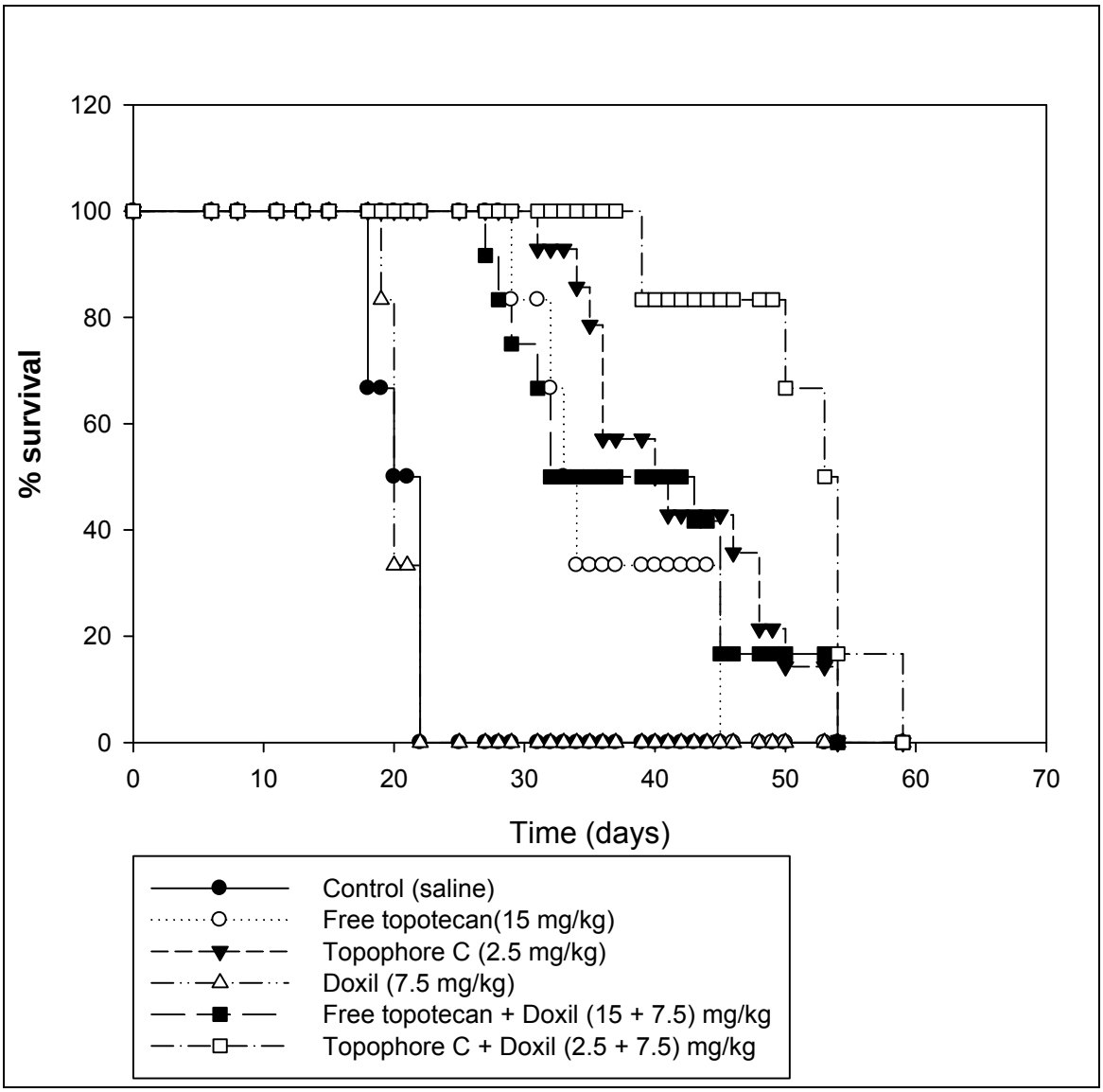


Figure 4.7: Kaplan-Meier survival plot of Ncr-Nude mice bearing intraperitoneally grown ES-2 clear cell carcinoma xenografts after i.v treatment (q7d x 3) with free topotecan, Topophore C and Doxil either as single agents or in combination as shown. Data points represent mean ± SD, ($n \geq 6$).

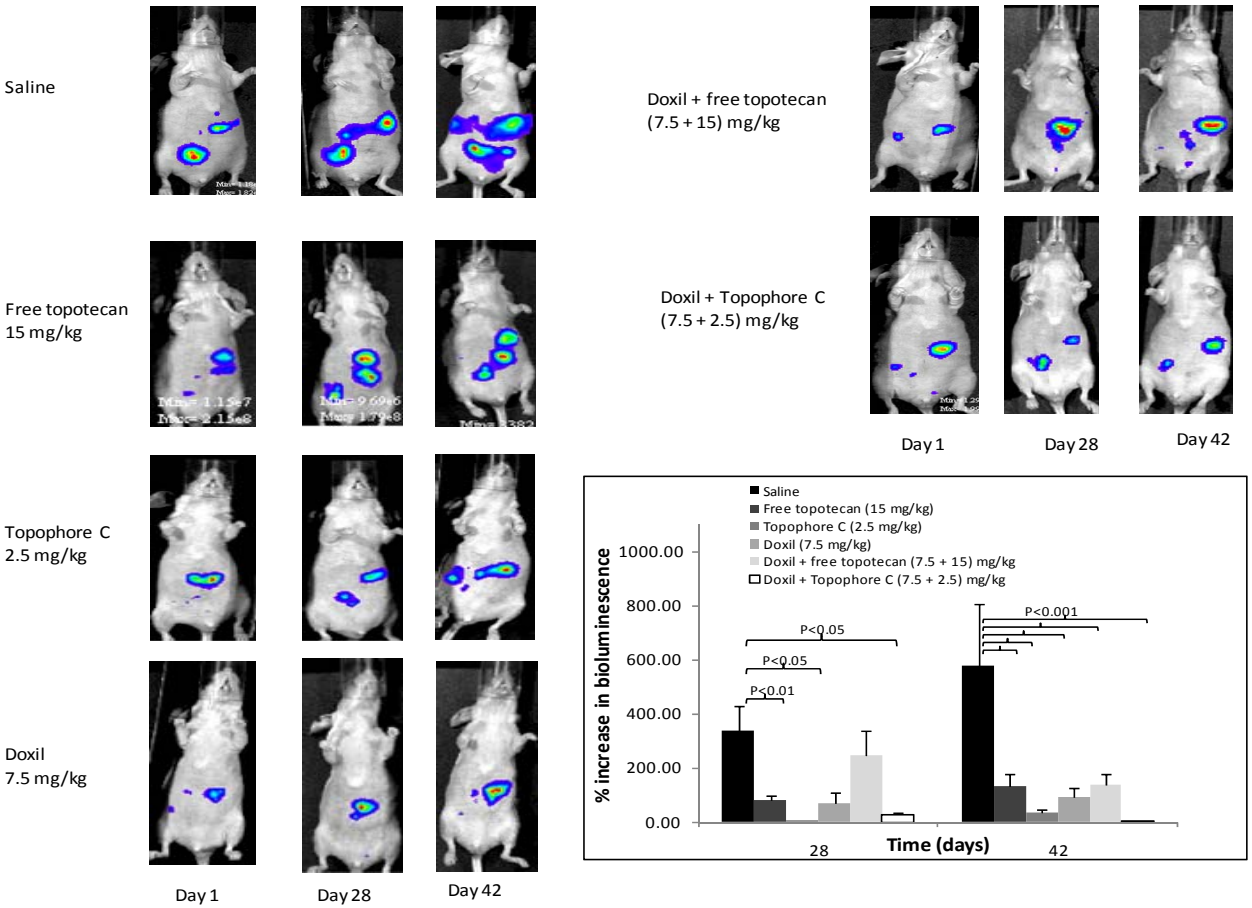


Figure 4.8: Tumor progression as determined by increase in bioluminescent signal in mice bearing intraperitoneally grown SKOV-3 serous adenocarcinoma xenografts. Mice were inoculated with luciferase modified SKOV-3 cells on day 0 and tumor measurements were conducted once a week following i.p. injection of luciferin solution. Treatment groups were administered intravenously on day 7 (q7d x3) with either free topotecan (15 mg/kg) or Topophore C (2.5 mg/kg,) or Doxil (7.5 mg/kg) either as single agents or in combination as shown and control mice were administered equivalent volume of saline. Data points represent mean ± SD (n = 6).

4.7 REFERENCES

Agarwal, R., Linch, M. and Kaye, S. B. (2006). "Novel therapeutic agents in ovarian cancer." Eur J Surg Oncol **32**(8): 875-86.

Ahmad, T. and Gore, M. (2004). "Review of the use of topotecan in ovarian carcinoma." Expert Opin Pharmacother **5**(11): 2333-40.

Baek, J. H., Kim, J. G., Jeon, S. B., Chae, Y. S., Kim, D. H., Sohn, S. K., Lee, K. B., Choi, Y. J., Shin, H. J., Chung, J. S., Cho, G. J., Jung, H. Y. and Yu, W. (2006). "Phase II study of capecitabine and irinotecan combination chemotherapy in patients with advanced gastric cancer." Br J Cancer **94**(10): 1407-11.

Bailly, C. (2000). "Topoisomerase I poisons and suppressors as anticancer drugs." Curr Med Chem **7**(1): 39-58.

Bally, M. B., Mayer, L. D., Loughrey, H., Redelmeier, T., Madden, T. D., Wong, K., Harrigan, P. R., Hope, M. J. and Cullis, P. R. (1988). "Dopamine accumulation in large unilamellar vesicle systems induced by transmembrane ion gradients." Chem Phys Lipids **47**(2): 97-107.

Bally, M. B., Nayar, R., Masin, D., Hope, M. J., Cullis, P. R. and Mayer, L. D. (1990). "Liposomes with entrapped doxorubicin exhibit extended blood residence times." Biochim Biophys Acta **1023**(1): 133-9.

Batist, G., Ramakrishnan, G., Rao, C. S., Chandrasekharan, A., Gutheil, J., Guthrie, T., Shah, P., Khojasteh, A., Nair, M. K., Hoelzer, K., Tkaczuk, K., Park, Y. C. and Lee, L. W. (2001). "Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer." J Clin Oncol **19**(5): 1444-54.

Berenbaum, M. C. (1989). "What is synergy?" Pharmacol Rev **41**(2): 93-141.

Burden, D. A. and Osheroff, N. (1998). "Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme." Biochim Biophys Acta **1400**(1-3): 139-54.

Carter, W. H., Jr. and Wampler, G. L. (1986). "Review of the application of response surface methodology in the combination therapy of cancer." Cancer Treat Rep **70**(1): 133-40.

Chan, S., Davidson, N., Juozaityte, E., Erdkamp, F., Pluzanska, A., Azarnia, N. and Lee, L. W. (2004). "Phase III trial of liposomal doxorubicin and cyclophosphamide compared with epirubicin and cyclophosphamide as first-line therapy for metastatic breast cancer." Ann Oncol **15**(10): 1527-34.

Chou, T. (1991). Synergism and Antagonism in Chemotherapy. San Diego, Academic Press.

Chou, T. C. (2006). "Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies." Pharmacol Rev **58**(3): 621-81.

Chou, T. C. and Talalay, P. (1984). "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors." Adv Enzyme Regul **22**: 27-55.

Creemers, G. J., Bolis, G., Gore, M., Scarfone, G., Lacave, A. J., Guastalla, J. P., Despax, R., Favalli, G., Kreinberg, R., Van Belle, S., Hudson, I., Verweij, J. and Ten Bokkel Huinink, W. W. (1996). "Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study." J Clin Oncol **14**(12): 3056-61.

Crotzer, D. R., Sun, C. C., Coleman, R. L., Wolf, J. K., Levenback, C. F. and Gershenson, D. M. (2007). "Lack of effective systemic therapy for recurrent clear cell carcinoma of the ovary." Gynecol Oncol **105**(2): 404-8.

D'Arpa, P. and Liu, L. F. (1989). "Topoisomerase-targeting antitumor drugs." Biochim Biophys Acta **989**(2): 163-77.

D. N.Waterhouse, J. K. (2008). Nanotechnology as an Enabling Approach to the Development of Fixed-Dose Combination Products for Treating Cancer, American Scientific Publishers.

Dadashzadeh, S., Vali, A. M. and Rezaie, M. (2008). "The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats." Int J Pharm **353**(1-2): 251-9.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Connolly-Ingram, C., Gabriel, B. S., Hann, B., Liu, B., Park, J. W., Hong, K., Benz, C. C., Marks, J. D. and Kirpotin, D. B. "Development of a highly stable and targetable nanoliposomal formulation of topotecan." J Control Release **141**(1): 13-21.

Drummond, D. C., Noble, C. O., Hayes, M. E., Park, J. W. and Kirpotin, D. B. (2008). "Pharmacokinetics and in vivo drug release rates in liposomal nanocarrier development." J Pharm Sci **97**(11): 4696-740.

Dupont, J., Aghajanian, C., Andrea, G., Lovegren, M., Chuai, S., Venkatraman, E., Hensley, M., Anderson, S., Spriggs, D. and Sabbatini, P. (2006). "Topotecan and liposomal doxorubicin in recurrent ovarian cancer: is sequence important?" Int J Gynecol Cancer **16 Suppl 1**: 68-73.

Emerson, D. L., Bendele, R., Brown, E., Chiang, S., Desjardins, J. P., Dihel, L. C., Gill, S. C., Hamilton, M., LeRay, J. D., Moon-McDermott, L., Moynihan, K., Richardson, F. C., Tomkinson, B., Luzzio, M. J. and Baccanari, D. (2000). "Antitumor efficacy, pharmacokinetics, and biodistribution of NX 211: a low-clearance liposomal formulation of lurtotecan." Clin Cancer Res **6**(7): 2903-12.

Frei, E. (1991). Clinical studies of combination therapy for cancer. San Diego, Academic Press.

Gabizon, A., Shmeeda, H. and Barenholz, Y. (2003). "Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies." Clin Pharmacokinet **42**(5): 419-36.

Ghesquieres, H., Faivre, S., Djafari, L., Pautier, P., Lhomme, C., Lozahic, S., Djazouli, K., Armand, J. P. and Raymond, E. (2006). "Phase I dose escalation study of pegylated liposomal doxorubicin (Caelyx) in combination with topotecan in patients with advanced malignancies." Invest New Drugs **24**(5): 413-21.

Gill, P. S., Wernz, J., Scadden, D. T., Cohen, P., Mukwaya, G. M., von Roenn, J. H., Jacobs, M., Kempin, S., Silverberg, I., Gonzales, G., Rarick, M. U., Myers, A. M., Shepherd, F., Sawka, C., Pike, M. C. and Ross, M. E. (1996). "Randomized phase III trial of liposomal daunorubicin versus doxorubicin, bleomycin, and vincristine in AIDS-related Kaposi's sarcoma." J Clin Oncol **14**(8): 2353-64.

Goff, B. A., Sainz de la Cuesta, R., Muntz, H. G., Fleischhacker, D., Ek, M., Rice, L. W., Nikrui, N., Tamimi, H. K., Cain, J. M., Greer, B. E. and Fuller, A. F., Jr. (1996). "Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease." Gynecol Oncol **60**(3): 412-7.

Gordon, A. N., Fleagle, J. T., Guthrie, D., Parkin, D. E., Gore, M. E. and Lacave, A. J. (2001). "Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan." J Clin Oncol **19**(14): 3312-22.

Hanahan, D., Bergers, G. and Bergsland, E. (2000). "Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice." J Clin Invest **105**(8): 1045-7.

Harasym T. O., T. P. G., Bally M., Janoff A (2006). *Fixed drug ratio liposome formulations of combination cancer therapeutics*. Liposome Technology. G. Gregoriadis. New York, informa healthcare. **III:** 25-48.

Harasym, T. O., Cullis, P. R. and Bally, M. B. (1997). "Intratumor distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes." Cancer Chemother Pharmacol **40**(4): 309-17.

Harasym, T. O., Tardi, P. G., Bally, M. and Janoff, A. (2006). Fixed drug ratio liposome formulations of combination cancer therapeutics. New York, informa healthcare.

Horowitz, N. S., Hua, J., Gibb, R. K., Mutch, D. G. and Herzog, T. J. (2004). "The role of topotecan for extending the platinum-free interval in recurrent ovarian cancer: an in vitro model." Gynecol Oncol **94**(1): 67-73.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M. J. (2008). "Cancer statistics, 2008." CA Cancer J Clin **58**(2): 71-96.

Jonsson, E., Fridborg, H., Nygren, P. and Larsson, R. (1998). "Synergistic interactions of combinations of topotecan with standard drugs in primary cultures of human tumor cells from patients." Eur J Clin Pharmacol **54**(7): 509-14.

Kerbel, R. and Folkman, J. (2002). "Clinical translation of angiogenesis inhibitors." Nat Rev Cancer **2**(10): 727-39.

Kurman, R. J. and Shih Ie, M. "The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory." Am J Surg Pathol **34**(3): 433-43.

Liu, J. J., Hong, R. L., Cheng, W. F., Hong, K., Chang, F. H. and Tseng, Y. L. (2002). "Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation." Anticancer Drugs **13**(7): 709-17.

Mahany, J. J., Lewis, N. and Heath, E. I. e. a. (2009). A Phase IB study evaluating BSI-201 in combination with chemotherapy in subjects with advanced solid tumors. Proc. Am. Soc. Clin. Oncol.

Main, C., Bojke, L., Griffin, S., Norman, G., Barbieri, M., Mather, L., Stark, D., Palmer, S. and Riemsma, R. (2006). "Topotecan, pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line or subsequent treatment of advanced ovarian cancer: a systematic review and economic evaluation." Health Technol Assess **10**(9): 1-132 iii-iv.

Mayer, L. D., Harasym, T. O., Tardi, P. G., Harasym, N. L., Shew, C. R., Johnstone, S. A., Ramsay, E. C., Bally, M. B. and Janoff, A. S. (2006). "Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice." Mol Cancer Ther **5**(7): 1854-63.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004). "Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity." Pharmacol Rev **56**(2): 185-229.

Ozols, R. F. (2000). "Optimum chemotherapy for ovarian cancer." Int J Gynecol Cancer **10**(S1): 33-37.

Ozols, R. F., Bundy, B. N., Greer, B. E., Fowler, J. M., Clarke-Pearson, D., Burger, R. A., Mannel, R. S., DeGeest, K., Hartenbach, E. M. and Baergen, R. (2003). "Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study." J Clin Oncol **21**(17): 3194-200.

Patankar, N., Strutt, D., Waterhouse, D. and Bally, M. (2010). "TopophoreC^{TM}: A liposomal nanoparticle formulation of topotecan for treatment of ovarian cancer. (submitted) " Journal of Controlled Release.

Pather, S. and Quinn, M. A. (2005). "Clear-cell cancer of the ovary-is it chemosensitive?" Int J Gynecol Cancer **15**(3): 432-7.

Pectasides, D., Pectasides, E., Psyrri, A. and Economopoulos, T. (2006). "Treatment issues in clear cell carcinoma of the ovary: a different entity?" Oncologist **11**(10): 1089-94.

Penson, R. T., Seiden, M. V., Matulonis, U. A., Appleman, L. J., Fuller, A. F., Goodman, A., Campos, S. M., Clark, J. W., Roche, M. and Eder, J. P. (2005). "A phase I clinical trial of continual alternating etoposide and topotecan in refractory solid tumours." Br J Cancer **93**(1): 54-9.

Ramsay, E. C., Dos Santos, N., Dragowska, W. H., Laskin, J. J. and Bally, M. B. (2005). "The formulation of lipid-based nanotechnologies for the delivery of fixed dose anticancer drug combinations." Curr Drug Deliv **2**(4): 341-51.

Sadzuka, Y., Hirotsu, S. and Hirota, S. (1998). "Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11." Cancer Lett **127**(1-2): 99-106.

Sugiyama, T., Kamura, T., Kigawa, J., Terakawa, N., Kikuchi, Y., Kita, T., Suzuki, M., Sato, I. and Taguchi, K. (2000). "Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy." Cancer **88**(11): 2584-9.

Swift, L. P., Rephaeli, A., Nudelman, A., Phillips, D. R. and Cutts, S. M. (2006). "Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death." Cancer Res **66**(9): 4863-71.

Swisher, E. M., Mutch, D. G., Rader, J. S., Elbendary, A. and Herzog, T. J. (1997). "Topotecan in platinum- and paclitaxel-resistant ovarian cancer." Gynecol Oncol **66**(3): 480-6.

Tardi, P., Johnstone, S., Harasym, N., Xie, S., Harasym, T., Zisman, N., Harvie, P., Bermudes, D. and Mayer, L. (2009). "In vivo maintenance of synergistic cytarabine:daunorubicin ratios greatly enhances therapeutic efficacy." Leuk Res **33**(1): 129-39.

ten Bokkel Huinink, W., Gore, M., Carmichael, J., Gordon, A., Malfetano, J., Hudson, I., Broom, C., Scarabelli, C., Davidson, N., Spanczynski, M., Bolis, G., Malmstrom, H., Coleman, R., Fields, S. C. and Heron, J. F. (1997). "Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer." J Clin Oncol **15**(6): 2183-93.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D. and Liu, L. F. (1984). "Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II." Science **226**(4673): 466-8.

Waterhouse, D. N., Gelmon, K. A., Klasa, R., Chi, K., Huntsman, D., Ramsay, E., Wasan, E., Edwards, L., Tucker, C., Zastre, J., Wang, Y. Z., Yapp, D., Dragowska, W., Dunn, S., Dedhar, S. and Bally, M. B. (2006). "Development and assessment of conventional and

targeted drug combinations for use in the treatment of aggressive breast cancers." Curr Cancer Drug Targets **6**(6): 455-89.

Zimmermann, G. R., Lehar, J. and Keith, C. T. (2007). "Multi-target therapeutics: when the whole is greater than the sum of the parts." Drug Discov Today **12**(1-2): 34-42.

Zoli, W., Ricotti, L., Tesei, A., Barzanti, F. and Amadori, D. (2001). "In vitro preclinical models for a rational design of chemotherapy combinations in human tumors." Crit Rev Oncol Hematol **37**(1): 69-82.

5. DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

The main objective of this thesis project was to develop a new and effective treatment strategy against recurrent ovarian cancer using a combination of chemotherapeutic drugs that are approved for use in the treatment of relapsed disease. This objective has been achieved to a significant extent after considering the overall results of the project.

The combination of Doxil® and Topophore C™ has shown significant efficacy in two different pre-clinical models of recurrent ovarian carcinoma. An important advantage of this combination is that improved therapeutic efficacy was achieved at lower and better tolerated therapeutic doses of the two agents. Results from experiments conducted in different parts of the project were discussed in detail separately in chapters 2, 3, and 4 of this thesis; the current chapter is dedicated to discussion of the key findings, limitations and possible modifications that can be applied to the future studies and overall significance of the presented work.

A broad objective of the experiments presented in chapters 2 and 3 together was to develop a lipid based nano-particulate formulation for topotecan utilizing the recently developed liposomal technology for irinotecan (Irinophore C™) (Abraham, Edwards et al. 2004; Messerer, Ramsay et al. 2004; Ramsay, Alnajim et al. 2006). Irinophore C™ has already demonstrated significant therapeutic activity in several pre-clinical cancer models and is currently being scaled up for assessment in clinical trials. If approved for clinical use, this product will be a major break-through in the field of cancer chemotherapy and a significant milestone in the area of cancer therapeutics using nanocarriers after clinical approval of Doxil® in 1995. Since formulation technology plays a vital role in the improved therapeutic outcomes achieved with Irinophore C™, it is believed that expansion of this product-specific technology to a platform technology that can be applied to other drugs will allow the utilization of several potent anti-

cancer drugs, the use of which is currently limited due to concerns such as toxicity or stability. The structural and physico-chemical similarity between irinotecan and topotecan was the basis to test the potential expansion of this technology for topotecan. Chapter 2 was dedicated to characterization of some of the crucial experimental variables associated with the drug loading process and various formulation parameters which influence the physical properties of Irinophore C™. Key outcomes from this part of the project helped in understanding the mechanistic aspects associated with the drug loading process and functional role of excipients more precisely and therefore will certainly help moving Irinophore C™ and the technology forward.

Experiments presented in chapter 3 were primarily focused on developing and optimizing topotecan LNP formulation using drug loading parameters defined for Irinophore C™. However, application of these parameters to topotecan LNP was not straightforward. Apart from modifications in experimental process variables like temperature and D/L, drug loading process for topotecan was noted to be sensitive to pH. Maintenance of neutral pH (7-7.5) during the drug loading process was very critical. This could be due to the complex ionization chemistry of topotecan. Topotecan possesses two ionisable groups; dimethylaminoethyl group has a pKa of 10.0 (Fassberg and Stella 1992) and phenolic hydroxyl group has a pKa of 6.5-7.0 (Fassberg and Stella 1992). Therefore, topotecan molecule is highly charged over a wide range of pH (particularly towards alkaline side) which makes it difficult to load through liposomal lipid membranes. As discussed in section 3.5.1 of chapter 3, this formulation was evaluated for higher drug accommodating capacity by increasing D/L. However, increasing D/L beyond 0.2 resulted in inconsistent and unstable loading (Figure 3.3 of chapter 3).

5.1.1 Potential of Topophore C^{TM}

Optimized topotecan LNP termed as Topophore C^{TM} exhibited greater than 95% encapsulation efficiency, narrow particle size distribution and good storage stability at 4ºC. In addition to these superior formulation characteristics, Topophore C^{TM} also showed better therapeutic activity compared to free topotecan in the pre-clinical models tested. Improved therapeutic activity of this formulation was attributed to altered pharmacokinetics and increased drug delivery to the tumor site. More importantly, improvement in the therapeutic activity was achieved at several folds lower dose of topotecan with no significant toxicity which makes this formulation highly suitable for use in the combination therapy that is becoming a mainstay of cancer treatment.

A combination of factors such as robust drug loading methodology, simple manufacturing method and lipid composition that has been successfully tried and tested with different small molecule drugs should allow easy translation of Topophore C^{TM} into clinic.

Recognizing the potential of topotecan, several nano-particulate drug delivery systems are currently being developed for topotecan in order to improve its therapeutic index (Drummond, Noble et al. 2010; Liu, Hong et al. 2002; Dadashzadeh, Vali et al. 2008). Although direct comparison of these systems has not been done till date as most are still in the early phase of development,

5.1.2 Limitations of Topophore C^{TM} and future improvements

In spite of definite improvement in the therapeutic index of topotecan; there is a scope for future improvements of Topophore C^{TM}. Pharmacokinetic assessments showed that Topophore C ^{TM} prolonged the plasma circulation time ($t_{1/2}$) of topotecan and also increased plasma AUC by several folds. However, these improvements were relatively small when compared to those

observed with Irinophore C™ over free irinotecan (Ramsay, Anantha et al. 2008) or Doxil® over free doxorubicin (Gustafson, Rastatter et al. 2002; Gabizon, Shmeeda et al. 2003). Relatively faster plasma clearance of liposomal topotecan is not uncommon and other liposomal formulations of topotecan that are currently under investigation have noted similar findings (Tardi, Choice et al. 2000; Liu, Hong et al. 2002). Possible reasons could be intrinsic low $t_{1/2}$ of topotecan or higher lipid membrane permeability of topotecan which is evident from its considerable oral bioavailability (23%) (De Cesare, Zunino et al. 2000). Apart from these physico-chemical characteristics of free drug, it is also important to note the difference in the administered dose of Topophore C™ and Irinophore C™. MTD of Topophore C™ in mice was noted as 5 mg/kg (equivalent to approximate lipid dose of 60 mg/kg); whereas Irinophore C™ can be administered at doses greater than 100 mg/kg (equivalent to approximate lipid dose of 500 mg/kg) to mice without significant toxicity (data not published).

It is known that significant portion of i.v. administered liposomes is taken up by macrophages and tissues of RES and thus cleared from the circulation. However, this uptake has been shown to be saturable with increased lipid doses (Proffitt, Williams et al. 1983). As described above, due to the lower MTD of Topophore C™, the dose of administered lipids is also relatively low. Therefore, lower plasma levels observed with liposomal topotecan may be the result of faster RES uptake.

One of the ways to solve this problem in future studies would be to increase the lipid dose of the product to partially saturate the RES. This can be done by simply mixing empty liposomes with topotecan loaded liposomes. However, this approach poses a risk of drug dilution. Another solution can be administering empty i.v. liposomes prior to administering the drug loaded product. However; these are only speculations and utmost care should be taken

while determining optimum lipid dose and its physiological consequences, as complete saturation of RES can result in lethally compromised immune system and therefore may cause other complications. Alternatively, plasma circulation time of liposomes can be improved by shielding them from opsonins and macrophages with the help of hydrophilic protective coating like PEG (stealth liposomes). However, benefits of PEG coating on liposomes are under debate due to issues like increased drug leakage, increased immunogenicity, compromised intracellular delivery etc. and therefore careful consideration should be given in balancing different factors.

5.1.3 Combination effect

One of the key objectives of this project was to study the combination effects of doxorubicin and topotecan in treating relapsed ovarian cancer. These effects were also studied with respect to increased exposure time of these drugs to the cancer cells.

Drug-drug interactions between topotecan and doxorubicin were studied using median effect principle (MEP) which is discussed in detail in chapter 1 (section 1.5.1). This method strongly suggests studying drug-drug interactions using a fixed ratio design as it allows measurements using entire dose response curve (for details regarding the applications and methodology of MEP, refer to Chou 2006). This design also increases cost-effectiveness of the experiment by reducing the number of data points (and therefore number of animals) required but is still be able to generate maximum useful information on the combination effects. This method also recommends that constant combination ratio should be carried out at equipotency ratio so that contribution of each drug in the combination effect would be equal. Hence, topotecan/doxorubicin interactions in this project were studied at $(IC_{50})/(IC_{50})$ ratios.

Overall results from the experiments conducted in the third part of this project (chapter 4) demonstrated that combination of topotecan and doxorubicin resulted in either synergistic or

additive interactions when tested against three different ovarian cancer cell lines and these beneficial effects were increased with increased exposure time. Further, *in vitro* observations were translated very well *in vivo* with the help of long circulating LNP formulations of topotecan (Topophore C™) and doxorubicin (Doxil®). Doxil® showed clear therapeutic advantage when combined with newly developed Topophore C™. These results satisfy the conditions of working hypothesis as stated in chapter 1.

One of the future directions for better understanding the combination effect can involve studying drug interactions at non-constant ratios, wherein concentration of one drug is kept constant and that of other is varied. This approach can be useful if the interaction between drugs used in combination are strongly dependent on the ratio. As long as m and Dm values for each drug in a combination are known, MEP method can still calculate CI value for each combination data point separately. However, if one decides to generate dose response curves for different possible ratios, then it can increase the size as well as the cost of experiments enormously. Use of drugs, combination effects of which are highly dependent on the ratio should be avoided in combination therapy as following a series of *in vitro* studies even if one is able to optimize an effective ratio, it is very difficult to maintain such a ratio following administering a combination *in vivo*. However, recent studies have shown partial success in achieving this type of control pre-clinically with the use of drug carriers (Mayer, Harasym et al. 2006); however, such combination will still be highly un-predictable to use in the clinic considering the highly unpredictable pre-clinic to clinic co-relation of the data. These concerns can further magnify in case of combinations involving more than two drugs.

Cell lines used in this work (ES-2, SKOV-3 and OVCAR-3) are representative of different types of ovarian cancer as explained before. These are also commonly used cell lines

for developing human ovarian cancer xenografts to study efficacy of various treatments and the data obtained from these models is considered a reliable pre-clinical data. However, it is interesting to note that creation of these types of cell lines (renewable) usually involves immortalizing and then expanding in the *in vitro* cell culture environment. It is difficult to preserve the immunophenotype and morphology of original tumor in the propogated cells. Human cancer tissue xenografts can also be prepared by using tumor tissues obtained directly from the operating room of surgically operated patient (primary cells) which may more closely represent human tumors with respect to their response to treatments. Ovarian cancer xenografts developed using this method by Lee et al have shown high engraftment rates and preservation of original tumor characteristics (Lee, Hui et al. 2005). Hence, confirming some of the key results from this project using these types of primary tumor models will be a good value addition in the future studies.

The success of effective liposomal drug delivery to the solid tumor regions via EPR effect depends on liposomal size (100-200 nm) as well as on the circulation half-life as discussed before in the introduction. This type of passive targeting can be enhanced by attaching a tumor specific ligand to liposomes, an approach commonly known as active targeting. Immunoliposomes prepared by attaching tumor specific antibodies on the liposomal surface have attracted much attention (Lasic, Papahadjopoulos et al. 1995. Park, Hong et al. 1997) in this area. However, merely delivering the drug to the tumor region is not enough for effective ani-tumor activity. Extravasation and targeting plays an important role in providing the macromolecules an access to the tumor site but in order to kill tumor cells, enacpasulated drugs should be able to penetrate into the cellular compartment of the cell. Recent approaches in order to improve the intracellular targeting of liposomal drugs involves use of targeting ligands specific to

internalizing receptors over-expressed on the tumor cells. Receptor-ligand complexes are then internalized through receptor mediated endocytosis. These receptors include cell surface determinants, growth factor receptors like CD-20, Her-2, folic acid, transferrin etc. (Maruyama 2010, Suzuki, Takizawa et al. 2008, Sapra, Tyagi et al. 2005). Liposomes modified by using such targeting ligands have been shown to be taken up preferentially by tumor cells in number of *in vitro* and *in vivo* studies (Gabizon, Harowitz et al. 2003, Park, Hong et al. 2002). However, translation of such effective pre-clinical data into clinical studies has been very challenging mainly due to concerns like *in vivo* stability, shorter circulation times of the ligand-liposomal constructs in the plasma etc. Thus, although tumor targeted liposomes hold a promising future in improving antitumor activity of liposomal drugs, their success largely depends on the robust basic formulation with desired pharmacokinetic characteristics that can withstand physiological conditions when administered *in vivo*. Topophore C^{TM} developed in this work relies on the passive targeting method in order to deliver the drug to the tumor region and the interstitial spaces. This formulation has shown to possess the desired pharmacokinetic characteristics and *in vivo* stability as discussed in chapter 3. Therefore, it will be a strong candidate to be evaluated for tumor or intracellular targeting purpose in the future studies.

5.2 SIGNIFICANCE OF THE WORK

As outlined in the specific aims, this thesis was driven by milestones which were achieved at different stages of the project. Divalent metal mediated active loading of drugs into liposomes is becoming an increasingly recognized methodology in the development of liposomal products. However, there is no clear understanding till date about the mechanism involved in the drug loading process. Results from the first part (chapter 2) of this thesis enhanced our

understanding of these systems and will hopefully lead to further development and improvement of liposomal delivery systems using these methodologies.

Successful development of a stable and effective Topophore C™ formulation is considered as another significant milestone of this project. This formulation has shown a remarkable improvement in the therapeutic activity when compared with current standard treatment option. This can be used as a key proof of concept data to test this formulation in other cancer models. Successful development of Topophore C™ also indicated potential future expansion of copper based drug loading methodology for other drugs.

Topophore C™ was developed with the intent of evaluating it in combination with Doxil® against ovarian cancer tumor models. However, this formulation has shown a significant promise even as a standalone treatment option in the preclinical models studied. Therefore future studies will involve utilization of the key data generated so far with this formulation with the aim of effective translation of this product into the clinic.

The third part of this thesis (chapter 4) outlines potential benefits of using topotecan and doxorubicin together against recurrent ovarian cancer and emphasizes the use of liposomal delivery systems to maximize the therapeutic benefits achieved using this combination. Future studies may involve evaluating this combination in other cancer models. Additionally, understanding the mechanisms behind the synergistic interactions observed between these two drugs may help in improving this combination further with the help of newer targeted drugs.

Together, these results and future findings will lead to a better understanding of the disease and therefore will lead to a novel treatment regimen that will not merely be palliative but will be used with a curative intent in treating relapsed ovarian cancer.

5.3 REFERENCES

Abraham, S. A., Edwards, K., Karlsson, G., Hudon, N., Mayer, L. D. and Bally, M. B. (2004). "An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes." J Control Release **96**(3): 449-61.

Dadashzadeh, S., Vali, A. M. and Rezaie, M. (2008). "The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats." Int J Pharm **353**(1-2): 251-9.

De Cesare, M., Zunino, F., Pace, S., Pisano, C. and Pratesi, G. (2000). "Efficacy and toxicity profile of oral topotecan in a panel of human tumor xenografts." Eur J Cancer **36**(12): 1558-64.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Connolly-Ingram, C., Gabriel, B. S., Hann, B., Liu, B., Park, J. W., Hong, K., Benz, C. C., Marks, J. D. and Kirpotin, D. B. "Development of a highly stable and targetable nanoliposomal formulation of topotecan." J Control Release **141**(1): 13-21.

Fassberg, J. and Stella, V. J. (1992). "A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues." J Pharm Sci **81**(7): 676-84.

Gabizon, A., Horowitz, A. T., Goren, D., Tzemach, D., Shmeeda, H., Zalipsky, S. (2003).” In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice.” Clin. Cancer Res. **9**(17):6551–6559.

Gabizon, A., Shmeeda, H. and Barenholz, Y. (2003). "Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies." Clin Pharmacokinet **42**(5): 419-36.

Gustafson, D. L., Rastatter, J. C., Colombo, T. and Long, M. E. (2002). "Doxorubicin pharmacokinetics: Macromolecule binding, metabolism, and excretion in the context of a physiologic model." J Pharm Sci **91**(6): 1488-501.

Harasym T. O., T. P. G., Bally M., Janoff A (2006). *Fixed drug ratio liposome formulations of combination cancer therapeutics*. Liposome Technology. G. Gregoriadis. New York, informa healthcare. **III:** 25-48.

Lasic, D. D. and Papahadjopoulos, D. (1995). “Liposomes revisited.” Science **267:**1275–1276.

Lee Cheng-Han, Hui X., Sutcliff M, Gout P. W., Huntsman D. G., Miller D. M., Gilks C. B, Wang Y.Z. (2005) “Establishment of subrenal capsule xenografts of primary human ovarian tumors in SCID mice: potential models.” Gynec. Onc. **96:** 48-55.

Liu, J. J., Hong, R. L., Cheng, W. F., Hong, K., Chang, F. H. and Tseng, Y. L. (2002). "Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation." Anticancer Drugs **13**(7): 709-17.

Maruyama K. (2010). “Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects.” Adv. Drug Deliv. Rev. Article in Press.

Mayer, L. D., Harasym, T. O., Tardi, P. G., Harasym, N. L., Shew, C. R., Johnstone, S. A., Ramsay, E. C., Bally, M. B. and Janoff, A. S. (2006). "Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice." Mol Cancer Ther **5**(7): 1854-63.

Messerer, C. L., Ramsay, E. C., Waterhouse, D., Ng, R., Simms, E. M., Harasym, N., Tardi, P., Mayer, L. D. and Bally, M. B. (2004). "Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer." Clin Cancer Res **10**(19): 6638-49.

Park, J. W., Hong, K., Kirpotin, D. B., Colbern, G., Shalaby, R., Baselga, J., et al. (2002). “Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery.” Clin. Cancer Res. **8**(4):1172–1181.

Park, J. W., Hong, K., Kirportin, D. B., Meyer, O., Papahadjopoulos, D., and Benz, C. C. (1997) “Anti-HER2 immunoliposomes for targeted therapy of human tumors.” Cancer Lett. **118:**153–160.

Proffitt, R. T., Williams, L. E., Presant, C. A., Tin, G. W., Uliana, J. A., Gamble, R. C. and Baldeschwieler, J. D. (1983). "Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles." Science **220**(4596): 502-5.

Ramsay, E., Alnajim, J., Anantha, M., Taggar, A., Thomas, A., Edwards, K., Karlsson, G., Webb, M. and Bally, M. (2006). "Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation." Pharm Res **23**(12): 2799-808.

Ramsay, E. C., Anantha, M., Zastre, J., Meijs, M., Zonderhuis, J., Strutt, D., Webb, M. S., Waterhouse, D. and Bally, M. B. (2008). "Irinophore C: a liposome formulation of irinotecan with substantially improved therapeutic efficacy against a panel of human xenograft tumors." Clin Cancer Res **14**(4): 1208-17.

Sapra, P., Tyagi, P., Allen, T. M. (2005). Ligand-targeted liposomes for cancer treatment. Curr. Drug Deliv. **2**(4):369–381.

Suzuki R., Takizawa T., Kuwata Y., Mutoh M., Ishiguro N., Utoguchi N., Shinohara A., Eriguchi M., Yanagie H., Maruyama K. (2008). “Effective anti-tumor activity of Oxaliplatin encapsulated in transferrin-PEG-liposome.” Int. J. Pharm. **346**: 143–150.

Taggar, A. S., Alnajim, J., Anantha, M., Thomas, A., Webb, M., Ramsay, E. and Bally, M. B. (2006). "Copper-topotecan complexation mediates drug accumulation into liposomes." J Control Release **114**(1): 78-88.

Tardi, P., Choice, E., Masin, D., Redelmeier, T., Bally, M. and Madden, T. D. (2000). "Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models." Cancer Res **60**(13): 3389-93.