DEVELOPMENT OF AN OPTIMIZED LIPOSOMAL FORMULATION OF A CAMPTOTHECIN TO IMPROVE TREATMENT OF NEUROBLASTOMA

by

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

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2016

ABSTRACT

Purpose: To develop an optimized liposomal formulation of topotecan for use in the treatment of patients with neuroblastoma.

Experimental design: Cytotoxic activity of both camptothecins (topotecan (Hycamtin) and irinotecan (Camptosar)) was determined against SK-N-SH; IMR-32 and LAN-1 neuroblastoma cell lines. Sphingomyelin (SM)/Cholesterol (Chol) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/Chol liposomes were prepared using extrusion methods and then loaded with topotecan using a method that relies on copper-drug complexation in combination with a transmembrane pH gradient. The influence of lipid composition and encapsulated drug-to-lipid ratio was assessed in-vitro in the presence and absence of serum. Dose-range finding studies were used to define maximum tolerated dose of the optimized liposomal formulation. Pharmacokinetic studies were completed to compare plasma elimination of topotecan following intravenous administration of the liposomal formulation or the currently used clinical product (Hycamtin). Studies were done in NRG mice bearing established subcutaneous tumours. The anti-tumour activity of the liposomal formulation was compared to Hycamtin when administered to NRG mice with established neuroblastoma tumours.

Results: Topotecan was significantly more effective than irinotecan when used to treat neuroblastoma cell lines (as determined by IC_{50}). Increased exposure time to topotecan further increased the drug potency against all neuroblastoma cell lines. In vitro studies showed that SM/Chol liposomes retained topotecan better than DSPC/Chol liposomes. Decreasing the drug-to-lipid ratio from 0.1 to 0.025(mol: mol) engendered significant increase in drug retention. The optimized SM/Chol liposomal topotecan formulation exhibited a 10-fold increase in plasma half-life and a 1000-fold increase in $AUC_{0\text{-}24h}$ when compared to Hycamtin administered at equivalent doses. When administered at 5mg/kg, SM/Chol liposomal topotecan was significantly more effective than Hycamtin administered at 2-times the dose. The liposomal formulation increased the life span of mice by 50% for the systemic tumour model and by 87% for the subcutaneous models.

Conclusion: Increased systemic drug exposure following administration of the optimized SM/Chol liposomal topotecan formulation produced superior response in subcutaneous and systemic models of neuroblastoma. In the future, this formulation will be assessed in combination with radiotherapy and immunotherapy treatment modalities currently used in neuroblastoma therapy.

PREFACE

All of the work was conducted in the Department of Experimental Therapeutics, BC Cancer Agency- Research Centre. I was the lead investigator responsible for all major areas of concept formation, data collection and analysis with the assistance of Experimental Therapeutic personnel: Malathi Anantha assisted with analytical methods for drug detection; animal work was performed by Investigational Drug Program animal tech group. Dr. B. Bally was the supervisor of this project and was involved through the project. All animal studies were completed under an animal care protocol that has been reviewed and approved by the Institutional Animal Care Committee (IACC) at UBC - protocol #A10-0171.

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LIST OF ABBREVIATIONS

[^{131}I]-MIBG	: ^{131}I-metaiodobenzylguanidine
4S	: 4 Special
ALK	: Anaplastic lymphoma receptor tyrosine kinase
ANOVA	: Analysis of Variance
AUC	: Area under curve
Chol	: Cholesterol
COG	: Children's Oncology Group
Cu	: Copper
DSPC	: 1,2-Disteraoyl-*sn*-glycerol-3-phosphocoline
EPR effect	: Enhanced permeability and retention
FDA	: US Food and Drug Administration
GD2	: Disialoganglioside
hNET	: Norepinephrine transporter
hr	: Hour
IC	: Inhibitory concentration
i.v	: Intravenous
LMV	: Large multilamellar vesicles
LOH	: Loss of heterozygosity
LSC	: Liquid scintillation counting
Min	: Minutes
MKI	: Mitosis karyorrhesis index
MPS	: Mononuclear phagocyte system
MR	: Mitotic rate
MYNC	: N-myc proto-oncogene protein
NB	: Neuroblastoma
PC	: Phosphatidylcholine
PEG	: Polyethylene glycol
PHOX2B	: Paired-like homeobox 2b
RES	: Reticuloendothelial system

SC	: Subcutaneous
SM	: Sphingomyelin
Tc	: Glass transition temperature
TOP 1	: Topoisomerase I enzyme

ACKNOWLEDGEMENTS

I would like to express my gratitude towards everyone who contributed to this thesis. First and foremost I would like to thank my supervisor Dr. Marcel Bally for his mentoring and support.I would like to thank members of my research committee Dr. Rebecca Deyell, Dr. Francois Benard and Dr. Paul Rennie for their support and for sharing their advice and expertise.A big thank you to Experimental Therapeutics lab members especially Malathi Anantha, Brent Sutherland and Hong Yang. Special thanks to entire IDP group, especially Dr. Nancy Dos Santos and animal techs: Dana Masin, Kirbee Parsons, Dita Strutt, Maryam Osooly and Nicole Wretham.Finally, my biggest thanks to my family- my husband Alex and my daughter Danielle. Thank you for your patience, love and support.

DEDICATIONS

This thesis is dedicated to all little patients who are fighting cancer.

1 INTRODUCTION

1.1 Project overview

Neuroblastoma is the most common extra-cranial solid tumour of infancy and one of the deadliest pediatric cancers. Although its hallmark is clinical heterogeneity, children with high-risk neuroblastoma have long-term survival rates below 50% despite intensive, multi-modality treatment approaches. Topotecan is a promising chemotherapeutic agent that is currently incorporated in first-line induction chemotherapy for children with high-risk neuroblastoma. Along with radiosensitizing properties, topotecan was shown to have single agent activity against neuroblastoma. However, rapid *in vivo* conversion of topotecan to an inactive lactone form and its fast plasma clearance, results in low bioavailability and decreased amounts of the drug reaching the tumour cells.

The goal of my studies was to select a camptotecin liposomal formulation for use in the development of a product candidate for treatment of neuroblastoma. The studies explored methods to obtain an optimized formulation that exhibits improved drug retention in hopes that this would result in improved efficacy.

1.2 Hypothesis

An optimized liposomal formulation of topotecan will be significantly more effective than the currently used clinical product (Hycamtin) when treating models of neuroblastoma. The specific objectives for the research contained in this thesis are as follows:

a. To improve topotecan retention in liposomal formulations by changing liposomal lipid composition and drug-to-lipid ratio;

b. To develop subcutaneous and systemic models of neuroblastoma;

c. To assess the activity of the optimized liposomal formulation of topotecan when used to treat models of neuroblastoma.

1.3 **Neuroblastoma**

Neuroblastoma is the third leading cause of childhood cancer mortality, and is the most common and the deadliest pediatric extra-cranial solid tumor (1, 2). It accounts for 8% of pediatric cancers (ages of 0-14) with more than 650 new cases diagnosed annually in North America (8), and 355 new cases were diagnosed in Canada between the years 2006-2010 (3). Neuroblastoma, a cancer of the sympathetic nervous system, arises from sympathoadrenal lineage of the neuronal crest during development (5, 6). Neuroblastoma originates from a pluripotent cell of the neural crest that has not undergone differentiation. During the normal process, neuronal crest derived cells give rise to several cell types depending on the inductive environment. Those include peripheral neurons, enteric neurons and glia, melanocytes, Schwann cells, and cells of the craniofacial skeleton and adrenal medulla (5, 30).

Neuroblastoma tumors can arise anywhere along paraspinal sympathetic ganglia, with most primary tumors arising within the adrenal medulla (5). This is a sporadic cancer where less than 2% of cases have a familial origin (11, 12). Familial predisposition is associated with germline mutations in signaling pathways that are important during development of sympatheticoadrenal lineage. These include mutations in PHOX2B (regulates differentiation pathway in the sympatheticoadrenal lineage of neuronal crest) and ALK (regulates the balance between proliferation and differentiation in the developing sympathoeticoadrenal lineage) (4).The median age at diagnosis is 9 months and 18 months for familial and sporadic cases, respectively (4). In sporadic neuroblastoma, several oncogenes have been identified as prognostic markers of aggressive disease phenotype: amplification of *MYCN* is present in 22%

of tumors and is independently prognostic of inferior survival outcomes. Somatic *ALK* activating mutations within the tyrosine kinase domain, or gene amplification is present in 9-14% of the sporadic cases and is also associated with inferior outcomes (1, 5). Additional genetic abnormalities associated with an aggressive clinical course of neuroblastoma are loss of heterozygosity (LOH) of 1p 11q, and gain of chromosome 17q (13-14, 28, 71).

1.4 **Neuroblastoma classification and treatment**

Neuroblastoma is a very heterogeneous disease with broad spectrum of clinical behavior (9-10,72). Some tumors will progress despite intensive multimodality treatment, while others undergo spontaneous regression without therapy, or mature into benign ganglioneuromas (13). Prediction of clinical outcome depends highly on a number of factors including age at diagnosis, stage of the disease, and several genetic features such as the presence of *MYCN* amplification that form the basis of current therapy risk stratification (4,72).

Recent studies showed that molecular characteristics of the individual tumors should also be taken into consideration when predicting clinical behavior. Neuroblastic tumors mainly consist of two cell populations: neuroblasts and Schwann cells. Shwannian stroma-poor tumors, are a morphological type associated with neuroblastoma. Stroma-poor, undifferentiated neuroblastoma is a subtype with very poor prognosis. Additional histological features used as predictive markers include the mitosis karyorrhexis index (MKI) (defined as the number of tumor cells in mitosis and in the process of karyorrhexis) and mitotic rate (MR). Undifferentiated tumors with amplified MYCN and high MKI, or tumours with high MR in older children are associated with aggressive clinical behavior (29, 73). DNA ploidy of the primary tumour is another factor considered for neuroblastoma risk-group assignment: hyperdiploid or near–triploid DNA index with no segmental aberrations, usually associated

with favorable clinical behavior, while diploid or tetraploid DNA content (frequently have chromosomal rearrangements) can predict more malignant set of the disease (14, 28). Shimada histology grading system is one of the classification systems used for prognostic grouping of neuroblastoma patients. Based on Shimada, patients younger than 18 months with low MKI, or age 18 months-5 years with low MKI and differentiating subtype are identified as favorable histology group. In contrast neuroblastoma with high MKI (any age), intermediate MKI (in patients 18 months-5 years) or patients with undifferentiated and poorly differentiated tumors (ages of 18 months- 5 years) or patients older than 5 years at the time of diagnosis are considered as unfavorable histology group (72, 73).

Around 50% of newly diagnosed neuroblastoma patients present with high-risk disease at the time of diagnosis, a condition associated with poor prognosis due to extensive tumor burden and disseminated metastasis. Patients younger than 18 months of age with limited metastasis (to liver, skin, bone marrow (<10% bone marrow involvement) and favorable tumor biology (absence of MYCN oncogene amplification and structural genetic abnormalities) have very good prognosis (Table 1) (1, 4, 6, 16-17). However, treatment of older children presenting with unfavorable prognostic markers remains one of the greatest challenges for pediatric oncologists (4, 5). For those high-risk neuroblastoma patients, current standard treatment includes induction chemotherapy including topotecan, surgery, high-dose myeloablative chemotherapy with autologous stem cell rescue and use of the differentiation agent 13-cis retinoic acid (7, 8). Following publication of Children's Oncology Group randomized phase III trial in 2010, immunotherapy with anti-GD2 antibody along with GM-CSF and IL2 has been incorporated, along with cis-retinoic acid, as the standard maintenance therapy for patients with high-risk disease. This antibody was approved by FDA March 2015,

under the commercial name Unitux (dinutuximab) (United Therapeutics) as part of first-line therapy for pediatric patients with high-risk neuroblastoma (18). Despite intensification of therapy 5-year event -free survival remains less than 50% for the high-risk neuroblastoma patients (4, 6, 8).

Table 1: Clinical outcome and standard therapy for neuroblastoma based on patient's age, stage of the disease and presence of MYCN

Risk Group	Tumour genomics	Stage	Age at diagnosis	Treatment	Survival Rate
Low	MYCN-non amplified; whole-chromosome gain	4S (localized primary tumours and metastasis limited to liver and skin , minimal bone marrow involvement)	< 18 months	Supportive care (unless symptomatic)	>91%
Low	MYCN-non amplified; whole-chromosome gain	Localized tumours	< 12 months	Surgery or observation (in perinatal neuroblastoma)	>95%
Intermediate	MYCN-non amplified: whole-chromosome gain	4 (Localized tumours with locoregional lymph-node extension)	< 18months	Surgery with moderate intensity chemotherapy (carboplatin, cyclophosphamide, doxorubicin or etoposide)	>89%
High	MYCN amplified	Locoregional	<21 years	Dose- intensive chemotherapy, surgery, radiotherapy, myeloblative chemotherapy with autologous stem-cell rescue, and, 13-cis-retinoic acid (isotrenion) immunotherapy (anti-GD2 antibody)	53%
	MYCN amplified	4 (Localized tumours with locoregional lymph-node extension)	< 18months		29%
	For both MYCN amplified and non-amplified		>18months and <21 years		31%
	MYCN- non amplified		>12 years		<10%

This thesis research is focused on a first step towards the development of a combination product for use in patients with high-risk neuroblastoma. The product would comprise a potent broad spectrum cytotoxic drug, a therapeutic radionuclide and a therapeutic antibody. This combination product would be designed around a lipid-based formulation technology and the first step in development of this refined product is the selection and optimization of the formulation for the broad spectrum drug.

1.5 Lipid based delivery systems for broad spectrum chemotherapeutic drugs

Use of nanoparticles for targeted drug delivery of antineoplastic agents is a proven strategy to improve efficacy and to reduce non-specific toxicities commonly associated with chemotherapeutic drugs. The biodistribution and pharmacokinetic profile of the associated drug is modified when delivered in a liposomal formulation and this can minimize the exposure to healthy tissues while increasing the amount of drug delivered to the tumour. Although those modifications could benefit any population of cancer patients, there is an increasing need for safer, more targeted therapeutics for pediatric patients. During the past few years, overall survival for pediatric cancer patients increased to 80%, however this has only been achieved through use of aggressive therapies combining chemotherapy and multimodality treatments (radiation and immunotherapy). Treatment, albeit beneficial, is associated with treatment-related late onset toxicities (35). Since lipid based carrier, such as liposomes, are an evolving drug delivery platform that has resulted in a number of approved products for use in cancer patients (see Table 2), this formulation is well suited for further development of products designed for pediatric patients (36,41,52).

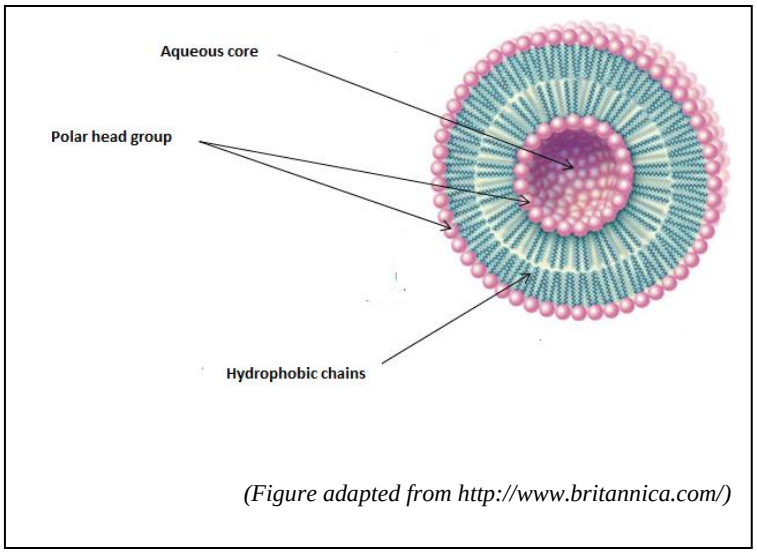
Table 2: Liposomal- based chemotherapeutic drugs approved by the US FDA

Product name	Active Ingredient	Approved indication
DaunoXome	Daunorubicin Citrate	Kaposi's sarcoma associated with HIV
Caelyx	Doxorubicin hydrochloride	Ovarian/breast cancers
Doxil		Ovarian/breast cancers, Kaposi's sarcoma associated with HIV
Myocet		Metastatic breast cancer
Depocyt	Cytarabine	Lymphomatous meningitis
Marqibo	Vincristine sulfate	Acute lymphoblastic leukemia

1.5.1 **Liposomes as drug carriers**

Liposomes are spherical structures (Figure 1), composed of lipid bilayer that formed upon hydration of amphiphilic lipids. Amphiphilic lipids have a dual preference for solvents: the hydrophilic head group is soluble in polar solvents and hydrophobic fatty acid chains that are soluble in nonpolar ones (37, 39-41). To decrease the interaction with an aqueous environment lipids, when hydrated in polar solutions, will spontaneously orient themselves such that the hydrophilic headgroup faces the aqueous solutions while the hydrophobic chains interact with themselves in a bilayer configuration. Due to their amphiphilic character a wide range of cargo molecules can be packaged into the liposomes, making them extremely flexible as a formulation system. Hydrophilic molecules can be loaded into the hydrophilic inner core, while hydrophobic drugs would incorporate into the hydrophobic bilayer (37, 39, 41).

Figure 1: Schematic representation of liposome



(Figure adapted from http://www.britannica.com/)

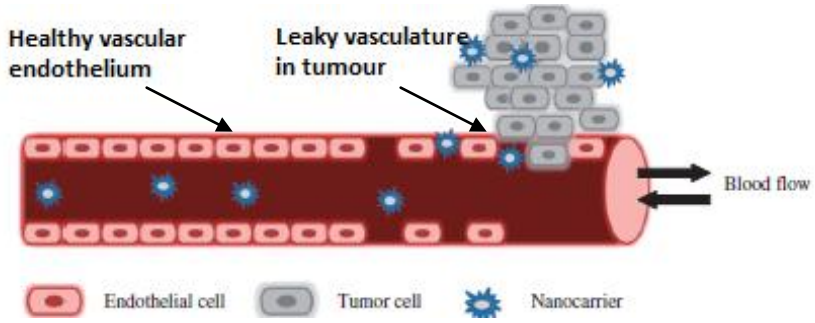
Encapsulation of chemotherapeutic agents into liposomes can be used to reduce the toxicity and improve efficacy; results that are achieved by liposome mediated changes in the biodistribution and pharmacokinetics of the drug. These modifications occur in several ways:

a. Reduction in distribution volume- most small-molecule antineoplastic agents have a large volume of distribution upon i.v administration, this results in exposure of healthy tissue to the drug increasing nonspecific toxicities.

b. The enhanced permeability and retention (EPR) effect- Encapsulation of the agents into liposomes reduces their transport across healthy vascular endothelium due the size limitations and this can result in lower drug accumulation in healthy tissues (Figure 2). Importantly, tumour vasculature is often immature, disorganized and contains large fenestrations between endothelial cells which increases capillary permeability to molecules in the blood compartment, including liposomes. In combination with impaired tumor lymphatics, this leads to accumulation of the liposomes in the sites of tumour growth. This is known as the enhanced permeability and retention (EPR) effect (35, 37, 39, 41). In addition to EPR effect,

accumulation of the carriers within the tumours can be enhanced by conjugation of ligands or specific binding molecules such as antibodies to the surface of the carriers- this is known as active targeting (37,41).

Figure 2: Schematic representation of enhanced permeability and retention (EPR) effect



(Figure adapted from Pretti Kumari, Balaram Ghosh&Swati Biswass. Nanocarriers for cancer-targeted drug delivery, Journal of Drug targeting (2016))

c. Protection from degradation – encapsulation of the drug protects the drug from enzymatic degradation as well as immunological and chemical inactivation including plasma protein binding. In an optimized liposomal formulation, the drug will be released from the carrier only when reaching the tumour and will remain protected within the liposomes remaining in the plasma compartment (37,41).

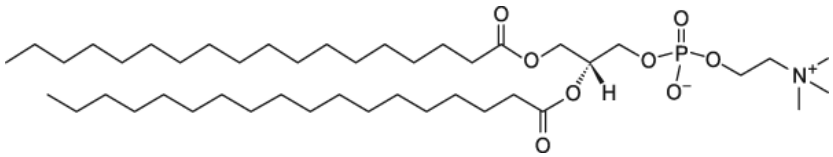
1.5.2 Phospholipids

The main components used in preparing liposomal formulations include phospholipids and cholesterol. Polyethylene glycol (PEG)-modified lipids are included for specific reasons outlined below. Phospholipids contain a hydrophilic head group and two fatty acyl chains joined to the head group by glycerol molecule. Sphingolipids utilize a sphingoid molecule to link the fatty acids to the head group (Figure 3). Most commercially approved liposomal

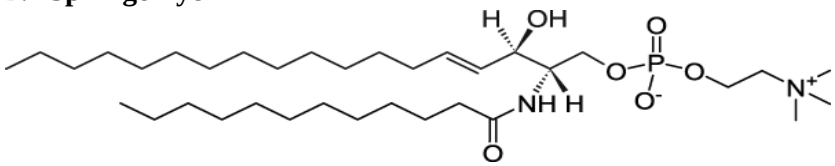
formulations use synthetic phospholipids or naturally occurring isolates. The majority of approved formulations contain lipids that have phosphatidylcholine (PC) as the head group (Figure 3). The lipid composition of liposomes, especially the properties of the phospholipid head group and its acyl chain composition, will determine the characteristics of the formulation; influencing drug retention, liposome plasma circulation time, liposome interaction with serum proteins and liposome elimination by the mononuclear phagocytic cell (MPS) system (35, 37, 42-43). The hydrophobic chains of the phospholipids play an important role in drug retention. Increasing the length of the acyl chains, raises the glass transition temperature (Tc), while the number of the unsaturations found in the acyl chains decreases the Tc (37). Below the Tc, phospholipid bilayer exists in a gel like phase, with acyl chains arranged in a tightly packed, highly ordered form. This reduces the permeability of the bilayer structure that encases the liposome. At temperatures above the Tc, the molecular motion of the acyl chains is increased resulting in increased permeability of the membrane. For this thesis research I explored how changes in liposomal lipid composition effects retention of the selected camptothecin (see below) being developed for use in treatment of patients with neuroblastoma.

Figure 3: Schematic representation of phospholipid and cholesterol molecules

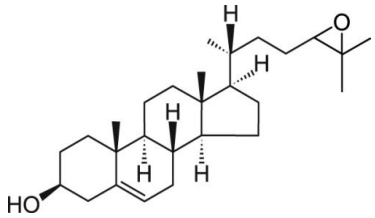
A. DSPC



B. Sphingomyelin



C. Cholesterol



(Figure adapted from http://www.avantilipids.com/)

1.5.3 Lipid composition of liposomal formulations described in this thesis

The liposomes used in this thesis were prepared with 1,2-Disteraoyl-*sn*-glycerol-3-phosphocoline (DSPC) or N-hexadecanoyl-D-*erythro*-sphingosylphosphorycholine (SM). Structure of both phospholipids is shown in Figure3. In addition to these lipids cholesterol was incorporated into the formulations at a mole ratio of 45%. Cholesterol is an organic molecule, steroid derivative, is an essential structural component found in cell membranes required to maintain membrane structure and fluidity (44). It has been suggested that incorporation of cholesterol into liposomal formulations modulates membrane fluidity around the Tc, resulting in increased stability of the bilayer (44). Additional benefit of cholesterol incorporation into the formulation is its ability to reduce serum protein binding to phospholipid membranes (44).

As suggested above, there are many different anticancer drugs that have been encapsulated in liposomes (Table 2). There is evidence to suggest that camptothecins are well suited for use with liposomal formulations and as indicated in the next sections, camptothecins are proving to be of interest in the treatment of neuroblastoma. The research in this thesis focused on liposomal formulations of camptothecins for this reason.

1.6 Camptothecins in the treatment of neuroblastoma

Recently topotecan was incorporated by the Children's Oncology Group (COG) as part of induction chemotherapy for high-risk neuroblastoma, in addition to being a key component of a front-line relapse regimen (15). This was driven by studies demonstrating clinical activity of topotecan as a single-agent against neuroblastoma (19-22). Incorporation of topotecan into treatment protocols was also shown to induce supra-additive levels of cytotoxicity when combined with ^{131}I-metaiodobenzylguanidine ([^{131}I]-MIBG) (23-24). This synergism is attributed to topotecan's ability to interfere with the DNA repair process and therefore enhance the toxicity of DNA- damaging radiation. [^{131}I]-MIBG, is a radiopharmaceutical that targets the norepinephrine transporter (hNET) highly expressed in 90% of NB tumors. Following binding and cell specific uptake, the radionuclide kills dividing cell populations (27).

Similar to topotecan, irinotecan has shown single agent activity against neuroblastoma (46). Combination studies of irinotecan with vincristine and [^{131}I]-MIBG, suggested synergism between the agents resulting in better outcomes for the patients (47). Irinotecan in combination with temozolomide is another standard relapse regimen, although is often complicated by irinotecan-induced diarrhea (74).

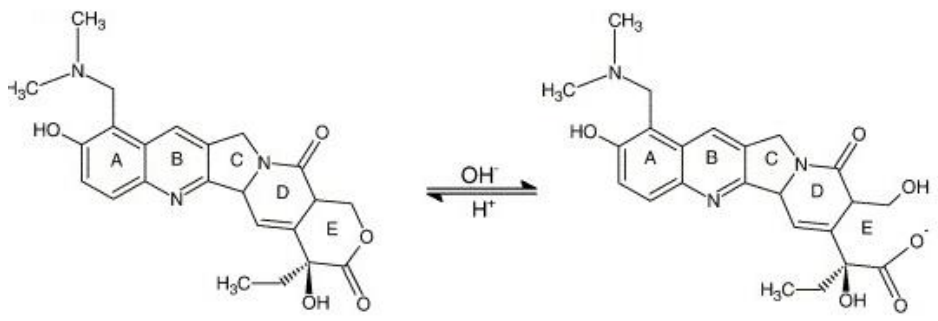
1.6.1 **Topotecan and Irinotecan**

Topotecan (9-[(dimethylamino)methyl]-10-hydroxy-camptothecin) and irinotecan (7-ethyl-10-[4-(piperidino)-1-piperedino]-carbonyloxycamptothecin) are synthetic, water-soluble analogues of camptothecin. The cytotoxic activity of these drugs is based on their ability to stabilize the cleavable complex between topoisomerase I (TOP 1) and DNA (31, 75). During DNA replication, TOP 1 forms a cleavable complex with DNA, relieving the torsonal stress that develops ahead of the replication (31, 75). This action allows rotation of the broken strand around the intact strand and re-ligation of new DNA strand, at this point TOP 1 is released. Camptothecins stabilize the TOP 1/DNA complex, turning it from cleavable complex to a stable covalent complex. This causes accumulation of non-lethal, reversible single-strand breaks. However, during further DNA replication, the replication fork collides with those complexes resulting in irreversible double-strand breaks and apoptotic cell death (31).

Topotecan has five ring heterocyclic structures with a α-hydoxylactone within its E-ring (Figure 4). The interaction of topotecan with topoisomerase I/DNA complex occurs through the closed lactone ring. However this ring structure undergoes a pH-dependent reversible hydrolysis reaction forming the non-active carboxylate (ring open) form at physiological pH. The closed ring form of topotecan is also required for the passive diffusion of the drug into tumor cells, thus the concentration of the non-hydrolized lactone ring determines the cytotoxic activity of the drug (31,32). Hydrolysis of the lactone ring occurs rapidly, with 30% of the drug administered converted to the non-active carboxylate form within 15 min following a 30 minute infusion (32).

Topotecan is the first camptothecin analogue to be approved for clinical use by FDA. The indications for use include ovarian cancer (metastatic carcinoma of the ovary) and small cell lung cancer (after failure of first line chemotherapy). Experimental uses include cervical cancer (Stage IV-B, recurrent or persistent carcinoma of the cervix), neuroblastoma, glioma, acute myelogenous leukemia, multiple myeloma, pancreatic cancer and retinoblastoma. Myelosuppression, primarily neutropenia, is the dose limiting toxicity. Additional toxicities include leucopenia, thrombocytopenia, anemia and respiratory adverse events (interstitial lung disease) (38).

Figure 4: Chemical structure of topotecan hydrochloride

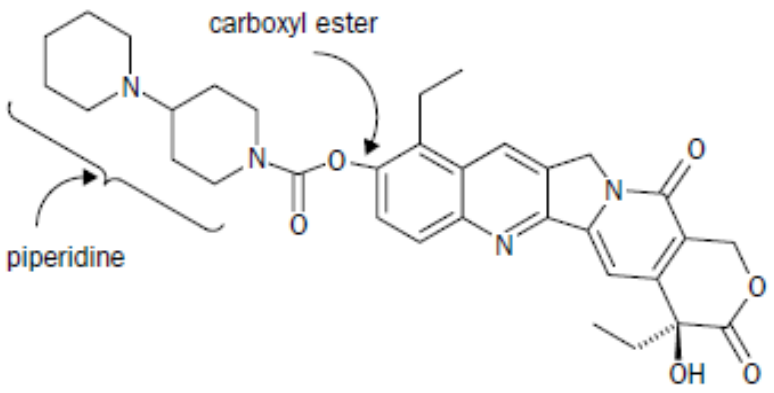


Lactone- active form **Carboxilate-inactive form**

(Figure Modified from: Zhengguang Li, et all . 2012)

Irinotecan is another water-soluble semisynthetic derivative of camptothecin. Solubility of irinotecan depends on its dipiperidino side-chain; however presence of this side chain reduces drug anticancer activity (31). This side chain is cleaved by carboxylesterases (present in the liver and gastrointestinal tract) to form SN-38 , metabolite with 1000-fold increased potency (9). Similar to topotecan therapeutic activity of irinotecan and SN-38 depends on an intact lactone ring (closed form) that undergoes a pH-dependent reversible hydrolysis into non-active carboxylate (ring open) form at physiological pH (9).

Figure 5: Chemical structure of irinotecan



Adapted from Joseph F.Pizzolato. 2003.

1.6.2 Development of lipid based formulations of camptothecins

A variety of lipid based formulations have been used to protect the camptothecin from hydrolysis. More specifically, several liposomal formulations of camptothecins have been described (49-51,65), and these are in addition to the two formulations developed using technology described in this thesis which relies on copper-camptothecin interactions: Topophore C™ (33,34) and Irinophore C™ (48). In general, the goal for preparing liposomal formulations of camptothecins concerns protection from hydrolysis at neutral pH.

Topotecan provides a good example camptothecin candidate to outline other benefits of liposomal formulation.

a. Due to its mechanism of action topotecan is most lethal during the S-phase of the cell cycle, similarly to cell cycle specific agents. Prolonged exposure time of the agent allows more cells to enter the cell cycle, increasing the cytotoxic potency of the agent. Topotecan AUC is about 53.1 ng*hr/ml and plasma half- life is relatively short - 2.76 hr. Encapsulation of topotecan into well designed liposomal

formulations can decrease the fast clearance of the drug, increasing the exposure of tumour cells to the agent.

b. Topotecan has a high volume of distribution (approximately 75 liter/m^2), this suggests that topotecan is preferentially localized in tissues that may be sensitive to the non-specific toxicities caused by this drug. Liposomal formulations rely, in part, on the EPR effect, decreasing healthy tissue drug exposure and increasing its accumulation into tumour cells.

c. As suggested already, hydrolysis of active lactone form of topotecan into nonactive carboxilate ring open form occurs rapidly, with 70% converted within 15 minutes after 30-min infusion. Lactone form is not only required for drug activity, but it is also necessary for its passive diffusion into the tumour cells. Loading of topotecan into carriers with acidic interior, stabilizes the active lactone form, protecting it from hydrolysis.

These benefits are also realized for other camptothecins, including irinotecan.

1.6.3 **Liposome preparation**

Several methods are available for preparation of liposomes and drug encapsulation. For my research I relied on use of lipid hydration techniques to generate MLVs, followed by size reduction through high-pressure extrusion (24). This was previously described by Boman et al (45) and is described in more detail in Section 2.4. Liposomal size was reproducable between different batches with a target size of 100 nm (± 20 nm). Nanocarriers in the size range of 20-200nm can extravasate throught fenestrated tumour associated blood vessels utilizing the EPR effect, while liposomes graeter than 400nm are more likely to be taken up

by phagocytic cells of the MPS system (41, 43). This suggests that 50 to 150nm is an optimal carrier size for intravenous administration of anticancer agents (41, 43).

1.6.4 **Drug loading**

Encapsulation of a selected drug into liposomes can be done using passive or remote loading (active loading) techniques. With *passive loading* the agent is added during the manufacturing process of the liposomes. This could be done by mixing the drug during preparation of the lipid film or by adding the drug to the aqueous buffer during film hydration. Lipophilic agents would be typically added during film preparation, relying on the ability of the compound to incorporate into the lipid bilayer. Hydrophilic, water soluble compounds can be added to the aqueous buffer during hydration of the lipid film, and the drug is then encapsulated within the core of the liposomes. The advantage of passive loading is its simplicity; however the yield of encapsulation is low, reflective of the trapped volume of the liposome and the liposomal lipid concentration. Low trapping efficiencies will result in a large portion of the added drug being discarded. When using *remote loading* (active loading) techniques, encapsulation efficiencies can be up to 100% (37, 69). Remote loading method uses various techniques to entrap the drug within preformed liposomes. The loading technique described in this thesis utilized pH gradient along with divalent metal complexation to encapsulate the selected camptothecin (68).

Several transition metals have been investigated for their potential to facilitate loading of topotecan and irinotecan into preformed liposomes. Cobalt (Co^{2+}) and zinc (Zn^{2+}) did not form strong complexes with the drug (70). In contrast, Cu^{2+} exhibited superior properties with regards to topotecan and irinotecan loading efficiency and stability of the preparation. For this method a transmembrane pH gradient was established using liposomes with encapsulated

copper by adding the divalent cationic ionophore A23187 (calcimycin). Incorporation of the ionophore into liposomal bilayer maintained the transmembrane pH gradient by facilitating the movement of two protons from the external buffer inward in exchange to one Cu^{2+} outward. Efficient encapsulation of topotecan and irinotecan into copper containing liposomes did not require utilization of A23187; however when copper complexation was combined with transmembrane pH gradient, substantial improvement in the drug retention was reported (34).

The research presented in this thesis describes a novel, optimized formulation of liposomal topotecan, with improved drug retention, and improved pharmacokinetic profile. In addition, I have established preclinically the potential for using this improved liposomal topotecan formulation for use in the treatment of neuroblastoma.

2 MATERIALS AND METHODS

2.1 Materials

1,2-Disteraoyl-*sn*-glycerol-3-phosphocholine(DSPC),N-hexadecanoyl-D-*erythro*-sphingosylphosphorycholine (SM) and Cholesterol were obtained from Avanti Polar Lipids, Inc. ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Life Science (Boston, MA), Pico-Fluor 40 scintillation cocktail was obtained from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Hycamtin® injection (topotecan) was purchased from BC Cancer Agency (Vancouver, BC), A23187 from Sigma-Aldrich (Oakville, ON, Canada). Methanol (MeOH), HPLC Grade was obtained from Alfa Aesar (Massachusetts, USA). Glacial acetic acid, Sucrose, HEPES, Sephadex G-50, EDTA, and all other chemicals came from Sigma-Aldrich (Oakville, ON, Canada).

2.2 Cell culture

IMR-32 and SK-N-SH neuroblastoma cells came from the American Type Culture Collection (ATCC, USA) and LAN-1 cells came from DSMZ (Braunschweig, Germany). IMR-32 and SK-N-SH maintained in EMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS)/ 2mM L-glutamine (Life Technologies) were used for in vitro and in vivo studies. LAN-1 were maintained in RPMI 1640 (Life Technologies) supplemented with 20% heat-inactivated FBS/ 2mM L-glutamine).

2.3 Cell viability assay

The cytotoxic activity of topotecan (Hycamtin) and irinotecan (Camptosar) against SK-N-SH, IMR-32 and LAN-1 neuroblastoma cell lines was measured by assessing changes in cell viability determined using PrestoBlue® Cell Viability Reagent (ThermoFisher Scientific,

Burlington, ON, Canada). This method assessed the ability of live cells to reduce the dye resazurin to resorufin, a red fluorescent compound. The amount of resorufin measured at an excitation wavelength of 544nm and emission wavelength of 590nm was determined on an FLUOstra OPTIMA (BMG Labtechnologies) Spectrophotometer. The concentration of drug that decreased the viability of the cells by 50% defined the IC_{50} of the drug. To evaluate the effect of exposure time, SK-N-SH; IMR-32; LAN-1 neuroblastoma cell lines were incubated with increasing concentrations of the drug for 1, 4, 8, 24, 48 or 72 hours. Following each time point, the drug containing medium was removed and replaced with 200µL of fresh medium. The cell viability after a total incubation time of 72h was then determined.

2.4 **Liposomes preparation**

Liposomes were prepared using an extrusion method described by Boman et al. (45). Briefly, the lipids (DSPC/Chol or SM/Chol; 55:45) were dissolved in ethanol (100mg lipid/ml) with ^{3}H-CHE, a non-exchangeable, non-metabolizable radiolabeled lipid marker. The lipid mixture was hydrated in 300mM $CuSO_4$ solution (1ml ethanol/ 5.66 ml buffer) preheated to 60˚C; resulting in a final ethanol concentration of 15% (v/v). Large unilamellar vesicles were generated using extrusion methods using two stacked polycarbonate filters of 0.1 and 0.08 µm pore size at 60˚C (Extruder™, Northern Lipids, Vancouver, BC). The size of the liposomes was assessed using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY), and was characteristically in the range of 100 ±20 nm. Tangential flow diafiltration (Watson Marlow 232 Pump) was used to remove ethanol. The resulting liposomal solution passed through a Sephadex G-50 column equilibrated with SHE buffer (300 mM Sucrose, 20mM HEPES and 15mM EDTA, pH 7.5).

2.5 Measurement of copper concentration

Copper concentration was determined using atomic absorption spectrometry (AA) (AANALYST 600 PerkinElmer Instruments, Woodbridge, ON). This instrument is equipped with transversely heated graphite atomizer (THGA) furnace with an AS-800 Autosampler. A hollow cathode lamp (Cu-LUMINA.HCL) was used as a light source for copper detection.

Liposomal 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.

2.6 Cryo-electron microscopy (CEM)

CEM analysis was performed using a Zeiss Libra 120 transmission electron microscope at the University of Uppsala, Sweden. Briefly, topotecan loaded liposomes were prepared as described above. In a controlled chamber for humidity and temperature (25°C), 1-2 uL of the sample were deposited on copper grids coated with a holey cellulose acetate butyrate polymer. Excess liquid was blotted away carefully with filter paper and then samples were quickly vitrified by plunging into liquid ethane. This was then transferred to liquid nitrogen to maintain the temperature below 1080K, which minimizes formation of ice crystals. Images were taken in a zero-loss bright-field mode and an accelerating voltage = 80 kV.

2.7 Analytical methods for quantification of topotecan

The drug was quantified using methods previously established in our laboratory. For *in vitro* studies drug concentration was determined by diluting samples (90% v/v) in acidified

methanol (3% v/v Acetic Acid, 97% v/v MeOH). Subsequently, the absorbance at 383 nm (Agilent/Hewlett Packard UV-spectrophotometer, model: 8453, Agilent Technologies, Mississauga, ON, Canada) was measured. For *in vivo* derived samples or samples containing >10% serum protein, drug levels were assessed by a High-Performance Liquid Chromatography (HPLC) method using Waters Alliance HPLC system equipped with a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 380 nm and an emission wavelength of 525 nm. Samples were mixed with 3% acidified methanol, centrifuged at 14000 rpm for 10 min to separate plasma proteins, and 10μL of an appropriately diluted supernatant was injected into a Water Symmetry Shield RP C_{18} column (5μm, 100 $A^{o,}$ 4.6 x 100 mm) adjusted to 55°C. The samples were maintained at 4°C before injection. Each sample was run for 10 min at flow rate of 1 ml/min, using mobile phase consisting of 30% solvent A (100% Methanol) and 70% solvent B (1% TEA in water with the pH adjusted to 6.4 with acetic acid). Topotecan in organs was analyzed by homogenising the organ in cold PBS and mixing the homogenate with cold 3% acidified methanol before centrifugation at 14000 rpm for 10 min. The resultant supernatant was processed for analysis by HPLC as described above.

2.8 **Topotecan encapsulation and release assays**

Topotecan was encapsulated into liposomes prepared in 300mM $CuSO_4$ (pH 3.5) and subsequently processed such that the external buffer was the SHE buffer. The transmembrane pH gradient was maintained by the addition of A23187 (0.5 µg per 1mg of lipid). The mixture was incubated at 60˚C for 15 min before the addition of topotecan. Immediately after the addition of topotecan the pH was adjusted to pH 7.5 with 1N NaOH. This mixture was incubated at 50 ˚C for 30 min, and then unencapsulated topotecan was removed by tangential

flow dialysis against PBS (pH 7.5). For studies measuring topotecan loading efficiency, at the indicated time point samples were passed through 1mL Sephadex G-50 spin columns equilibrated with PBS. Topotecan in the eluent (liposome associated drug) was measured by the UV Spectroscopy assay described above. Liposomal lipid in the eluent was estimated by measuring ^{3}H-CHE by liquid scintillation counting (LSC).

For release assays, liposomes were diluted to a final topotecan concentration of 1.09 µmol/mL with PBS (pH 7.5) and then 200 µL of this sample was mixed with 1mL FBS. The resulting mixture was incubated at 37˚C and at the indicated time points, 100 µL aliquots were fractioned on 1mL Sephadex G-50 spin columns. Liposomal lipid in the eluent was estimated by measuring ^{3}H-CHE by LSC. Topotecan was determined using high-performance liquid chromatography (HPLC) as described above.

2.9 **Pharmacokinetic and Biodistribution Studies**

The selected formulation was diluted to the appropriate concentration in PBS (pH 7.5) such that the specified dose (mg/kg) could be administered in a volume of 10 µL/gm body weight. NRG male mice (6-8 weeks) were injected subcutaneously (right ventral flank region) with LAN-1 cells mixed in matrigel (2.5×10^{6} cells per animal). When the tumour size was approximately 100-200 mg (as measured using a caliper where the measured dimensions (mm) were converted to tumor weight (mg) using length x (width^{2}) ÷ 2), the animals were randomized one day before initiation of treatment (intravenously (iv) dose of 5 mg/kg). At selected time points the animals were terminated by isoflurane followed by CO_2 exposure and blood was collected by cardiac puncture and placed into EDTA containing microtainers. Plasma was separated by centrifuging samples at 2500 rpm for 15 minutes at 5˚C. The concentration of topotecan and liposomal lipid in the plasma samples were determined as

described above. The plasma AUC and half-life of topotecan were determined using PK Solution 2.0, Noncompartmental Pharmacokinetics Data Analysis software. Harvested tissues were placed into pre-weighed containers, weighed and frozen until analyzed for drug and liposomal lipid. A portion of the homogenized tissue (10% weight/volume) was processed for measuring topotecan, and another was prepared for measuring ^{3}H-CHE. The topotecan levels were determined as described above. To measure ^{3}H-CHE, 200 µL of tissue homogenate was mixed with 500 µL of Solvable™ (PerkinElmer) and then heated at 50˚C overnight before addition of 50 µL 200mM EDTA and 200 µL 30% H_2O_2. Five mL of Pico-Fluor 40 scintillation cocktail was added, and ^{3}H-CHE was measured using LSC.

2.10 Evaluation of toxicity of free and SM/Chol liposomal topotecan

Dose range finding studies were used to define tolerability of the selected formulation. Tumour free NRG mice (6-8 weeks) were injected i.v. using a Q7Dx3 (one injection/week for three weeks) schedule and the health status of the animals was monitored following an established Standard Operating Procedure. In particular, signs of ill health were based on body weight loss, change in appetite, and behavioral changes such as altered gait, lethargy and gross manifestations of stress. When signs of severe toxicity were present, the animals were terminated (isoflurane overdose followed by CO_2 asphyxiation) for humane reasons. Necropsy was performed to assess other signs of toxicity. The animals were monitored up to two weeks after administration of the last dose and those responsible for monitoring were blinded to the treatment groups.

2.11 **Antitumor activity in murine models of neuroblastoma**

Anti-tumour activity was evaluated in a subcutaneous (sc) and systemic model of neuroblastoma established in NRG male mice (6-8 weeks). The sc model was established as described above and treatment was initiated when the tumour size was between 50-150 mg. Tumour size, as measured using a caliper where the measured dimensions (mm) were converted to tumor weight (mg) using the equation length x (width^2) ÷ 2, was determined every other day until the tumor size exceeded 800mg (the defined humane endpoint) or when the tumour ulcerated.

Systemic model was achieved by intracardiac injection of the cells. Animals (8 mice/ group), were anesthetized using isoflurane. 1mL syringe attached to 26G needle was inserted at 30 degree angle, immediately caudal to the xyphiod process, aiming towards the left shoulder of the animal. Slight negative pressure was placed on the syringe upon entry and the needle was slowly moved forward until blood appeared in the hub of the needle. 1.5×10^6 LAN-1 cells were injected slowly in a volume of 100 µl media.

On day 14, the animals were given the specified formulations i.v. (Q7Dx3 schedule) at the indicated drug dose. Health status of the animals was monitored on a daily basis, more if deemed necessary, for signs of morbidity. In particular, signs of ill health were based on body weight loss, change in appetite, behavioural changes such as altered gait, lethargy and gross manifestations of stress.

When the animals reached a defined humane endpoint they were terminated (isoflurane followed by CO_2 asphyxiation) and a necropsy was performed to assess other signs of tumour progression as noted above. The time of death was recorded as the following day. All animal studies were completed under an animal care protocol reviewed and approved by the

University of British Columbia's Animal Care Committee (ACC). The studies met current guidelines of the Canadian Council of Animal Care.

2.12 **Statistical analysis**

All statistical data was collected using GraphPad Prism (San Diego, CA). Long-rank test was used to compare overall survival (OS), differences between the groups were considered significant if $p<0.01$. One way ANOVA was used to compare Median Survival Time (MST) in various treatment groups against appropriate controls. Differences between the groups were considered significant if $p<0.05$.

3 RESULTS

3.1 Topotecan is more potent than irinotecan when used against neuroblastoma cell lines

The cytotoxic activity of irinotecan and topotecan was assessed in three neuroblastoma cell lines: IMR-32, SK-N-SH and LAN-1 cells and the results are summarized in Figure 6A and B, respectively. Topotecan was 150- to 300-fold more potent than irinotecan when considering the IC_{50} of the drugs. The IC_{50} values reported here for topotecan (3 to 30 nM) are consistent with those previously reported (0.71-489 nM) (53). The remaining studies focused on topotecan because of its enhanced activity when compared to irinotecan.

The activity of topotecan is highly dependent on exposure time and this is illustrated by the data summarized in Fig. 6C and D. For all 3 neuroblastoma cell lines evaluated, the extended exposure to topotecan resulted in significant decreases in IC_{50} concentrations. Specifically, for LAN-1 cells as the exposure time was increased from 4 hours to 72 hours the IC_{50} of topotecan decreased from almost 1µM to 0.03µM, a 30-fold decrease in IC_{50}. Similar decreases in IC_{50} were observed for SK-N-SH and IMR-32 neuroblastoma cells. A primary justification for formulating topotecan into nanoscaled drug delivery systems is the potential that increased topotecan exposure time engendered by the drug delivery system should result in a formulation that is more effective.

Figure 6: Topotecan is more potent that irinotecan when used against IMR-3, SK-N-SH and LAN-1 neuroblastoma cell lines.

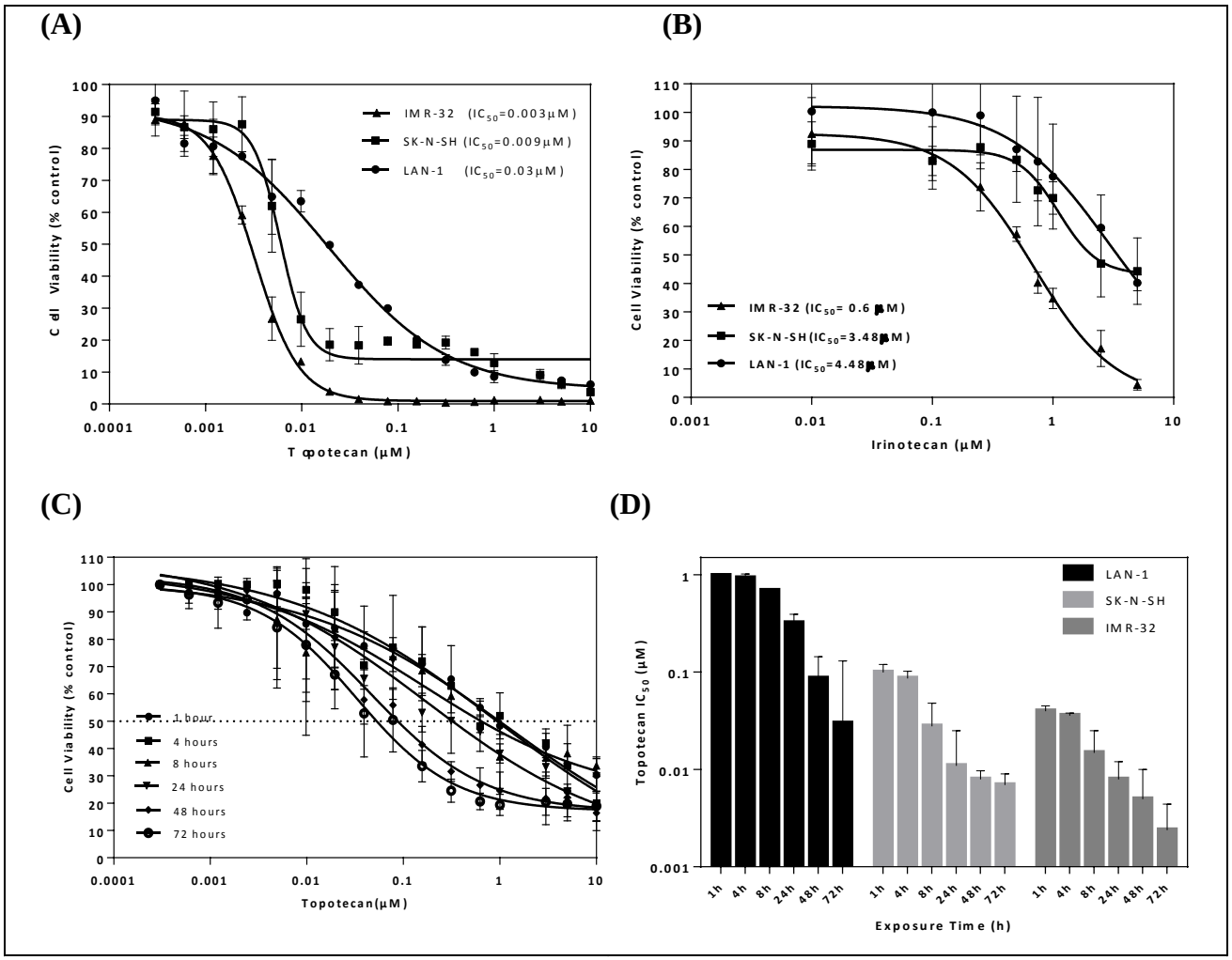


Figure 6: Dose-response curves for topotecan (panel A) and irinotecan (panel B) when added to neuroblastoma cell lines was determined using PrestoBlue®. The IC_{50} for the drugs are indicated. Changes in LAN-1 cell viablity as function of different topotecan exposure times (panel C) was determined as described in the Method. The mean topotecan IC_{50} as a function of topotecan exposure time for each cell line is summarized in panel D. Results are compared to untreated controls and presented as the mean ± SD determine in triplicate at least three times.

3.2 **Optimization of liposomal topotecan formulation**

Topophore C™ (a DSPC/Chol liposomal formulation of topotecan prepared at a final drug-to-lipid ratio of 0.1 (mol: mol) has been described previously (34). This formulation exhibited significant antitumor activity in models of ovarian cancer, however was significantly more toxic than the clinical formulation (Hycamtin) and it released associated topotecan rapidly, with more than 98% drug loss within 8 hours following intravenous administration. The studies summarized below sought to improve the topotecan retention in the liposomes by changing the lipid composition and drug-to-lipid ratio. The goal was to establish that improved drug retention enhances the circulation lifetime of topotecan, decreases the toxicity of the liposomal formulation and achieves further enhancements in therapeutic activity. Sphingomyelin (SM) has been used in previous liposomal anticancer drug formulations, in part because it is more stable than DSPC (39,54). Specifically, sphingomyelin lacks ester-linked acyl chains that are present in DSPC, as the aliphatic chain of sphingomyelin are amide linked. This property decreases SM susceptibility to hydrolysis or enzymatic degradation (54). In addition it was shown to enhance drug retention in cholesterol containing liposome because of an affinity form between SM and cholesterol. Sphingomyelin can form intermolecular hydrogen bonds with the neighboring cholesterol molecule, generating more tightly packed carriers with reduced drug leakage rate (55,56).

Topotecan was encapsulated in SM/Chol (55:45, mol: mol) liposomes using the same encapsulation method developed for Irinophore C™ and Topophore C™; a method that relied on use of copper complexation with the camptothecin as well as an established transmembrane pH gradient maintained by the addition of the divalent metal ionophore A23187. Topotecan loading efficiency for the SM/Chol (Figure 7A) and DSPC/Chol (Figure 7B) topotecan

formulations prepared to achieve a final drug-to-lipid ratio 0.1 was equivalent at the 3 loading temperatures tested (20, 50 and 60°C). Optimal loading was achieved when using an incubation temperature of 50˚C where >98% of the added topotecan became associated with the liposomes within 5 minutes. Stability of these formulations was assessed *in vitro,* by comparing drug release rate in the presence of fetal bovine serum (80%) over an incubation time period of a 24h. The results, summarized in Fig. 7C, indicate that topotecan retention was better for the SM/Chol liposomes when compared to the DSPC/Chol liposomes. After 8 hours at 37˚C, the SM/Chol formulation retained more than twice the amount of topotecan when compared to the DSPC/Chol liposomes.

Figure 7: The SM/Chol liposomal topotecan formulation retains topotecan better than DSPC/Chol liposomal topotecan.

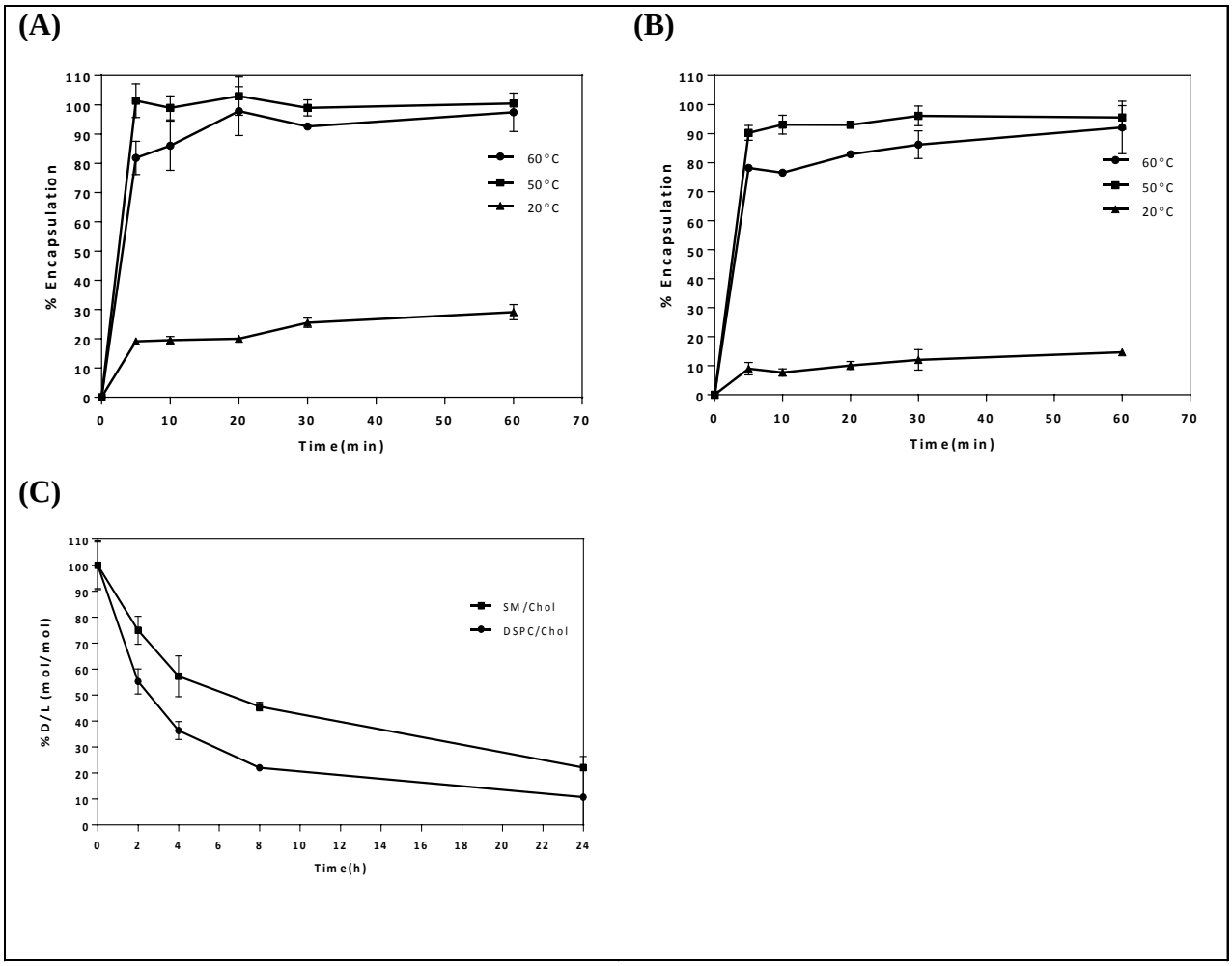


Figure 7: The SM/Chol liposomal topotecan formulation retains topotecan better that DSPC/Chol liposomal topotecan. The liposomes were prepared with unbuffered 300mM copper sulfate (pH3.5) and the divalent cation ionophore A23187 was added to help maintain the pH gradient following addition of topotecan added (0.1 mole topotecan per mole liposomal lipid) at time zero followed by an immediate adjustment of solution pH to 7.5 as described in the Methods. The amount of liposome associated topotecan was determined at the indicated time points as described in the Methods. The results for SM/Chol (panel A) and DSPC/Chol (panel B) liposomes represent the mean ± SD for experiments repeated at least three times. In vitro topotecan release from SM/Chol and DSPC/Chol liposomes (loaded with topotecan using an incubation temperature of 50°C for 30 min) was determined following incubation of the indicated formulation in 80% FBS at 37˚C as described in the Methods. The amount of retained topotecan (% of initial drug (D) to lipid (L) ratio) was determined over a 24 hour time course where the data points represent the mean ± SD for experiments done in duplicate and repeated 3 times (panel C).

Previous studies suggested that increasing the amount of encapsulated topotecan (i.e. increases in final drug-to-lipid ratio) resulted in a formulation that released associated topotecan more rapidly. This was contrary to previous studies suggesting that drug retention was increased in formulations exhibiting higher drug to lipid ratios; an effect thought to be due to drug precipitation within the liposome core (56). For the loading method described here, faster release rates are believed to be due to loss of the transmembrane pH gradient (57) as well as loss of encapsulated copper needed to complex topotecan. To investigate this further SM/Chol liposomes were prepared at a 0.1 and 0.025 drug-to-lipid ratio (mol:mol) and the results have been summarized Figure 8. The drug loading rate at 50°C was comparable for both drug-to-lipid ratios (Fig. 8A), however the rate of drug dissociation at 37°C in the presence of 80% FBS was significantly slower. There was <10% loss of encapsulated topotecan over 24h under these *in vitro* conditions for the 0.025 drug-to-lipid ratio formulation as compared to >80% for the formulations prepared at 0.1 drug-to-lipid ratio. The two liposomal formulations exhibited the same size as determined by Phase Analysis Light Scattering (see Methods) (Fig. 8C). The amount of retained copper in these formulations was measure by atomic absorbance (see Methods) and, as expected, the copper to liposomal lipid ratio, was reduced 7-fold for the formulations prepared at the 0.1 drug-to-lipid ratio whereas the 0.025 drug-to-lipid formulation lost only about 50% of the initial copper levels (Fig. 8D). For the 0.025 drug-to-lipid formulation it can be estimated that the topotecan to copper molar ratio is 0.2, i.e. there is a 5 fold molar excess of copper to topotecan. In contrast the copper to topotecan ratio in the 0.1 drug to lipid formulation is 2, i.e there is a 2-fold molar excess of topotecan compared to copper. Cryo-electron microscopy (CEM) was performed as described in the Methods and revealed that the liposomal formulations of topotecan, regardless of final

drug-to-lipid ratio, exhibited a fine needle-like electron dense structure within the liposomes. As suggested by the representative micrographs shown in Fig. 8E, the SM/Chol liposome without encapsulated topotecan appeared more spherical then formulations containing topotecan. The presence of the electron dense needle-like crystal is comparable to what was reported previously for the DSPC/Chol topotecan formulations (58).

Figure 8: Liposomal topotecan formulations prepared at a 0.025 drug-to-lipid mol ratio retained drug better than liposomes prepared at a 0.1 drug-to-lipid mol ratio.

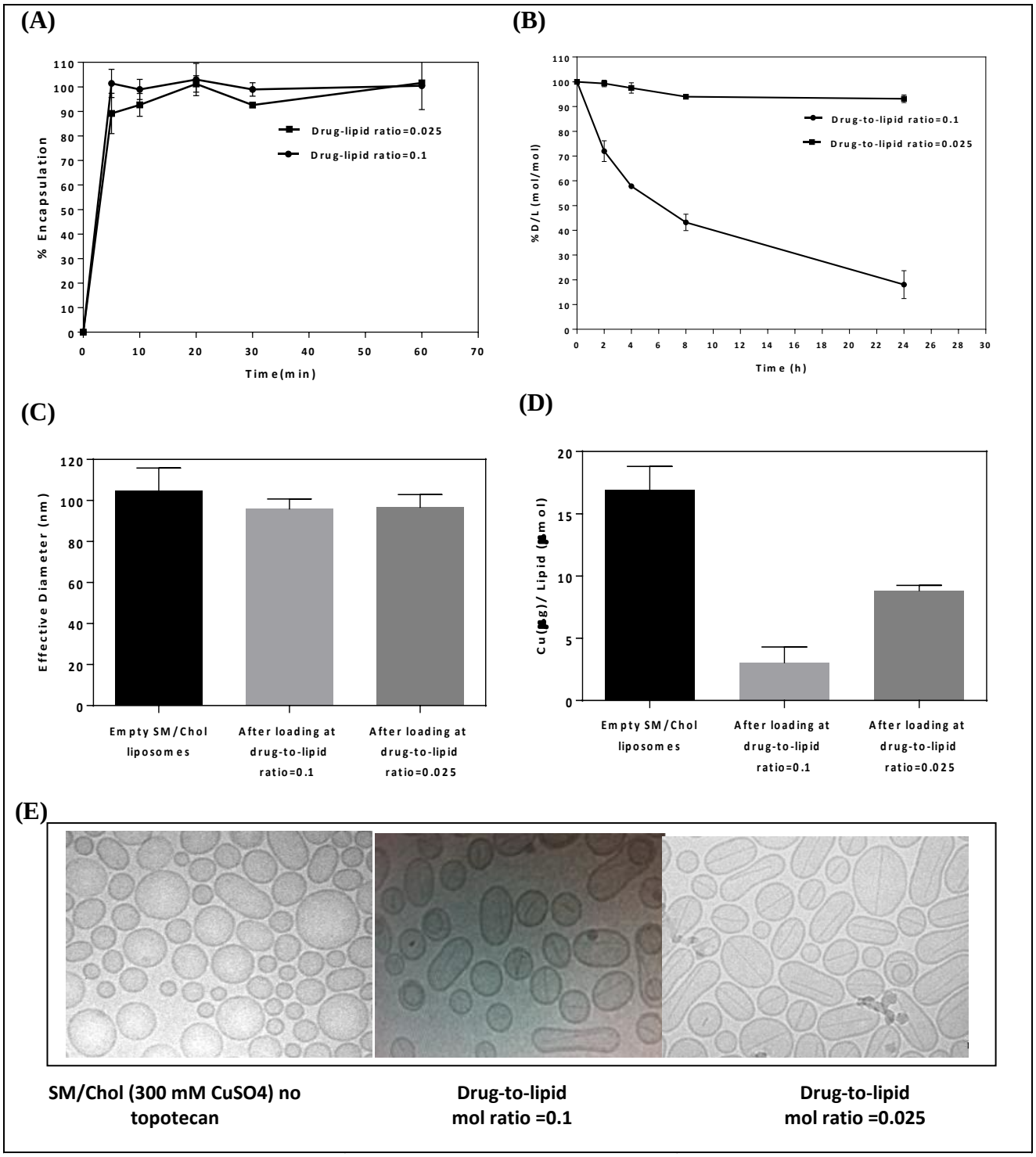


Figure 8: Topotecan was loaded into SM/Chol liposomes at the indicated drug-to-lipid ratio using an incubation temperature of 50°C (panel A). The effect of drug to lipid ratio on topotecan release from SM/Chol liposomal formulations following incubation in 80% FBS at 37˚C over 24 hours is shown in Panel B. Decreases in the drug-to-lipid ratio represents loss of topotecan from the liposomes over time and each data point represent the mean ± SD of experiments repeated at least three times. The size of SM/Chol liposomes with and without encapsulated topotecan (drug-to-lipid mole ratios of 0.1 and 0.025) is shown in panel C; where size was determined using Phase Analysis Light Scattering as described in the Methods. The amount of liposome associated copper (µg copper/ µmole lipid) before and after topotecan encapsulation (drug-to-lipid mole ratios of 0.1 and 0.025) is shown in Panel D, where copper was measured using AAS as described in the Methods. Representative cryo-electron microscopy (CEM) images of SM/Chol liposomes before and after topotecan encapsulation (drug-to-lipid mole ratios of 0.1 and 0.025), panel E, were obtained as described in the Methods.

3.3 In Vivo Characterization of the SM/Chol 0.025 Topotecan-to-Lipid Mole Ratio Formulation.

The formulation optimization efforts summarized above suggest that optimal retention of topotecan in liposomes can be achieved using SM/Chol liposomes prepared at a 0.025 drug to lipid mole ratio. The loading efficiency is always >98% when the drug is incubated with SM/Chol liposomes at 50°C and, although not emphasized above, the loading is critically dependent on ensuring that the external pH after addition of topotecan is maintained at 7.5. Should the pH drop below 7, then the loading efficiency will decrease dramatically. The resulting liposomes were used for the *in vivo* studies summarized below.

Pharmacokinetic and limited biodistribution data for Hycamtin (the clinical product) and SM/Chol liposomal topotecan given as single i.v doses of 5 mg/kg are summarized in Figure 9. This study was conducted in NRG mice bearing established sc LAN-1 neuroblastoma tumours. The amount of the drug and/or liposomal lipid (measured with [3H]-CHE as a liposomal lipid marker, see Methods) was measured at the indicated time points. Following injection of Hycamtin, >99% of the injected drug was eliminated from the plasma

compartment within 1h (Fig. 9A). The topotecan concentration in the plasma was < 0.04 µM 2h after administration and at time points beyond 4h topotecan levels were below the detection limits of the assay used. In contrast, the topotecan levels in the plasma compartment following administration of the SM/Chol liposomal topotecan formulation were detectable over the full 24h time course. The level measured at 24h was greater than that measured at 2h for animals injected with Hycamtin. The difference between the formulations is emphasized by differences in plasma $AUC_{0\text{-}24h}$ for Hycamtin (0.4 µg*hr/ml) and SM/Chol liposomal topotecan (463 µg*hr/ml); where there was an increase in $AUC_{0\text{-}24h}$ of >1000 fold (Table 3). Liposomal lipid elimination following injection of the SM/Chol liposomal topotecan formulation is summarized in Fig. 9B. Greater than 10% of the injected liposomal lipid dose was still in the plasma compartment at 24h. Since greater than 99% of the topotecan was eliminated at this time point, these data indicate that the vast amount of liposome associated topotecan was released from the liposomes over 24 h. This is illustrated by the calculated drug-to-lipid ratio data summarized in Fig. 9C. These data suggest that approximately 90% of the associated drug is released from the SM/Chol liposomes within 8h. The level of topotecan measured at 8 hours represents a 10-fold improvement over the previously described DSPC/Chol formulation (34).

Following administration of SM/Chol liposomal topotecan there was also a significant increase in topotecan accumulation in the sc LAN-1 tumours when compared to animals given Hycamtin (Fig. 9D). 2h following administration of Hycamtin the topotecan levels in the tumour were just at the detection limits of the assay used while at the same time point the topotecan levels were more than 100-fold higher in animals given the SM/Chol liposomal topotecan formulation. There was a 25-fold increase in tumour $AUC_{0\text{-}24h}$ when using the

liposomal formulation. Table 3 compares levels of topotecan in the liver and adrenal gland, tissues known to be common sites of neuroblastoma growth. In both tissues there were significant increases in topotecan levels over time following administration of SM/Chol liposomal topotecan compared to Hycamtin. Topotecan was still detectable in the tumour 24h after the injection, and for up to 8h in the adrenal gland and the liver.

Table 3: Comparison of topotecan plasma and tissue AUC $_{0\text{-}24H}$ following administration of Hycamtin or the SM/Chol liposomal topotecan formulation.

Tissue	Hycamtin AUC $_{0\text{-}24H}$ (µg*hr/ml)	SM/Chol liposomal topotecan AUC $_{0\text{-}24H}$ (µg*hr/ml)
Plasma	0.4	463
Tumour	1	25.168
***Liver**	5	33
***Adrenal gland**	1.6	10.1

** Tissues commonly associated with neuroblastoma metastasis.*

Figure 9: Administration of SM/Chol liposomal topotecan, in comparison to Hycamtin, enhances drug exposure in the plasma compartment, tumour, liver and adrenal gland.

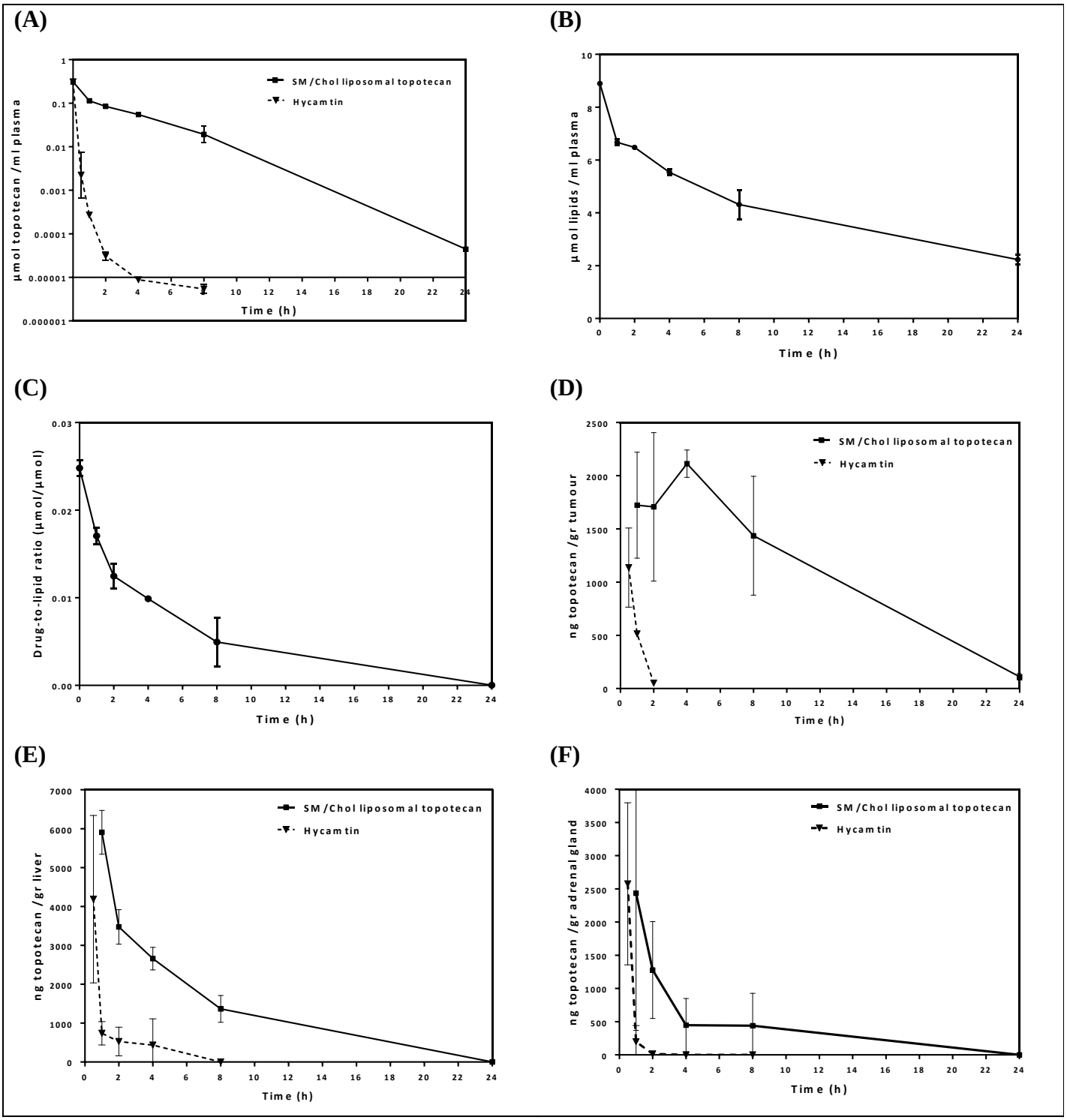


Figure 9: Pharmacokinetics and biodistribution of topotecan and liposomal lipid were assessed after a single 5mg Topotecan/kg dose administered as the SM/Chol liposomal topotecan formulation or Hycamtin. The specified formulations were administered iv into NRG mice with established sc LAN-1 tumours. Plasma topotecan levels following administration of Hycamtin (panel A, filled triangles) and SM/Chol liposomal topotecan (panel A, filled circles) were determined by HPLC analysis as described in the Methods. Plasma liposomal lipid levels following administration of SM/Chol liposomal topotecan are shown in panel B, where liposomal lipid was measured using [^{3}H]-CHE as a liposomal lipid marker as described in the methods. The calculated change in drug-to-lipid ratio in the plasma compartment following administration of SM/Chol liposomal topotecan is shown in panel C. Topotecan levels in tumour, liver and adrenal gland after administration of Hycamtin or the SM/Chol liposomal topotecan formulation are shown in panel D, E and F, respectively. Data points represent mean ± SEM obtained using at least three animals per group.

3.4 Antitumor activity of SM/Chol liposomal topotecan and Hycamtin in animals with established sc and systemic LAN-1 neuroblastoma

Prior to initiating efficacy studies, dose range finding studies in tumour free NRG mice were completed to establish tolerability. The formulations were given i.v. Q7D x 3 and health status of the animals was assessed as described in the Methods. The results have been summarized in Table 4. At 10mg/kg, the SM/Chol topotecan formulation caused significant weight loss (~12%) following the first treatment. The mice recovered within 6 days and no further signs of treatment related morbidity were noted. The maximum feasible dose of Hycamtin was 10 mg/kg and it appeared to be better tolerated then the liposomal formulation as judged by body weight loss. However, there were signs of toxicity comparable to that noted in animals given SM/Chol liposomal at the 10 mg/kg dose. Based on these data, the equitoxic doses were defined as 10 mg/kg and 7.5 mg/kg for Hycamtin and SM/Chol liposomal topotecan, respectively.

Table 4: Tolerability studies in NRG mice following administration of Hycamtin or SM/Chol liposomal topotecan (Q7D X 3).

Schedule	Formulation	Dose (mg/kg)	Major signs of toxicity[3]
(Q7D x 3)	**SM/Chol liposomal topotecan**[1]	2.5	No weight loss
		5	No weight loss
		7.5	1-5% weight loss (4)
		10	5-12% weight loss (2), sunken eyes, scruffy, skin tented, diarrhea (1)
	Hycamtin	10[2]	1-5% weight loss (1),hyperactive, sunken eyes, scruffy, skin tented, hunched, diarrhea, loss of righting reflex(1).

[1] Prepared at 0.025 drug-to-lipid ratio (mol:mol), particle size ~100nm.
[2] 10 mg/kg was the Maximum Feasible Dose (MFD) of the clinical product available.
[3] Established using a Standard Operating Procedure designed to assess signs of toxicity. The staff recording the signs of toxicity were blinded to the treatment groups.

The LAN-1 neuroblastoma model development studies are summarized in Figure 10 which summarizes the survival curves for untreated animals and representative micrographs confirming the presence of an undifferentiated neuroblastoma with varying degree of stroma and the presence of Homer-Wright pseudorosettes characteristic of neuroblastoma. For the sc model, the Kaplan-Meier survival plot were defined based on the humane endpoint for tumor growth (≥800 mg) and suggested a median survival time of 30 days (Fig 10B). All animals in the study reached the humane end point by day 35. The systemic LAN-1 neuroblastoma model was generated by intracardiac injection of the LAN-1 cells as described in the Methods. Following intracardiac injection of LAN-1 neuroblastoma cells, all animals reached their humane endpoints by day 50. Necropsy of the animals indicated that the tumour growth was largely confined to the livers which were enlarged (liver weighs were 3-times that of control NRG mice) and infiltrated with metastatic nodules.

Figure 10: LAN-1 neuroblastoma model development studies.

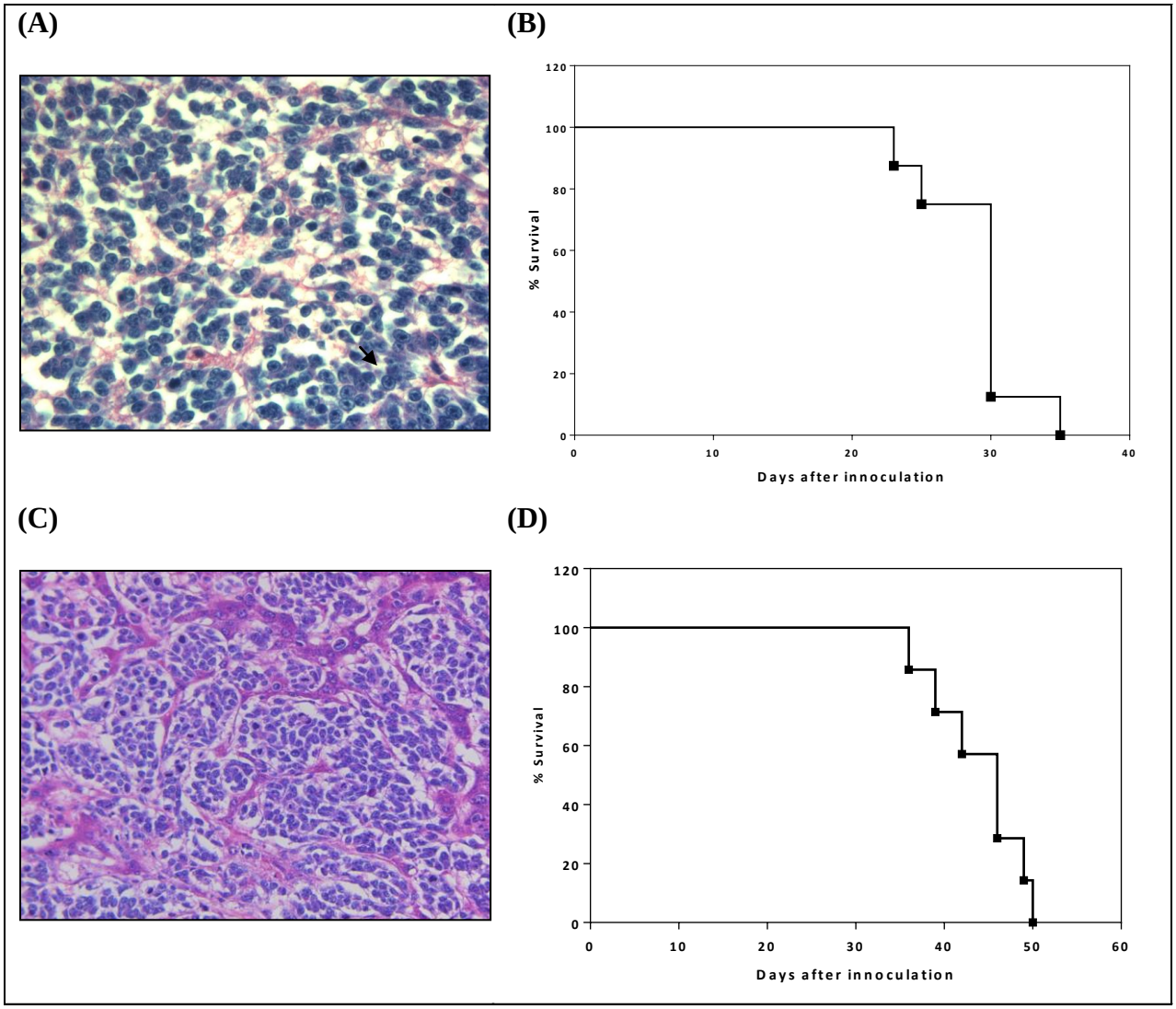


Figure 10: LAN-1 neuroblastoma model development studies. Hematoxylin and Eosin staining of tumours harvested after subcutaneous injection of LAN-1 neuroblastoma cells (panel A) show Homer-Wright pseudorosettes (black arrow) characteristic of neuroblastoma. Kaplan-Meier survival plot for animals bearing subcutaneous LAN-1 tumours, where the humane endpoint was defined by tumours exceeding 800mg (panel B). Kaplan-Meier survival plot for animals given i.c. injections of LAN-1 cells, where the humane endpoints were defined by body condition score, weight loss and behavioural changes (see Methods) (panel D). Animals that succumbed to tumour progression following ic injection of LAN-1 cells exhibited large livers with numerous associated tumours. A hematoxylin and eosin stain section of liver associated tumours is provided in panel C.

The antitumor actity of topotecan administered (i.v., Q7D x3) as the SM/Chol liposomal formulation or Hycamtin was evaluated when treatment was initiated on day 14 (first treatment) and survival was measured based on defined humane endpoints as described in the Methods. The results, summarized in Figure 11, indicate in both models that the therapeutic activity of the SM/Chol liposomal topotecan was significantly greater ($p<0.001$) than Hycamtin administered at 2-times the dose. The median survival time (MST) for control animals was 30 and 45 days for the sc and systemic models, respectively. NRG mice bearing sc neuroblastoma tumours treated with SM/Chol liposomal topotecan at 5mg/kg exhibited an 87% increase in MST compared to a 58% increase in life span for Hycamtin administered at 10 mg/kg. For the NRG mice with the systemic neuroblastoma model, treatment with SM/Chol liposomal topotecan at 5 mg/kg increased the MST by 50%, with some of the animals surviving up to 92 days. In contrast, when treated with Hycamtin at its maximum feasible dose (10mg/kg) the % increase in MST was 39%.

Figure 11: SM/Chol liposomal topotecan exhibits improved therapeutic activity compared to 2-times the dose of Hycamtin.

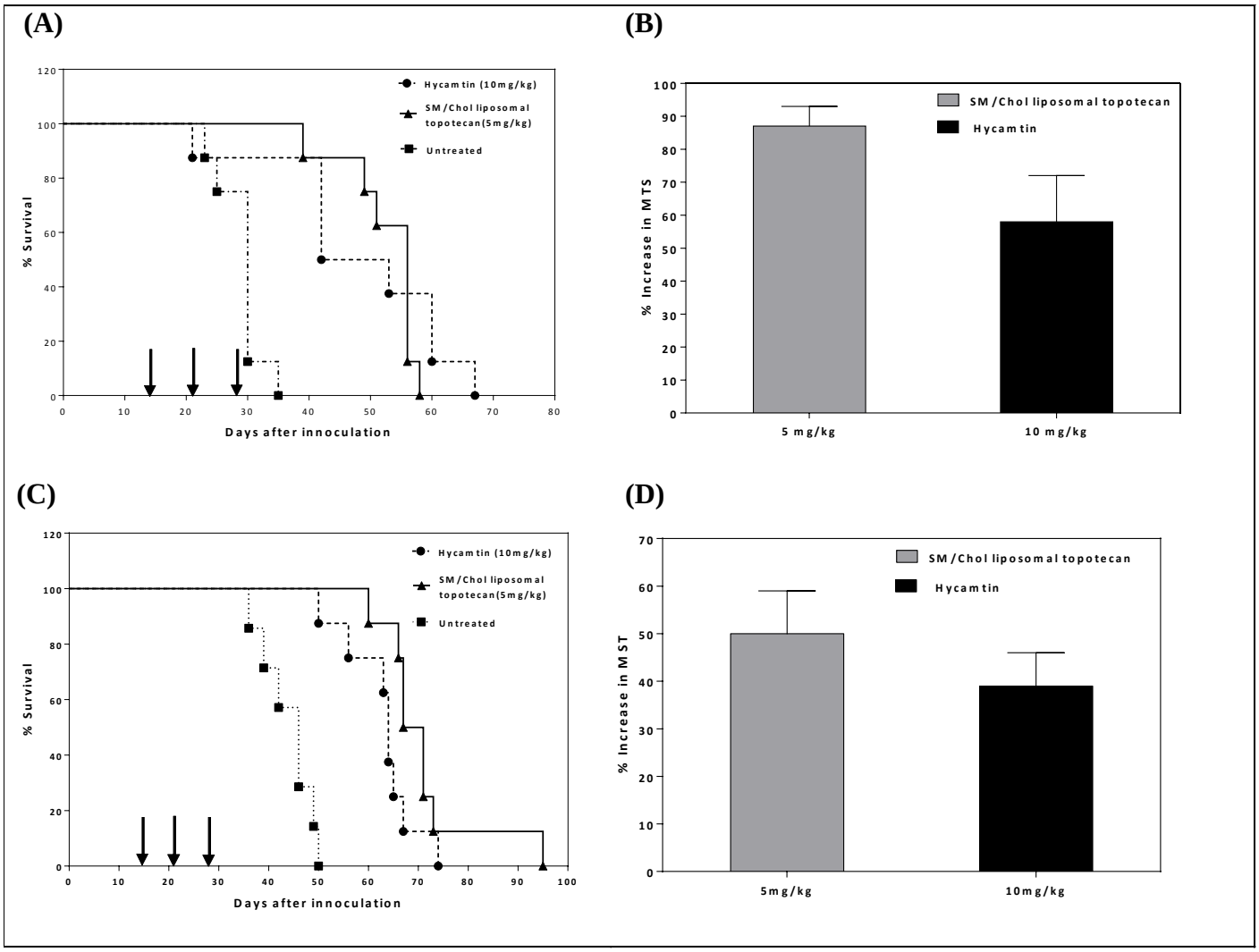


Figure 11: The anti-tumour activity of topotecan was assessed against the sc (panels A and B) and systemic (panels C and D) LAN-1 neuroblastoma models following i.v administration of SM/Chol liposomal topotecan (5mg/kg) or Hycamtin (10 mg/kg) where the drug was administration intravenously on day 14, 21 and 28 (arrows). Kaplan-Meier survival plots (A, C) and the percent increase in Median Survival Time (MST) (B, D) are shown. Survival curves were determined based on when the mice reached a humane endpoint as defined in the Methods. The day of death was recorded one day following euthanasia. The efficacy studies were completed using groups of 8 mice per dose tested; long-rank test was used to compare overall survival (OS) ($p<0.001$); One way ANOVA was used to compare MST ($p<0.05$).

It should be noted that efficacy studies with SM/Chol liposomal topotecan were conducted at 10 mg/kg also, however increase in the dose did not engender further increases in therapeutic activity when compared to the 5 mg/kg dose (results not shown), suggesting that the lower dose may represent the maximum efficacious dose for these models. Notably, the 5mg/kg dose of SM/Chol liposomal topotecan caused no signs of morbidity in the dose range finding studies (see Table 4).

4 DISCUSSION

Neuroblastoma is the most common malignancy during early childhood (30). Hycamtin is one of the drugs that is routinely incorporated into the treatment protocols for this aggressive cancer (19, 20, 22). Topotecan is cell cycle specific agent and is most lethal during the S-phase, and optimal activity is achieved when the drug exposure time increases (31, 59). This effect is exemplified by the data summarized in Figure 6. Prolonged exposure time to the drug allows a greater proportion of cells to enter the S-phase, consequently enhancing the therapeutic activity of the drug. Enhanced exposure times can be achieved using a number of approaches including drug infusions (60,61), more frequent dosing (i.e metronomic dosing (62-64)) and nano-scale drug delivery systems such as the liposomal formulations described here. This discussion will focus on comparing these approaches and the therapeutic potential of the liposomal formulation to be adapted for use in a combination setting with therapeutic antibodies targeting GD2 as well as radioactive metaiodobenzylguanidine (MIBG).

As noted in the introduction, topotecan's activity depends on maintaining an intact lactone ring. The importance of the lactone form is not only for the drug's ability to interact with the topoisomerase I/ DNA complex, but it is also necessary in order to cross tumour cell membranes through passive diffusion (32). The lactone ring of topotecan undergoes a reversible pH-dependent hydrolysis into the carboxylate (ring open) form of the drug, which is charged and less able to cross cell membranes. The clinical product (Hycamtin) is provided at a sufficiently low pH to maintain the drug in the lactone form. However, the plasma concentrations of the inactive carboxylate form of topotecan exceeds the lactone form within 15 min after completing the 30 minute infusion typically used when administering the drug (32). Investigators have used continuous infusion methods to administer topotecan with

improved response rates in some cancers (60) however this likely a consequence of maintaining therapeutically active levels of the lactone form of the drug over extended time periods and are not due to changes in the stability of the drug. In contrast, liposomal formulations of topotecan, as described here, will maintain the drug in the lactone form for extended time periods following administration. This is due to the fact that encapsulated drug in the plasma compartment is maintained in a low pH environment. It should be noted that a previous clinical study in children with relapsed/refractory tumors, including those with neuroblastoma, concluded that 21-day continuous infusion of topotecan provided limited therapeutic benefit (61). Based on improved chemical stability of topotecan when using the liposomal formulation, it is hoped that treatment outcomes could be improved if the candidate formulation is used to treat neuroblastoma patients.

Several liposomal formulations of topotecan have been described (49-51,65), and all demonstrate that topotecan is retained in its active lactone form following administration. The formulation approach described here is unique because the encapsulation and drug retention is dependent, in part, on copper encapsulated in the liposomes (22). Previous studies demonstrated that when compared to formulations without copper, the liposomal formulations prepared with copper exhibit better drug retention. The formulation modification described in this report relied on use of SM/Chol to engender further decreases in the rate of topotecan dissociation from the liposomes and a reduction in the topotecan to lipid ratio, which effectively meant that greater levels of copper were retained in the formulation. The resulting formulation is less toxic than the previously described liposomal topotecan formulation and also exhibited enhanced circulation longevity. The SM/Chol liposomal formulation increased

topotecan plasma $AUC_{0\text{-}24h}$ by 1000-fold when compared to the $AUC_{0\text{-}24h}$ of Hycamtin and by 3-fold when compared to the previously described Topophore C™ formulation (22).

This is the first report assessing the therapeutic activity of liposomal topotecan in models of neuroblastoma. Activity was established in sc and systemic models of neuroblastoma established in NRG mice following inoculation of LAN-1 cells. SM/Chol liposomal topotecan administered at 5 mg/kg was more effective then Hycamtin given at 10 mg/kg. Although an improvement, the magnitude of the effect was not as great as was expected from the changes in topotecan exposure achieved in the plasma compartment and tumour. The results suggest that the maximum efficacious dose of SM/Chol liposomal topotecan is significantly lower than the maximum tolerated dose, indicative of an improved therapeutic index for the liposomal formulation. It can be suggested that further improvements in therapeutic potential will require the use of this formulation in combination with other targeted therapeutics. This is consistent with the pioneering efforts of Ponzoni et al. who described the use of anti-GD2 antibody targeted liposomes (66) and his more recent efforts combining these immunoliposomes with siRNA therapeutics as well as ALK inhibitors (67). The novel SM/Chol topotecan formulation has considerable pharmaceutical potential, and future studies will be assessing its activity in combination with [^{131}I]-MIBG and Unitux; studies that will assess whether the additional therapeutic agents exhibit improved therapeutic activity when associated with liposomal topotecan as opposed to simply being used in combination.

5 FUTURE DIRECTIONS

The novel SM/Chol topotecan formulation described herein has considerable pharmaceutical potential. This formulation showed significant antitumor activity in models of neuroblastoma and should be further investigated in wider panel of neuroblastoma cell lines, including patient derived xenografts.

To assess the full potential of SM/Chol topotecan formulations, various dosages and schedules of the treatment should be investigated, including metronomic administration. Our preliminary data showed (Fig.12) an improvement in antitumour activity of both Hycamtin and SM/Chol topotecan formulation when administrated in a lower dose of 2mg/kg 3 times a week (M, W, F x3). At this dose both formulations were well tolerated with no signs of toxicity or weight loss.

Figure 12: Therapeutic activity of SM/Chol liposomal topotecan and Hycamtin following metronomic administration.

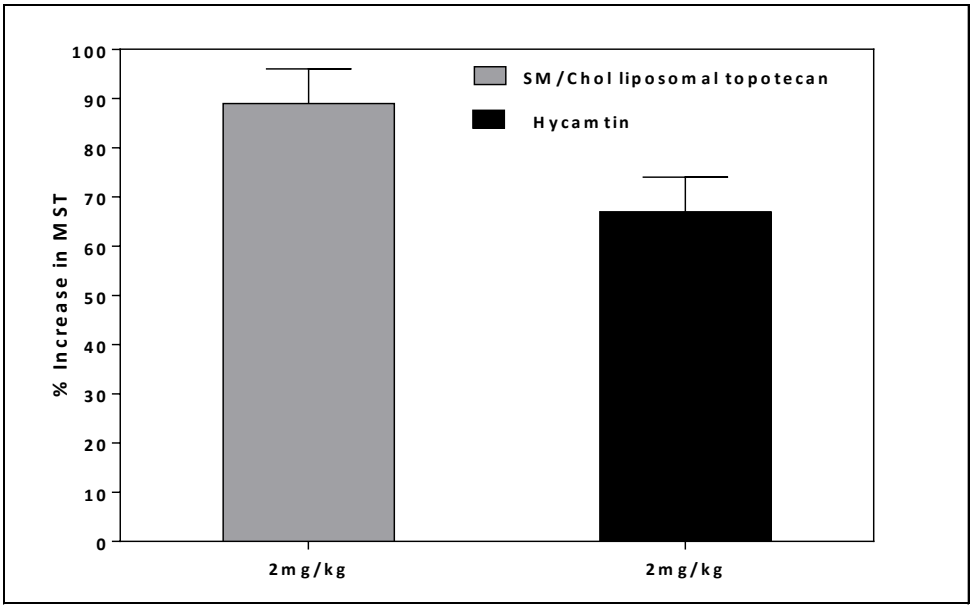


Figure 12: Percent increase in Median Survival Time (MST) of NRG mice bearing systemic LAN-1 neuroblastoma tumours, following i.v administration of SM/Chol liposomal topotecan or Hycamtin at equivalent dose of 2mg/kg at (M.W,F x3).

As discussed in the introduction, new targeted therapies are currently being incorporated into neuroblastoma treatment protocols. Those include radiotherapy using [^{131}I]-MIBG and immunotherapy with anti-GD2 antibody. Combination therapy comprising of anti-GD2 and SM/Chol topotecan should be investigated for synergism, assessing the SM/Chol topotecan formulation in combination with free or covalently associated anti-GD2 antibody. Conjugation of anti-GD2 to the SM/Chol topotecan may improve the biodistribution of the antibody, increasing its accumulation in the tumour and reducing systemic toxicity (severe pain) caused by undesirable antibody bonding to the peripheral nerves. Due to topotecan radiosensitizing properties, SM/Chol topotecan should also be evaluated in combination with [^{131}I]-MIBG, as well as in combination studies including both [^{131}I]-MIBG and anti-GD2 antibody.

It is anticipated that concurrent use of radiotherapy in combination with conventional therapeutics as well as a biological agent will result in optimal therapy that should be tested in in-vivo models of NB.

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