COPPER-BASED THERAPEUTICS: CREATING A FORMULATION PLATFORM TO FACILITATE DEVELOPMENT OF AN EMERGING DRUG CLASS

by

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Abstract:

Copper-based therapeutics (CBTs) are a promising class of drug candidates that have been shown to have anti-cancer activity. One of the major challenges associated with developing this emerging class of medicines, however, is their poor aqueous solubility (< 1 mg/mL). This has impeded the transition of these agents from preclinical candidate drugs to lead candidates suitable for use in humans. This thesis describes a novel method for preparing copper-based therapeutics suitable for parenteral administration. The method involves synthesis of the copper complexes in the core of liposomes (nanoscaled spherical structures with an aqueous core surrounded by a lipid bilayer). The method provides a simple, transformative solution enabling the development of CBTs as viable candidate anti-cancer drugs—a brand new class of therapeutics for cancer patients.

The potential of this technology was exemplified with several compounds. Diethyldithiocarbamate (DDC) is the primary metabolite of disulfiram, an approved drug for alcoholism that is being repurposed for cancer. The anti-cancer activity of DDC is dependent on complexation with copper ($Cu(DDC)_2$), which is a highly insoluble complex. An injectable $Cu(DDC)_2$ formulation was prepared through synthesis of $Cu(DDC)_2$ inside the aqueous core of liposomes, and the resultant formulation exhibited significant therapeutic activity in two different rodent models of cancer. Clioquinol (CQ) is an approved anti-microbial agent that has potential anti-cancer activity. The activity of CQ is enhanced in some cancer cell lines when complexed to copper. The copper-CQ complex ($Cu(CQ)_2$) is sparingly soluble in aqueous solution but it is demonstrated that $Cu(CQ)_2$ can be synthesized inside liposomes. The therapeutic activity of the resultant formulations was, however, not significant. In an effort to identify cancer indications that may benefit most from treatment with CBTs, the therapeutic potential of DDC, pyrithione (Pyr), plumbagin (Plum), 8-hydroxyquinoline (8-HQ) and CQ copper complexes was determined in a panel of cancer cell lines that differed in their sensitivity to cisplatin. $Cu(DDC)_2$, $Cu(Pyr)_2$, $Cu(Plum)_2$ and $Cu(8\text{-}HQ)_2$ showed IC_{50} values less than that of cisplatin in all tested cell lines. This work suggests that future studies with CBTs for cancer should focus on platinum refractory cancers.

Lay Summary:

Copper complexes have been shown to be anti-cancer, anti-microbial, anti-viral and anti-inflammatory agents. While this highlights the benefits of these types of compounds, their insolubility has thwarted their transition to clinic. This thesis describes a method for solving this challenge through the synthesis of copper complexes inside liposomes (tiny bubbles composed of fats). Through this technology it was possible to evaluate the copper complexes of diethyldithiocarbamate (a metabolite of the anti-alcoholism drug disulfiram) and clioquinol (an FDA approved anti-fungal drug) as anti-cancer agents. These are the first studies to use these copper complexes in animal models, contributing valuable insight into possible approaches to treating cancer with copper-containing medicines. A platform technology has been developed which provides a transformative solution to solve solubility challenges of metal complexes, thus enabling the development of this novel class of drugs for the first time.

Preface:

The studies presented in this dissertation were conceived and designed by M. Wehbe and MB. Bally.

Chapter 1 was adapted from the following review and perspective: Wehbe M, Leung A, Abrams M, Orvig C and Bally MB (2017) **A perspective – Can copper complexes be developed as a novel class of therapeutics?** *Dalton Transactions (In Press)*. Wehbe M, Chernov L, Chen K, and Bally MB (2016). **PRCosomes: pretty reactive complexes formed in liposomes.** *Journal of Drug Targeting*. 24(9):787-96. Both manuscripts were organized, written and edited by Wehbe M. The perspective was edited by Leung, A, Abrams M, Orivg C and Bally MB. Figures 1.1, 1.2 and 1.6 were illustrated by Leung A and conceived by Wehbe M, Leung A and Bally MB. The review was edited Chernov L, Chen K and Bally MB. Figures (1.4 and 1.5) were all prepared and illustrated by Wehbe M.

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Studies in Chapter 4 are published in the following article: Wehbe M, Anantha M, Shi M, Leung AWY, Dragowska WH, Sanche L and Bally MB (2017). Development and optimization of an injectable formulation of copper diethyldithiocarbamate, an active anti-cancer agent. *International Journal of Nanomedicine.* Wehbe M performed all experiments in Figures 4.1, 4.3, 4.5, 4.6, 4.7 and 4.8 with assistance from Anantha M (analytical support). Figure 4.2 was produced by Wehbe M, Leung A and Dragowska W. Leung A conducted the reactive

oxygen species assay and performed the Western blot with Wehbe M. The flow cytometry experiment was conducted by Wehbe M with Dragowska W and Leung A. The design and formulations were prepared by Wehbe M while Shi M performed the convection enhanced delivery study at the Université du Sherbrooke as shown in Figure 4.4. Sanché L contributed to the design of the convection enhanced delivery study and editing of the manuscript. This publication was written by Wehbe M with editing by Bally MB.

The research studies in Chapter 5 have been submitted as a manuscript: Wehbe M, Malhotra A, Anantha M, Lo C, Backstrom I, Dos Santos N and Bally MB (2017). **Repurposing the topical antifungal clioquinol for the treatment of cancer.** All $Cu(CQ)_2$ experiments were performed by Wehbe M with assistance from Anantha M (analytical support) and Malhotra A . The cell culture experiments were conducted by Wehbe M, the U251 and MV-4-11cell lines were performed by Lo C and Backstrom I. The model development for the A2780-CP model was conceived by Wehbe M with Dos Santos N. This publication and figures were written and prepared by Wehbe M with editing by Bally MB.

Studies in Chapter 6 are published in: Wehbe M, Lo C, Leung A, Dragowska WH, Ryan G, Bally MB (2017). *Copper complexes of bidentate ligands exhibit potent anti-cancer activity regardless of platinum sensitivity status.* ***Investigational New Drugs (In press).*** **Wehbe M selected copper complexes and designed experiments. Wehbe M and Leung A selected the cancer cell lines for these studies. The screen was conducted by Lo C with assistance from Wehbe M. Data analysis was performed by Wehbe M and Lo C with assistance from Dragowska and Ryan G. The liposomes were prepared by Wehbe M for the *in vivo* study. This publication and figures were written and prepared by Wehbe M with editing by Dragowska W and Bally MB.**

This thesis was written by Wehbe M with editing provided by Burt H, Ensom M, Webb M, Yip S and Bally MB.

The Animal Care Committee at the University of British Columbia examined and approved the use of animals for the studies in this thesis under the following Animal Care Certificates: A10-0171 and A14-0290.

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List of Abbreviations:

^{3}H-CHE	^{3}H-cholesteryl hexadecyl ether
AAS	Atomic absorption spectroscopy
AD	Alzheimers disease
ALDH1	Acetaldehyde dehydrogenase 1
ATSM	Diacetyl-2,3-*bis*(N^4-methyl-3-thiosemicarbazone)
AUC	Area under the curve
BD	Biodistribution
Bpy	2,2 -bipyridine
BTIC	Brain tumour initiating cell
BTZ	Bortezomib
CBDCA	Cyclobutane dicarboxylic acid (Carboplatin)
CBT	Copper-based therapeutic
CC	Copper chaperone
CDC	Copper drug complex
CDDP	*cis*-diamminedichloroplatinum (II) (Cisplatin)
CED	Convection enhanced delivery
CEM	Cryo-electron microscopy
Chol	Cholesterol
CLC	Copper ligand complex
COPD	Chronic obstructive pulmonary disease
CQ	Clioquinol
CTR1	Copper transporter 1
CTR2	Copper transporter 2
Cu	Copper
$CuSO_4$	Copper sulfate
DC-Chol	3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DDC	Diethyldithiocarbamate
DMSO	Dimethyl sulfoxide
DMT	Divalent metal transporter
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOX	Doxorubicin
DSF	Disulfiram
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG_{2000}	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[carboxy(polyethylene glycol)-2000]
DSPG	1,2-distearoyl-***sn***-glycero-3-phospho-(1'-***rac-***glycerol)
EDTA	Ethylenediaminetetraacetic acid

GTSM	Glyoxal-bis(N4-methylthiosemicarbazone)
H4ML	2-acetylpyridine-4-methylthiosemicarbazone
HBEpC	Human bronchial epithelial cells
HD	Huntington's disease
HIF-1	Hypoxia inducible factor - 1
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HQ	Hydroxyquinoline
HSA	Human serum albumin
I.V.	Intravenous
Mg	Magnesium
Mn	Manganese
MST	Median survival time
MTD	Maximum tolerated dose
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffered saline
Phen	1,10-Phenanthroline
PK	Pharmacokinetic
Plum	Plumbagin
Pt	Platinum
Py	Pyrrolidine dithiocarbamate
Pyr	Pyrithione
QELS	Quasi-electric light scattering
ROS	Reactive oxygen species
Sala	N-(2-hydroxybenzyl)-D,L-alanine
SEM	Standard error of the mean
Ser	L-serine
SH	Sucrose Hepes
SHE	Sucrose Hepes EDTA
SM	Sphingomyelin
SOD	Superoxide dismutase
SD	Standard deviation
Tdp	2-[(2-(2-hydroxyethylamino)- ethylimino)methyl]phenol
TEA	Triethanolamine
TETA	1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid)
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
Zn	Zinc

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To my family,

I only did this

to be the favourite.

1. Introduction

1.1 Thesis Overview

Liposomes, small vesicular structures comprising a lipid bilayer surrounding an aqueous core, have emerged as a successful drug delivery platform, whereby associated drugs or drug candidates are improved in terms of their stability, biodistribution and ultimately therapeutic potential[1, 2]. Liposomes have been used to formulate a variety of agents including those used to treat cancer[3-5], microbial infections[6, 7], inflammatory conditions[8, 9], etc [10, 11]. The methods used to prepare and characterized liposomal drug formulations have proven to be robust, and many formulations have successfully transitioned from the research lab to the clinic[2]. To date, much of the focus on development of liposomal drugs has been directed to reformulation of approved anti-cancer agents. Further, part of the success of these formulations was due to the development of robust ways to prepare liposomes at a scale and in a manner suitable for use in humans. In this context, a method, whereby dried lipids are hydrated in a solution of choice and then extruded under moderate pressure through filters which have defined pore sizes has been developed [12]. The liposomes produced in this way exhibit a mean diameter that is close to the size of the pores, i.e. if the pore sizes are 0.1 μM, then the resultant liposomes will be about 0.1 μM. Further, a method, referred to as remote loading, facilitated the encapsulation of selected drugs into preformed liposomes[13-15]. As exemplified by drugs such as doxorubicin, vincristine, and irinotecan, they can be added to the outside of liposomes exhibiting a transmembrane pH gradient (inside acid)[14, 16, 17]. These drugs have protonizable amine groups which at pHs close to the pKa are maintained in a neutral form. The uncharged form of the drug can cross the liposomal membrane but once in the acidic interior the drug becomes protonated, charged and membrane impermeable. Greater than 98% of the drug added to the liposomes can become trapped inside using this remote loading methods[13]. A number of different methods have been used to generate transmembrane pH gradients including encapsulation of a low pH buffer (e.g. citrate buffers at pH 4.0)[14, 18]; ammonium sulfate solutions (the ammonium ion can leave the liposomes leaving behind a proton and thus generating a pH gradient)[19, 20];

and the use encapsulated divalent metals (Mn^{2+}, Mg^{2+}, Cu^{2+}, etc) in combination with a divalent metal ionophore (e.g. A23187 aka- Calcimycin)[21, 22] which exchanges a metal ion for 2 protons and thus generates a pH gradient. Previous studies, however, have shown that certain drugs with functional groups capable of coordinating with divalent metals can be used to achieve drug encapsulation through the formation of a metal complex[23, 24]. These studies have been conducted using drug agents with both protonizible amine and metal binding functional groups. Thus, there exists a potential for using metal complexation reactions to associate compounds of interest inside liposomes which offers an opportunity to formulate drug candidates that were previously considered unsuitable for liposome formulations through remote loading.

The research summarized in this thesis explored the potential for using metal complexation reactions to prepare formulations of novel agents with anti-cancer activities. More specifically, the thesis research focuses on the development of copper-based therapeutics (CBTs), which define any compounds with functional groups that can bind Cu(II) and exhibit some therapeutic activity in particular for the treatment of cancer. There already exists a plethora of agents developed through the use of inorganic chemistry approaches to create compounds with anti-cancer activity. Many of these are consider class IV compounds in the biopharmaceutical classification system; exhibiting low permeability and low solubility. Class IV compounds are perhaps the most challenging to develop for therapeutic applications and would typically not be consider for liposomal formulations. The research described in this thesis suggests that CBT specifically and perhaps metal-based therapeutics in general can be formulated in liposomes. **It was hypothesized that CBTs can be synthesized inside liposomes and that the resulting formulations would be suitable for further pharmaceutical development as a novel class of anti-cancer agents**. For the first time, it is shown that copper complexes can be synthesized inside liposomes, where a reaction occurs between encapsulated copper and a compound with known metal binding groups that have been added to the outside of the liposome. The use of CBTs targeting cancer required the development of strategies to formulate these sparingly soluble compounds in a manner suitable for parenteral administration. This

approach has solved the solubility challenges faced when considering the development of CBTs.

The thesis research was built around four experimental aims:

Aim 1: *To determine if CBTs can be synthesized inside liposomes which contain copper and to determine if the method defined a platform approach for developing CBT formulations suitable for intravenous administration.*

Aim 2: *To evaluate the therapeutic potential of a novel copper diethyldithiocarbamate formulation discovered as a result of efforts designed to repurpose disulfiram, for use in the treatment of cancer.*

Aim 3: *To evaluate the anti-cancer potential of clioquinol, a topical antifungal, when prepared as a copper drug complex.*

Aim 4: *To determine whether CBTs are effective in platinum resistant cancers.*

1.2 Copper-Based Therapeutics as an Alternative to Platinum

In the 1970s, platinum-based complexes emerged as the focus of metal-based synthesis efforts owing to the widely successful drug cisplatin (CDDP). This effort spawned the development of carboplatin (CBDCA), oxaliplatin and other platinum agents that exhibit potent anti-cancer activity when used as a single agent and in combination [25]. Efforts focused on developing copper-based therapeutics (CBTs) have been directed, in part, towards replacing platinum therapeutics, which are incredibly successful in cancer therapy but do suffer from major drawbacks [26, 27]. The use of platinum, which is not native to the human body, results in many serious adverse effects such as nephrotoxicity [28], neurotoxicity[29] as well as ototoxicity and myelosuppression [30]. Furthermore, the development of platinum resistance has created a major clinical problem [31]. Platinum-based therapeutics such as CDDP[32], CBDCA[33, 34] and oxaliplatin[35] are best known for their DNA damaging abilities[30, 36]. It is important to note that oxaliplatin is the only platinum agent that is able to overcome platinum resistance through the formation of DNA adducts that are distinct from those formed with cisplatin and carboplatin [37, 38]. In contrast, copper-complexes developed as anti-cancer agents have been noted to induce cancer cell death through a variety of mechanisms including proteosome inhibition,[39-42]

generation of reactive oxygen species (ROS) [43-45] and DNA damage [46, 47], amongst others.

Therefore, there has been a great deal of interest in using copper (Cu) in place of platinum as it would be better tolerated due to the existence of natural biological pathways to regulate Cu levels and to detoxify the metal where necessary. Recently, Cu has re-emerged as the focus of inorganic synthesis programs wherein Cu-complexes have been prepared for different indications including cancer[48-50], viral infections [51], inflammatory diseases[52, 53], microbial infections[54-57] and cosmetic applications[58, 59]. Although there has been some success with Cu(II)-indomethacin complexes (currently used in veterinary medicine as an anti-inflammatory drug) [53, 60, 61], no CBT has been approved for medical use in humans. Despite the increase in the number of articles describing Cu-complexes as therapeutics, there has been limited transition from *in vitro* to pre-clinical *in vivo* studies[62].

Although not a single copper complex has gained regulatory approval for human use it should be noted that the casiopeínas are a class of copper complex compounds (specifically, casiopeina III-ia (aqua, 4,4-dimethyl-2,2′-bipyridine, acetylacetonato copper(II) nitrate)) that have entered Phase I clinical trials in Mexico[63]. In general, this class of compounds exhibits very low aqueous solubility. While it is relatively easy to perform *in vitro* experiments using solvent systems such as dimethyl sulfoxide (DMSO; a class 3 solvent not suitable for parenteral injection in concentrations >0.05%/day)[64] the poor aqueous solubility creates a pharmaceutical barrier for further pre-clinical and clinical evaluations. As noted in section 1.4, Cu(II) interacts with N, O and/or S binding moieties and this interaction results in the removal of two hydrogen bond-acceptor groups from the system, leading to a net-increase in hydrophobicity. Thus, many CBT candidates are insoluble in aqueous solutions at therapeutically relevant concentrations.

To address the solubility issue associated with CBTs, classical medicinal chemistry approaches have been used; approaches that involve chemical synthesis of more water soluble analogues of the CBTs of interest [65]. The other approach involves a discovery that is the topic of this thesis; whereby the synthesis of CBT of interest can be done within liposomes (Figure 1.1) [66]. Essentially the liposomes serve as nanoscale reaction vessels

supporting the synthesis of the Cu-complexes in a form that keeps the complex in a solution that is suitable for administration (referred to as Metaplex™ complex synthesis).

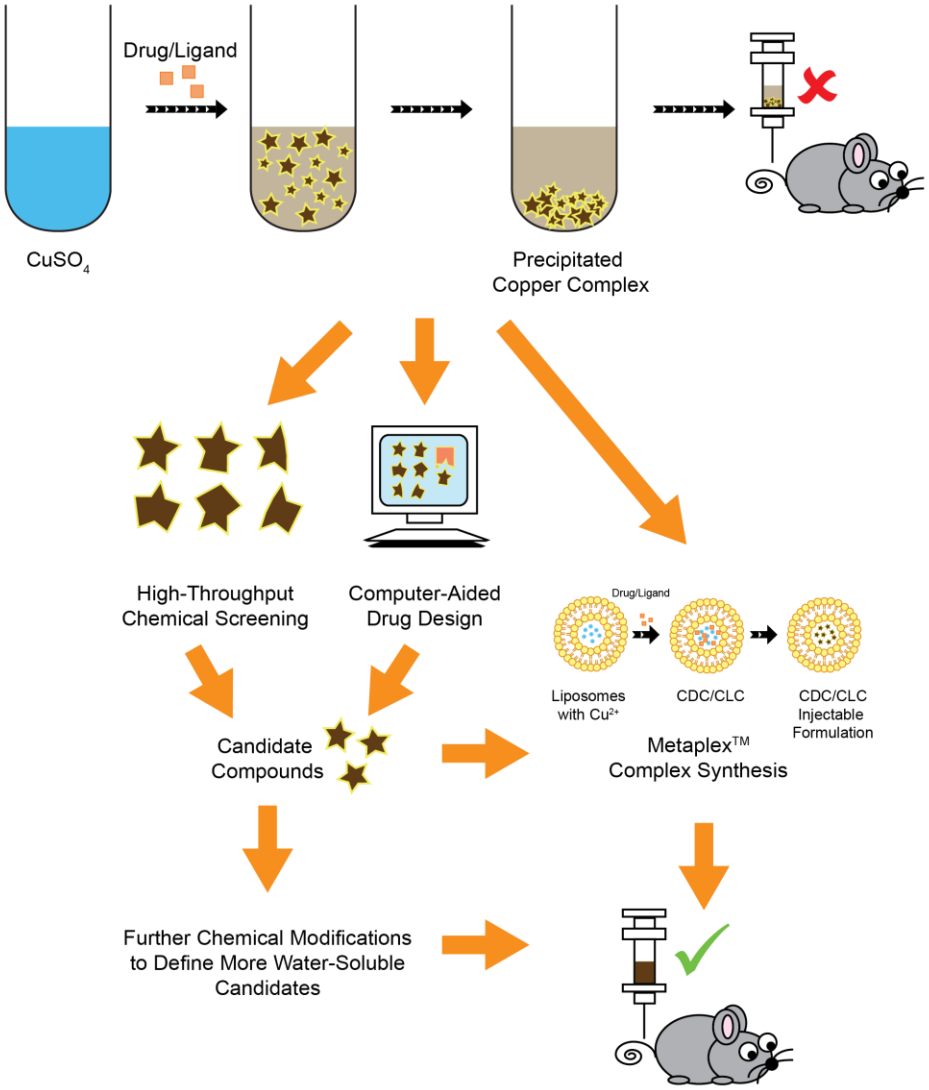


Figure 1. 1 Overcoming the pharmaceutical challenge of developing copper-based therapeutics. This figure illustrates the major solubility issue associated with most copper/metal complexes. The problem of metal complexes being insoluble in aqueous solutions under physiological conditions can be addressed via two main methods: modification of chemical structure or synthesis of the metal complex within lipid-based nanoparticles. High-throughput medicinal chemistry can be performed in two ways: 1) chemical synthesis of analogue libraries of the complex of interest followed by high-throughput screening for biological activity, 2) or computer-aided drug design of analogues of the complex of interest using techniques such as molecular

docking, followed by high-throughput screening for biological activity. This result of these processes is a list of candidate drugs that may or may not be soluble in aqueous solutions. Insoluble drug candidates would require further chemical modifications until a more soluble and at least equally active candidate can be identified. In contrast, the use of the Metaplex™ technology requires no chemical modification of the metal complex. It involves the preparation of copper/metal-containing liposomes by adding the appropriate drug/ligand to liposomes which contain entrapped copper/metals. Depending on liposomal lipid composition, temperature and copper to drug/ligand ratio, this process will result in the generation of the copper drug complex/copper ligand complex inside the liposomes; forming a solution that is suitable for parenteral administration. Metaplex™ can also be used to prepare formulations of poorly water soluble drug candidates identified from medicinal chemistry-based screen thus substantially reducing the amount of time required to generate a lead candidate for *in vivo* testing.

1.3 Copper in Biology

1.3.1 The Importance of Copper Homeostasis

The dietary requirement of copper is only 0.7-0.9 mg/day and yet it is vital for the growth and development of all aerobic organisms [67, 68]. Cu is a key player in a diverse number of biological pathways owing in part to its presence as either a reduced (+1) or oxidized (+2) state[67]. Deregulation of Cu homeostasis has led to a better understanding of the significance of Cu in human physiology[69]. For instance, X-linked mutations in *ATP7A*, which codes for a Cu-transporting ATPase, lead to the production of a truncated, dysfunctional enzyme, resulting in poor distribution of copper and is linked to a fatal condition known as Menkes Syndrome. Since *ATP7A* is responsible for the delivery of copper as co-factors and regulators of certain enzymes as well as the efflux of Cu from cells, the loss of *ATP7A* function leads to accumulation of Cu in certain tissues such as the small intestine and systemic Cu deficiency, leading to neurodegeneration and growth abnormalities [70-72]. If diagnosed early, neurodegeneration from Menkes Syndrome can be prevented and the disease can be managed through parenteral administration of Cu-histidine. Nonetheless, the treatment outcome is largely dependent on the residual activity of the mutant *ATP7A* enzyme and the time of initial treatment [73, 74]. In contrast, Wilson's disease is a disorder caused by mutations in *ATP7B*, which is primarily expressed in the liver and brain. Dysfunctional *ATP7B* results in impaired excretion of excess Cu from the body and increased accumulation leading to tissue damage particularly in the liver, brain, kidneys, and cornea[75]. Wilson's disease is usually treated with chelation therapy with agents such as penicillamine and trientine[70]. In recent years, treatment with zinc has been introduced as zinc can induce metallothionein, a protein that binds to copper in the

gut and facilitates copper excretion[69, 76, 77]. The life-threatening impact of Cu deficiency and overload found with Menkes and Wilson's disease, respectively, exemplifies the importance of Cu homeostasis to human health.

1.3.2 Cellular Components for Copper Regulation

It is known that Cu is a co-factor for metalloenzymes including superoxide dismutase, cytochrome C oxidase, tyrosinase, peptidylglycine alpha-amidating mono-oxygenase, and lysyl oxidase[78, 79]. The diverse role of Cu in biology is in part a result of its existence as either reduced Cu(I) or oxidized Cu(II) with each oxidation state having a preferred affinity for different coordinating groups[67]. Cu(I) is known to bind thiol and thioester groups whereas Cu(II) prefers coordinating with oxygen and nitrogen groups[80]. Therefore, the different oxidation states can interact with a variety of proteins that drive a multitude of biochemical reactions. Given its many biological roles, Cu levels are tightly regulated at all levels including cellular uptake, distribution, detoxification, and elimination [79, 81]. These mechanisms are vital to maintaining Cu homeostasis and avoiding Cu associated toxicities. Cu transporters are present on cell surfaces to mediate the influx and efflux of Cu [79, 82, 83]. Further, copper chaperones (CC) are present to deliver the imported Cu to specific target proteins within the cell [84]. Figure 1.2 summarizes the trafficking of Cu and the cellular components involved in regulating Cu levels within a cell.

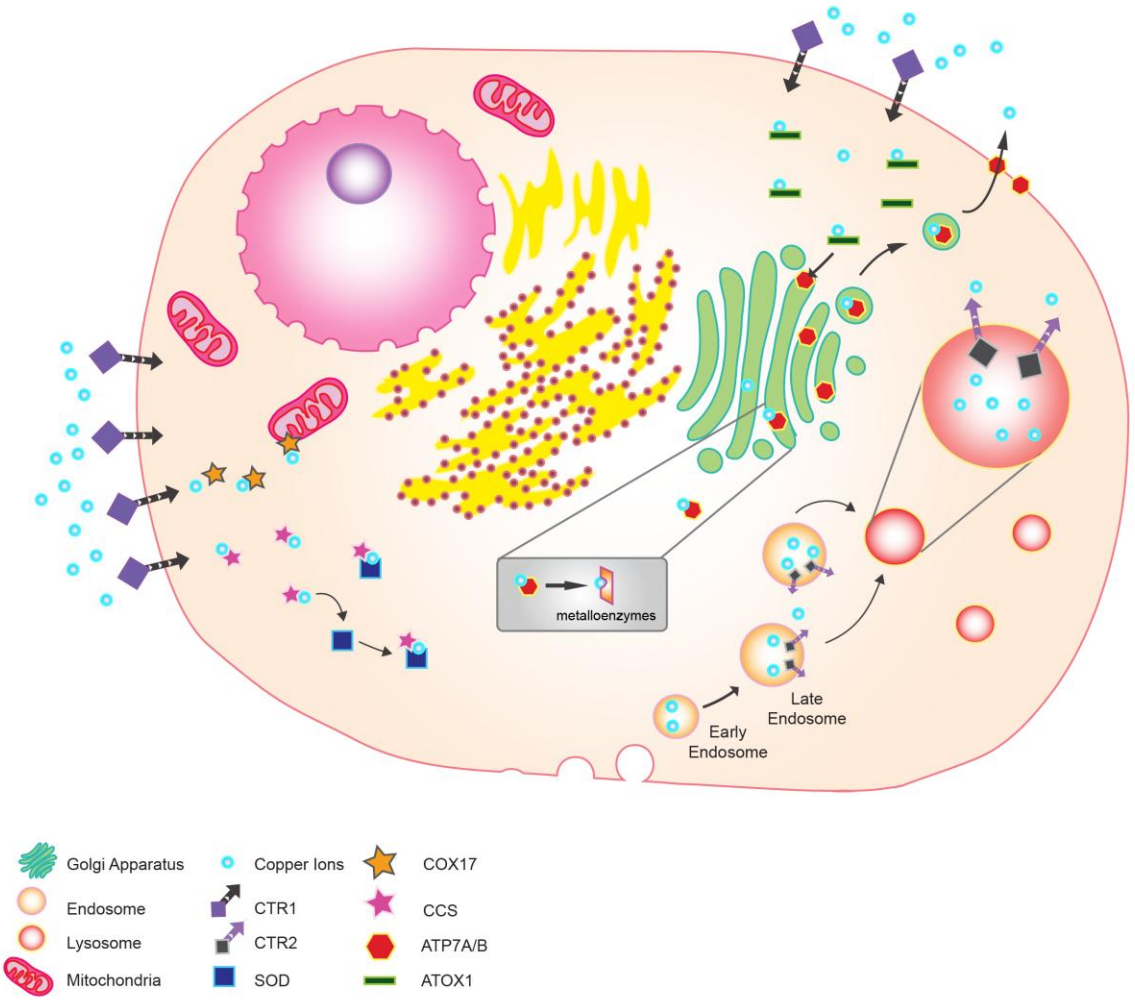


Figure 1. 2: Cellular regulation of copper levels. CTR1 is the copper transporter that is primarily responsible for delivering copper into the cell. Once copper enters the cell, it is bound to chaperones for further distribution. For instance, ATOX 1 is responsible for delivering copper to the copper efflux pumps ATP7A/B, which are localized to the trans-golgi apparatus where copper is transferred to metalloenzymes that require copper as a co-factor. If copper levels are high, ATP7A/B will redistribute to the cell surface via the secretory pathway where copper is pumped out of the cell. Intracellular copper can also bind to COX17, a chaperone which is important for transporting copper to the mitochondria. This is vital to organisms that require aerobic respiration to survive as the copper here is crucial to the proper functioning of the cytochrome c oxidase and the electron transport chain. Finally, CTR2, another copper transporter, resides in the late endosomes and lysosomes. This transporter aids in the regulation of copper levels inside the cytosol.

Copper transporter 1 (CTR1) contains 3 transmembrane domains and is believed to be the primary protein responsible for the import of dietary Cu [85, 86]. When Cu levels are elevated, CTR1 proteins at the plasma membrane are endocytosed and degraded to prevent Cu overload. A second isoform of the copper transporter known as CTR2 has been identified. The function of CTR2 remains to be fully elucidated. Nonetheless, it has been shown that while CTR1 is primarily expressed on cell surfaces, CTR2 is localized to endosomes and lysosomes, and partially to the plasma membrane[83, 87, 88]. It is postulated CTR2 is important for regulating Cu levels in the cytosol and hence plays a role in intracellular Cu homeostasis [89]. Additionally, some have suggested that the divalent metal transporter 1 (DMT1), which is typically involved the iron transport, may also be involved in Cu influx [90, 91]. While Cu uptake through CTR1 and 2 is an energy independent process, the efflux of cellular Cu requires the hydrolysis of ATP [83, 92, 93]. Two well-characterized Cu efflux proteins are known as *ATP7A* and *ATP7B* [93, 94]. *ATP7B* is highly expressed in the liver and expressed at lower levels in other organs; whereas, *ATP7A* is expressed in most tissues but its function is most important in the small intestine [94]. The *ATP7A* gene is encoded on the X-chromosome whereas *ATP7B* is found on chromosome 13. As indicated above, mutations in these genes are associated with Menkes and Wilson's disease, respectively [95-97]. Normally, *ATP7A* and *ATP7B* reside at the trans Golgi network (TGN) where Cu is incorporated into Cu-dependent enzymes [98]. *ATP7A*, particularly in the small intestine, is important for trafficking Cu out of the Golgi and into portal circulation, allowing systemic distribution of Cu. In the liver, *ATP7B* is responsible for incorporating Cu into ceruloplasmin, an enzyme that is important for regulating iron levels [99, 100]. When the intracellular Cu level reaches a threshold, the efflux pumps are redistributed to plasma membrane where their primary role is copper excretion from the cell.

CCs are responsible for trafficking Cu intracellularly and also for ensuring that the cell is not exposed to highly reactive Cu ions in transit which could result in the generation of ROS [101]. ATOX1 was the first chaperone discovered as a cytosolic protein that binds and delivers Cu(I) ions to ATP7A/B [93, 102, 103]. Another CC known as CCS (copper chaperone for superoxide dismutase) forms a heterodimer with Cu/Zn superoxide dismutase (SOD) and is essential for activating mammalian SOD [93]. Similarly, COX17 is

the CC that delivers Cu to the mitochondria; this chaperone is required for the activation of cytochrome c oxidase (COX), which is essential for aerobic respiration [104]. Other chaperones such as COX1, 2, 11 and 17, as well as SCO1 and SCO2, are also involved in the assembly of COX [104, 105]. CCs such as ATOX1 and COX17 are essential as knockout mice with loss of *Atox1* or *Cox17* were embryonically and perinatally lethal [93, 98, 106].

Cu is capable of coordinating with N, O and S groups in general and these interactions are involved in many biochemical reactions [67]. Additionally, Cu can displace other metals such as Zinc (Zn) in metalloproteins which could alter the protein-metal structure rendering the metalloenzyme inactive [107].Thus, it is reasonable to expect that in the presence of excess Cu, this affinity could result in the formation of undesirable coordinated systems. The transition between Cu(I) and Cu(II) can result in the generation of hydroxyl radicals which can damage proteins, nucleic acids and lipids [67, 108, 109].

1.4 Copper Chemistry

Research in Cu chemistry and drug synthesis is dominated by compounds using the most common oxidation states [62]. Cu(I) ions have a d^{10} configuration and are able to form complexes with ligands that have "soft" donor characteristics such as those containing thioether S, P, and aromatic nitrogen groups [48]. These complexes tend to exhibit a linear, trigonal or tetrahedral geometry [110, 111]. Cu(II) ions have a d^9 configuration and complexes with coordination geometry, favour 4-6 coordinate geometries (square-planar, trigonal bipyramidal, and octahedral) and allow for a large variety of ligands with different denticity and sizes [48, 111]. The ligands for Cu(II) are favoured towards N,O,S donors [112]. The stability of divalent metal complexes can be predicted to an extent using the Irving-Williams series[113]; Thus, Cu(II) is a good choice of metal as the complexes would be more stable in comparison to other potential metal species.

There are several excellent reviews, including those written by Santini et al.[62], Marzano *et al.*[48] and Iakovidis *et al.*[112] that discuss different ligands and functional groups that form Cu- complexes along with their various *in vitro* activities. These reviews are extensive and truly highlight the vast therapeutic opportunity for Cu-complexes in respect to both ligand heterogeneity and therapeutic application diversity. A familiarity

with copper chelating groups is required to fully appreciate the range of binding partners that are possible (Figure 1.3). These can be adapted and altered for different disease indications or targets.

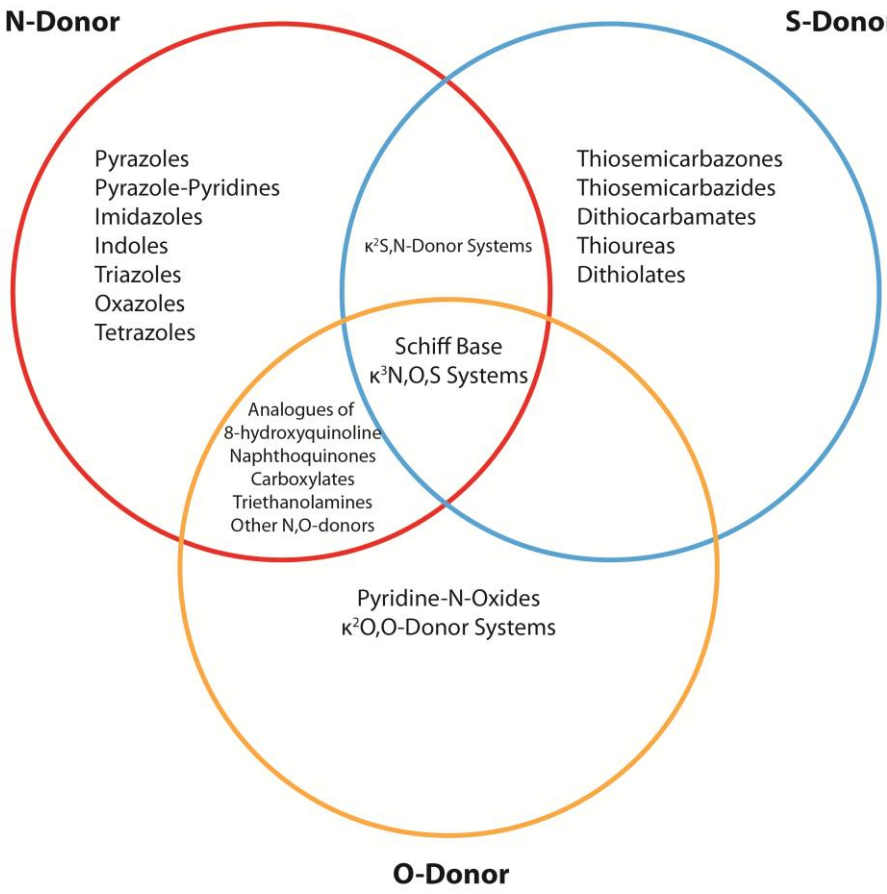


Figure 1. 3: Venn diagram showing the possible copper chelating functional groups composed of nitrogen (N-donor), oxygen (O-donor) and sulfur (S-donor) atoms. Copper binding groups can contain a single donor type or a mixture of N, O and S. The illustration was constructed based on a review article by Santini *et al.*[62]

1.5 Therapeutically Active Copper Complexes as Anti-cancer, Anti-Inflammatory, and Antimicrobial Agents

The therapeutic window for anti-cancer agents has always been dependent on the selectivity of these agents for the cancer cells over normal, healthy cells. While Cu chelation may reverse the metal imbalance and remove the tumour's growth advantages provided by elevated Cu levels, another approach is to administer agents that upon Cu binding, would elicit potent cytotoxic effects. As an example, disulfiram (DSF), an FDA-approved agent for

the treatment of alcoholism, has garnered much attention in recent years as a Cu-activated anti-cancer agent [114]. While DSF alone has little effect against cancer cells, its use in the presence of Cu *in vitro* has been shown to have IC_{50} values in the nano-molar range against a wide range of cancer cell lines [66, 115, 116]. *In vivo*, the administration of DSF into animals with subcutaneous breast cancer xenograft models known to have abnormally high levels of Cu resulted in decreased tumour growth [117, 118]. Some have suggested that the formation of a Cu-DSF complex as the means by which cytotoxicity is achieved. However, a thorough examination of DSF metabolism has led to the conclusion that the Cu-DSF complex does not exist [115, 119]. Lewis *et al*. have deduced that even in aqueous solution, DSF does not form a copper complex but rather it degrades into diethyldithiocarbamate (DDC), which reacts with Cu to form copper diethyldithiocarbamate ($Cu(DDC)_2$) [120]. Dithiocarbamates are a known class of Cu chelators and they have been shown to be very active *in vitro* against a variety of cancer cell lines [65]. Interestingly, the mechanism of action of $Cu(DDC)_2$ was found to be proteasome inhibition, but this action was through a mechanism that was distinct from bortezomib (BTZ), an FDA-approved proteasome inhibitor for cancer treatment [42, 121, 122]. It should be noted that clinical trials have attempted to use DSF for the treatment of liver tumours with adjuvant Cu given in the form of copper gluconate [123].

Aside from generating novel chemotherapeutics by metal coordination of existing drugs, researchers have also begun to explore the utility of metal coordination in the development of new drugs. For example, with the emergence of drug-resistant *Plasmodium falciparum*, there is an urgent need for new anti-malarials. Sekhon and Bimal have described various metal complexes that have demonstrated anti-malarial activity [124]. One example is the Cu(II)-complex with pyridine-2-carboxamidrazone. This copper complex has exhibited enhanced anti-malarial activity *in vitro* compared to the parent compound [125]. Inflammatory diseases, such as rheumatoid arthritis, are another area that could benefit from treatment with Cu-complexes. While multiple non-steroidal anti-inflammatory drugs (NSAIDs) are available in the market, they are associated with renal, gastrointestinal, and cardiovascular toxicities [53]. Over the past two decades, there is increasing evidence that Cu-complexes of NSAIDs are more effective and less toxic

compared to Cu or the parental NSAID compound used alone [53]. In many cases, chronic inflammation such as asthma, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis are associated with reduced superoxide dismutase (SOD) activity, leading to chronic oxidative stress and inflammation [53, 88, 126]. Although the exact anti-inflammatory mechanisms of Cu-NSAIDs remain to be elucidated, these Cu-NSAIDs have exhibited marked SOD-like anti-inflammatory activity which differentiates them from their parent compounds [53, 127, 128]. This SOD-mimetic activity of Cu-NSAIDs may also be useful in the treatment of cardiovascular diseases and skin disorders [53, 127, 129].

With the emergence of multi-drug resistant pathogens and a lack of novel effective antibiotics, metal complexes have also become of interest as antibacterial agents. As an example, Beeton *et al.* have synthesized nine Cu(II)-complexes of the aromatic ligand 1,10-phenanthroline and tested their antimicrobial and antibiofilm activity [130]. These Cu-complexes have demonstrated better antimicrobial activity than the free ligand against both Gram-Positive and Gram-Negative bacterial strains, as well as enhanced antibiofilm activity compared to the standard drug vancomycin against a clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA). Facing a similar multi-drug resistant problem in the clinic, the need for novel tuberculosis drug has led to the synthesis of Cu-complexes of pyruvate-isoniazid conjugate analogues and isoniazid-derived hydrazones which have demonstrated greater anti-tubercular activity than isoniazid *in vitro*[130, 131].

Finally, while Menkes Syndrome and Wilson's disease are known to be associated with Cu deficiency and overload, respectively, there is now strong evidence that disruption of biometal homeostasis plays an important role in certain neurodegenerative diseases including Alzheimer's (AD) and Huntington's Disease (HD) [132]. Formation of plaques comprising the misfolded amyloid-β (Aβ) protein in the brain is characteristic of AD. Several studies have demonstrated that AD patients have abnormally high levels of Cu in the amyloid plaques and these metal-bound Aβ proteins are more prone to self-aggregation [133], which correlates with increased oxidative stress and more severe disease progression [134]. Similarly, Cu accumulation has also been detected in the brains of HD patients and the metal has been found to promote the formulation of mutant Huntington protein aggregates, potentiating disease progression [135]. As such, CQ and later, PBT2, a second generation 8-HQ analogue, have been used to treat AD and HD [136-138]. PBT2

became the drug of choice as it is easier to synthesize, more soluble, and exhibits greater permeability across the blood-brain barrier [138]. It is important to note that while many consider this copper chelation therapy, the role that these agents play is to correct the metal imbalance in the brain. Unlike Wilson's Disease, the Cu accumulation occurs extracellularly rather than intracellularly in AD and HD. Trafficking of Cu with PBT2 reduces extracellular Cu levels and increases intracellular bioavailability, which leads to delayed protein aggregation and allows for activation of neuroprotective pathways [138]. Unfortunately, PBT2 has failed to meet the efficacy goals for both AD and HD in phase 2 clinical trials [139]. It has been suggested that successful treatment of AD and HD would require targeting multiple hallmarks of these diseases [140].

1.5.1 Copper Chelation Therapy

Copper chelation is a standard approach for treating Wilson's disease, wherein drugs/chelators are administered to bind Cu, resulting in the formation of complexes that can be eliminated through the kidneys [141-143]. In the last two decades, there is increasing evidence that many cancer patients have elevated levels of Cu within sites of tumour growth and plasma (when compared to plasma levels of Cu in healthy subjects) [144, 145]. Gupte and Mumper have written a comprehensive review demonstrating the link between Cu imbalance and cancer [144]. It is also known that one of the hallmarks of cancer is induction of angiogenesis [146-148]. Cu is required for the activation of hypoxia-inducible factor 1 (HIF-1), a major transcription factor regulating the expression of vascular endothelial growth factor (VEGF), which is primarily responsible for angiogenesis [147, 149]. Additionally, angiogenin is a potent inducer of blood vessel formation which binds to the endothelial cell receptors. It has been shown that the presence of Cu can lead to an increase in angiogenin binding to endothelial cells by more than 4-fold [150]. Thus, copper chelators can be used as part of a "de-coppering" approach to treat cancer similar to what has been done for Wilsons' disease [151]. Copper chelation has been shown to selectively kill colon cancer cells via ROS generation and a similar strategy has been proposed to be effective for targeting cancers, particularly melanoma, that harbour oncogenic *BRAF* mutations [44, 152]. Multiple clinical trials are ongoing investigating the

effectiveness of combining copper chelators such as tetrathiomolybdate and penicillamine with standard chemotherapy or radiotherapy; however, given the heterogeneous nature of cancer, it is unlikely that Cu depletion alone will be sufficient to cure the disease [153].

1.5.2 Copper Radiopharmaceuticals

^{64}Cu has garnered significant attention in the context of radiopharmaceuticals applications, owing in part to its utility in molecular imaging and targeted radiation therapy [154, 155]. Other Cu radioisotopes, including ^{60}Cu, ^{61}Cu, ^{62}Cu and ^{67}Cu have been reviewed by Asabella *et al.* Of these, ^{67}Cu is the only other isotope that can be used for radiation therapy [155]. Production of ^{64}Cu is through a ^{64}Ni(p,n)^{64}Cu reaction and is obtained as $^{64}CuCl_2$ [156]. The radioisotope possesses a half-life of 12.7 h and decays by positron emission (17.8%) and beta decay (38.4%).[157] These decay properties render it a useful isotope for PET imaging and/or radiotherapy.[154] Due to the lack of kinetic stability of Cu(I) complexes and the rarity of Cu(III), the radiopharmaceutical field is dominated by Cu(II) complexes [154]. Classes of Cu chelators such as sarcophogine[158], cross-bridged tetraamine[159-161] and thiosemicarbazone[162, 163] ligands have been developed. As an example, the ligand ATSM (diacetyl-2,3-*bis*(N^4-methyl-3-thiosemicarbazone)) forms a Cu(II) complex useful as a marker of chronic hypoxia. Lewis *et al*. performed a side by side comparison of ^{64}Cu/^{60}Cu-ATSM and they showed equivalent patterns and magnitude of tumor uptake. These authors, however, concluded that ^{64}Cu-ATSM was better due to lower noise in images [164]. One major limitation of Cu-ATSM is that the kinetics of uptake appears to be cell dependant and thus this complex may not be relevant for all cancers [165].

Conjugation of ^{64}Cu to biologic agents has been of great interest. In this context, the most widely used chelators are based on tetraazamacrocylic[154, 166] ligands (typically DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid)). This chelator class can be successfully conjugated to antibodies, protein and peptides but transchelation was noted to be a problem *in vivo* [167]. The conjugation of ^{64}Cu through DOTA has been performed and evaluated for cetuximab (EGFR antibody) [168, 169], trastuzumab (HER2 antibody) [170]

and Abegrin (human integrin αvβ3 antibody) [171]. The small cyclic peptide RGD which targets human integrin αvβ3 was conjugated to ^{64}Cu through DOTA and a novel bifunctional chelate 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid [172]. Octreotide, a peptide targeting the somatostatin receptor, was conjugated to ^{64}Cu through TETA and was used to detect neuroendocrine tumours [173]. Additionally, a dextranated and DTPA-modified nanoparticle was labeled with ^{64}Cu and used for imaging by Nahrendorf *et al*.[174]. To date, nothing has transitioned into humans due to challenges linked to poor *in vivo* stability and poor biodistribution attributes [166].

1.6 Classification of Copper Complexes

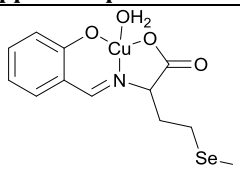
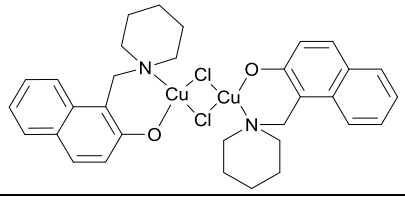
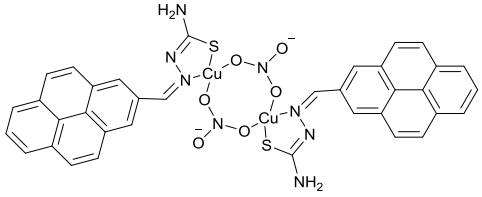
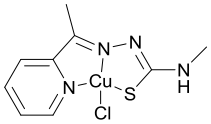
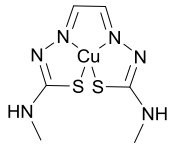
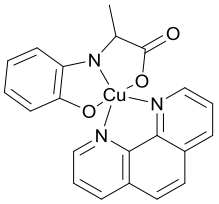
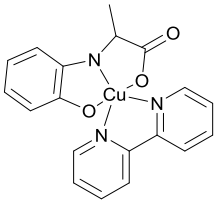
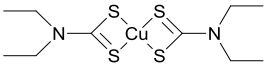
The information summarized above and in other more comprehensive review articles highlight the therapeutic potential of CBTs. When considering these, it is useful to define the complexes into two categories: copper-ligand complexes (CLCs) and copper-drug complexes (CDCs). In general, CDCs typically may not require Cu for activity whereas CLCs are significantly more active as the Cu-complexes. Table 1.1 and Table 1.2 provide examples for each of these two classes of Cu-complexes which are further described below.

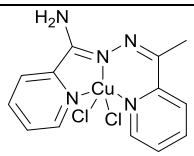
1.6.1 Copper-Ligand Complexes (CLCs)

CLCs are CBTs comprising ligands that have little or no therapeutic activity at relevant concentrations (e.g. IC_{50} > 10 μM). Examples of CLCs include Cu-(N-salicylidene-selenomethionine) [175], Cu-(NCI-109268) [39], and Cu(1-pyrenethiosemicarbazone) [176]. Cu-(N-salicylidene-selenomethionine has been shown to act as an antimicrobial agent while the latter two CLCs have demonstrated anti-cancer activity. Similar to the platinum-based chemotherapies, the ligands attached can alter the activity of the drug but the ligand itself does not have any therapeutic activity in the absence of the metal. A number of CLCs, including ligands like ethyl 2-[bis(2-pyridylmethyl)amino] propionate (ETDPA) [177], 2-[(2-(2-hydroxyethylamino)- ethylimino)methyl]phenol (tdp) [178], 1,10-phenanthroline-L-serine (Phen-Ser) [98], and 2-acetylpyridine-4-methylthiosemicarbazone (H4ML) [179], have been identified as DNA binding and cleavage agents, making them suitable anti-cancer drug candidates. Although these CBTs have been

characterized extensively and evaluated *in vitro*, no *in vivo* study with these compounds has been published.

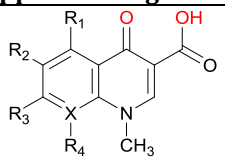
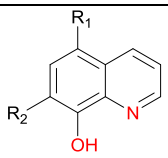
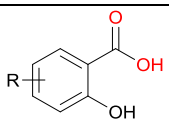
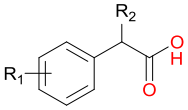
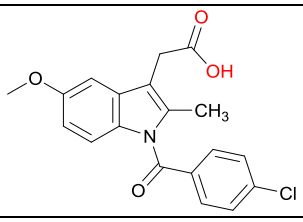
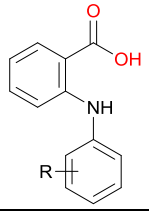
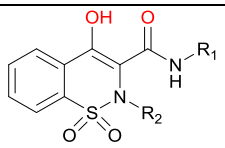
Table 1. 1: Examples of copper ligand complexes with antimicrobial and anti-cancer activity

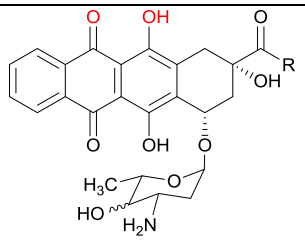
Drug/Ligand(s):	Copper Complex Structure:	Indication(s):	Ref:
(A) N-salicylidene-selenomethionine		Antimicrobial	[175]
(B) NCI-109268		Anti-cancer	[39]
(C) PyTSC		Anti-cancer	[176]
(D) 2-acetylpyridine-4-methylthiosemicarbazone		Anti-cancer	[179]
(E) GTSM		Anti-cancer / Antimicrobial	[180-182]
(F) SAla and phen		Antimicrobial	[183]
(G) SAla and bpy		Antimicrobial	[183]
(H) Diethyldithiocarbamate		Anti-cancer	[184]

(I) APPC		Anti-cancer	[185]

APPC:N1-(2-acetylpyridine)pyridine-2-carboxamidrazone
Bpy: 2,2 -bipyridine
GTSM: glyoxal-bis(N4-methylthiosemicarbazone)
Phen: 1,10-Phenanthroline
PyTSC: 1-pyrenethiosemicarbazone
SAla; N-(2-hydroxybenzyl)-D,L-alanine

Table 1. 2: Examples of drug classes which form copper drug complexes

Chemical Class:	Indication(s):	Copper Binding Moiety:	Example(s):	Ref:
Quinolones	Antimicrobial		ciprofloxacin, norfloxacin, cinoxacin	[186-189]
8-Hydroxyquinoline and derivatives	Antimicrobial/ Anti-cancer		8-hydroxyquinoline, clioquinol	[190-193]
Salicylate derivates	Anti-inflammatory		salicylic acid, diflunisal	[186, 194]
Propionic acids	Anti-inflammatory		ibuprofen, fenoprofen, naproxen, ketoprofen	[186, 195]
Heteroarylacetic acids	Anti-inflammatory		indomethacin	[61, 186]
Anthranilic Acid derivatives	Anti-inflammatory		mefenamic acid, diclofenac,	[186, 195-197]
Oxicam derivatives	Anti-inflammatory		meloxicam, piroxicam,	[186, 198, 199]

Anthracycline	Anti-cancer		daunorubicin, doxorubicin, idarubicin	[200, 201]

*Copper binding moiety labelled in red

To identify novel antibiotics, Haeili *et al.* performed a high-throughput screen using Cu-complexes against MRSA [180]. The bactericidal activity of the copper bis(thiosemicarbazonato) complexes identified were copper-dependent. In another study, complexes of N-(2-hydroxybenzyl)-D,L-alanine (SAla) and 1,10-phenanthroline (phen) or 2,2 -bipyridine (bpy) were found to have antibacterial/antifungal activity[183] when screened against nine bacterial and nine fungal pathogens using ampicillin and amphotericin B, respectively, as positive controls. The ligands alone did not show any growth inhibition whereas the CLCs show activity against a variety of bacterial and fungal pathogens. Despite *in vitro* activity of these compounds, among others [54, 57, 99, 202, 203], there does not appear to be any further study of these compounds in relevant disease models. The pharmacological properties, such as tolerability or pharmacokinetics, of these CLCs have yet to be explored.

1.6.2 Copper-Drug Complexes (CDCs)

Cu(II) is able to coordinate with a large variety of functional groups and these groups are present in many drugs already approved by the FDA. Some examples include Cu-complexes of ciprofloxacin [189], isoniazid [204, 205], doxorubicin [205], indomethacin [61, 206] and clioquinol[190, 207]. For these agents, Cu is not required for the drug to exert its activity, however the Cu-drug interactions can be used to alter the solubility of the drug (i.e. making an insoluble drug more soluble by Cu coordination) [208]. The resulting complex defines a new formulation; one that will have differing pharmacokinetic properties and associated changes in therapeutic activity [53]. The Cu-indomethacin complex, for example, has been shown to have an enhanced activity as a topical anti-inflammatory in comparison to indomethacin [209]. This can be attributed, in part, to a

differentiation in the physico-chemical properties of the copper complex [208]. Interestingly, Okuyama *et al.* hypothesized that Cu(indomethacin)$_4$ is able to activate Cu-dependent opioid-receptor which may help to explain the enhanced anti-inflammatory activity [210]. Cu-complexes have been made with other NSAIDs, including diclofenac [196, 211], fenoprofenate [212], mefenamic acid[197] as well as others [53, 186, 210]. Although many of these Cu-NSAID complexes have demonstrated some success in relevant animal models, none have been approved for human use.

Zinc pyrithione, which is used in dandruff shampoos, is an example of an agent with antimicrobial activity [213]. The ligand pyrithione on its own has no activity. Interestingly, zinc pyrithione and copper pyrithione complexes have been shown to have anti-cancer activity as well [214, 215]. Additionally, copper complexation can alter the mechanism of action of the associated drug. Other examples would include Cu-complexes of the 8-HQ class of drugs which are typically used as anti-fungals or antimicrobials [55]. When these drugs are complexed to Cu, they exert novel anti-cancer activities [216]. Clioquinol (CQ), an analogue of 8-HQ which is traditionally used as an antibiotic, has demonstrated enhanced activity when complexed with Cu as an anti-cancer agent [217]. As suggested above for Cu(DDC)$_2$, the principal mechanism of activity for Cu-CQ and Cu-8-HQ involves proteasome inhibition [216]. These compounds have also been shown to behave as metal ionophores [218]. Previous reports have shown that copper-complexed 8-HQ is at least 10 times more cytotoxic than the drug given alone when tested against HeLa and PC3 cell lines over 48 hr time course [219]. The Cu-8HQ complex has also been tested on bacteria and has shown some success as a more potent antibiotic compared to its parent compound [55, 220].

Schimmer *et al.* demonstrated that CQ has Cu dependent and independent mechanisms of action when added to cancer cells [191]. Thus, an important consideration when attempting to repurpose drugs such as CDCs is that the inherent mechanism of action of the metal-free drug may not play an important role if the metal complex stays intact *in vivo* but this activity may become apparent should the complex fall apart.

1.7 Approaches to Solve Copper Complex Solubility Challenges:

1.7.1 High-Throughput Screening

High-throughput screening (HTS) is ubiquitously used in the pharmaceutical industry as a means to identify candidate compounds for development. This approach has been used to identify candidate compounds selected on the basis of phenotypic screens; where measurable changes in cell appearance (e.g. autophagy)[221] linked to a particular behaviour (e.g. cell survival)[222] or biochemical screens [223]; where inhibition of a purified target protein is the goal. HTS have been used to indentify active anti-cancer and anti-microbial copper complexes. A HTS was performed by Feldman *et al.* wherein ~320,000 compounds were screen to identify caspase inhibitors [224]. This screen led to the discovery of a new class of allosteric copper-based caspase inhibitors (NSC321205, NSC277584, NSC321206, and NSC310547) with submicromolar IC_{50} values. A screen was also devised to identify copper-activated ligands that were efficacious in methicillin-resistant *Staphylococcus aureus* (MRSA) [180]. The compounds were tested against the bacteria in media containing trace levels of copper or media supplemented with 50 μM copper using optical density as a marker for bacterial cell growth. This screen indentified the bis(thiosemicarbazones) ligands (GTSM, PTSM, ATSM) as active in MRSA in the presence of copper. It should be noted that upon further study of this class of copper complexes as antibacterial agents solubility issues were identified making it difficult to test the activity of these copper complexes in the absence of DMSO [225]. Thus, while the HTS approach allows for the identification of candidate compounds further chemical modifications may be required to create chemical entities with improved solubility while retaining activity. Such traditional medicinal chemistry programs are highlighted by the studies described by Wang *et al.* who were examining the development of analogues of two popular Cu-complexes that have garnered much attention over recent years.[65] The first is DDC. The $Cu(DDC)_2$ complex suffers from solubility issues [42, 184, 226], so this group chose to make analogues of DDC, maintaining the dithiocarbamate backbone and altering the end groups from ethyl groups to functional groups that would make the compound more water soluble. Interestingly, they were able to increase the water solubility of the

complex but this came at the expense of therapeutic activity; the more water soluble compounds were less active. The chemical modification altered the ability of the complex to fit into the 19S cap of the proteasome; an interaction that is crucial for its activity [184]. This chemical modification approach was also used with 8-HQ [193], where 64 analogues of 8-HQ were synthesized and screened for proteasome inhibition. The authors attempted to increase the solubility of the complex by substituting the hydroxyl group with N-phenylacetamide, which resulted in decreased potency. It was found, in this case, that the polarity of the substituent played a role in 8-HQ-induced cytotoxic activity.

1.7.2 Computer-Aided Drug Design

Computer-aided drug design is a computational approach which allows for the development of molecular entities which are specific to a structure of interest. This approach is limited in that it does require the three-dimensional structure of the target of interest. This can be achieved through bioinformatic methods, which predict the structure based on an amino acid sequence, or alternatively, a crystal structure determined by X-ray crystallography methods or single-particle electron cryo-microscopy. One example in the CBT literature using such methods is described by Zuo *et al.* who utilized docking analysis of the proteosome to analyze the binding affinity of four amino acid Schiff base-Cu(II) complexes [227]. This approach allowed the authors to molecularly model the copper complexes and compare the binding to that of BTZ, the clinically available proteosome inhibitor. Another example concerns human serum albumin (HSA) which has a well defined crystal structure and has been used in molecular docking analysis. Li *et al.* examined how four Schiff-base copper complexes would interact with the protein [228]. HSA plays an important role in drug transport and distribution and thus through docking analysis it was possible to gain an understanding of how the copper complex behaves *in vivo*. Owing to the success of DNA damaging agents in treatment of cancer some researchers have directed their efforts on the creation of copper complexes with DNA intercalation capabilities [229, 230]. This was accomplished through the use of docking analysis used to examine how the copper complexes interact with DNA. This computer-aided approach allows one to directly compare where in the active site of the complexes are binding and additionally one could

predict how alterations of the compound may affect binding affinity. Again, if the candidate compounds identified prove to be pharmaceutically challenging because of their solubility properties, more classical medicinal chemistry programs can be applied to define more water soluble compounds.

1.8 Nanoscale Synthesis Reactions Inside Liposomes

As noted already, this thesis describes a new approach which allows for the formulation of CBTs inside liposomes. This method is reliant on metal complexation reactions. Provided that the reaction does not impact the integrity of the liposome, the resulting product will be suitable for administration. In this context, it is important at this stage in this introduction to provide a brief description of liposomes and how the approaches developed in my thesis can be differentiated from previous disclosure involving the use of encapsulated metal ions.

1.9 Liposomes

Liposomes represent one of the most successful nanoparticle platforms with numerous products already approved by the FDA [11]. Since the original structure was described by Bangham, liposomes as a technology have clearly blossomed with the success of liposomal doxorubicin formulations (Doxil® [5],and Myocet® [231]) and several new iterations of liposomes (e.g. thermosensitive [232] and combiplex formulations [233]) which are being produced today. They are typically composed of phospholipids and cholesterol and form spontaneously when hydrated. Liposomes have become a go-to technology useful for many different therapeutics including anti-cancer, antibiotic, natural products and NSAIDs [17, 234-236]. Much of the success of liposomes as drug delivery vehicles has to do with advancements made in manufacturing methods. Today, liposomes can be created in large batches and candidate drug loading is efficient when using active "remote" loading processes reliant on pH gradients (Figure 1.4A) or metal complexation (Figure 1.4B) [23, 237-239]. Technologies such as extrusion and more recently the development of microfluidic devices (e.g. NanoAssemblr) have allowed for the reproducible production of liposomes with defined attributes [12, 240, 241].

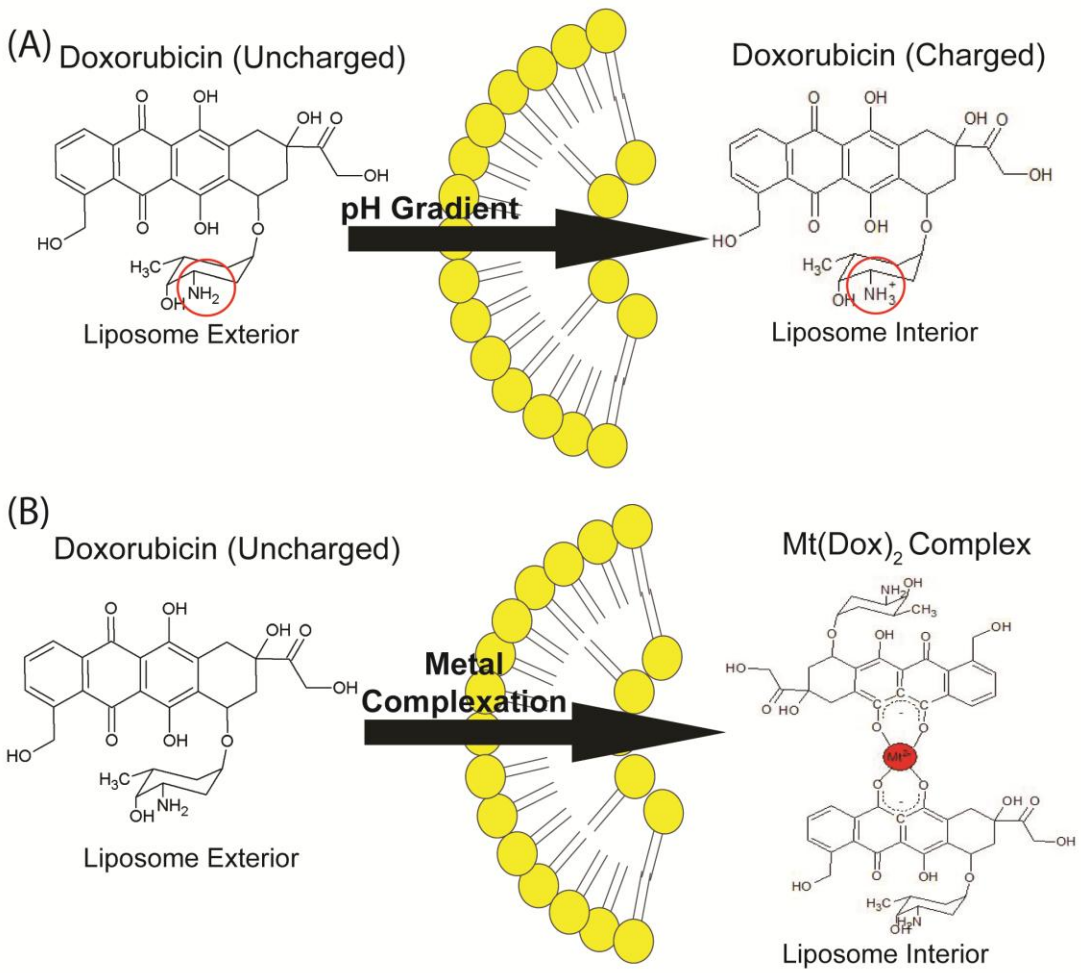


Figure 1. 4: Doxorubicin possesses structural motifs that facilitate remote loading in liposomes through use of transmembrane pH gradients and metal complexation. (A) pH gradient loading: uncharged doxorubicin on the liposome exterior crosses the lipid bilayer and becomes charged in the low pH environment. The charged form of doxorubicin is less membrane permeable and remains encapsulated. (B) Metal complexation loading: doxorubicin on the exterior of the liposomes crosses the lipid bilayer in its neutral form and forms complexes with the encapsulated divalent metal (e.g. Mn^{2+}, Cu^{2+}).

1.10 Remote Loading Using pH Gradients

Many factors contribute to the success of liposomes as a platform for drug delivery. In this context, the development of remote candidate drug loading processes was a significant "game changer"; allowing for a shift away from passive encapsulation approaches. This process allowed candidate drugs to be loaded into pre-formed liposomes with high trapping efficiency, minimizing waste of costly drugs and eliminating the need to work with drugs during liposomal preparations. One driving force for remote loading relies on the presence of transmembrane pH gradients (acidic interior) (Fig.1.5). Candidate drugs (e.g. doxorubicin) with weakly basic amines ($R\text{-}NH_3^+$) could be added to the outside of liposomes and, at appropriate pHs, the neutral form of the candidate drug would redistribute across the lipid bilayer and in the low interior pH the candidate drug becomes protonated. The protonated form of the drug is much less membrane permeable and becomes trapped in the liposomes. This approach used liposomes with encapsulated citrate buffer (pH 4.0, Figure 1.5A) or ammonium sulfate (pH 5.5, Figure 1.5B). Despite the limitation that remote loading using pH gradients works for only a subset of drug structures, for those candidate drugs with appropriate chemical attributes the method facilitates efficient trapping (>98%). The loading efficiency eliminates waste and allows for practical scale-up processes. Remote loading of drugs with a protonizable amine function, as one example, has already been widely discussed [13, 15, 237]. Hence, the focus here will be on the use of metal based loading methods, and differentiation between metal complexation reactions from pH gradient loading to remote load candidate drugs into preformed liposomes.

As suggested above, another variation of remote loading was developed that relied on the use of liposomes with encapsulated divalent metal-containing solutions. For example, unbuffered $MnSO_4$ solution (pH 3.5) could be trapped inside liposomes and following the addition of a divalent cation ionophore (e.g. A23187, an ionophore which exchanges metal ions for protons) a pH gradient could be generated to engender drug loading (Figure 1.5C). This was first reported in publications using doxorubicin. What was interesting about doxorubicin as a drug choice was the fact that it can form dimeric Mn-

$(Dox)_2$ complexes (Figure 1.5D). Studies demonstrated remote loading of doxorubicin into liposomes with entrapped Mn^{2+} even in the absence of transmembrane pH gradients. Metal complexation reactions can be used as an alternative to encapsulate drugs into pre-formed liposomes. In this thesis, copper-dependent loading methods are differentiated from pH-driven loading processes where the metal ions are removed from the interior of the liposomes by addition of A23187. While this method has been effective, it is limited to drugs with the protonizable amine functions.

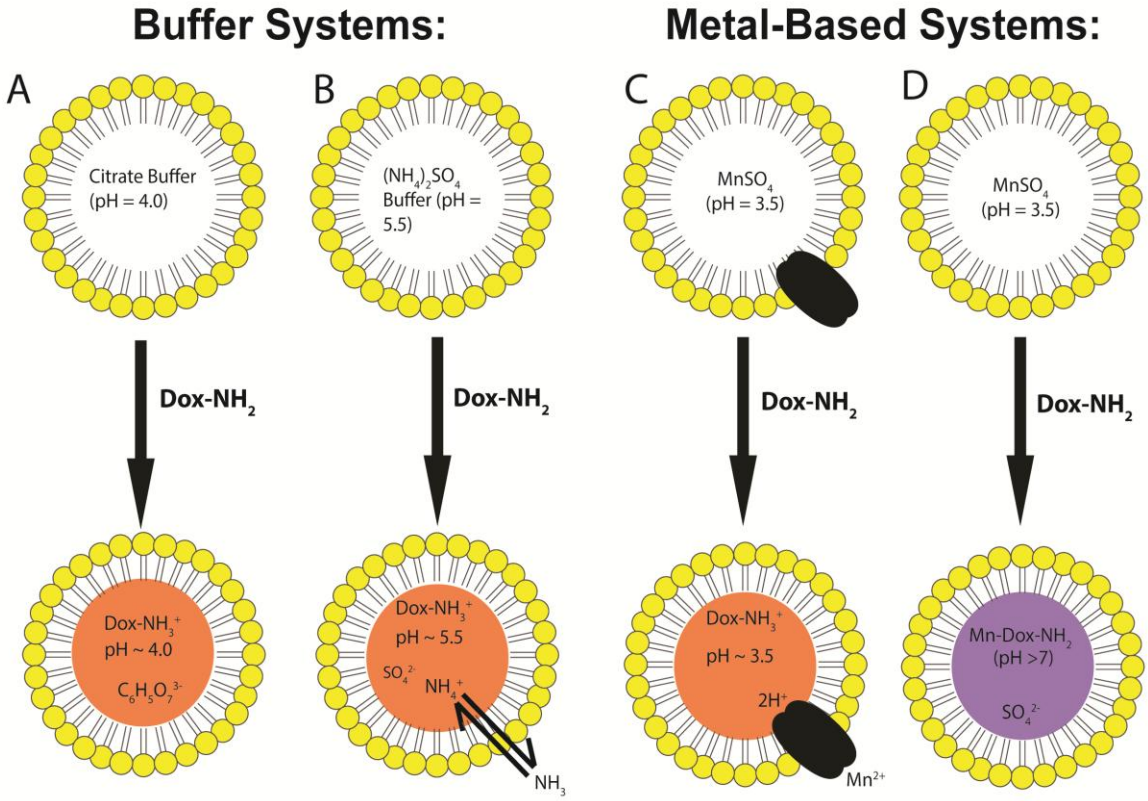


Figure 1. 5: Remote loading approaches to encapsulate doxorubicin into liposomes. Liposomes can be prepared in (A) citrate (pH 3.5) or (B) ammonium sulfate (pH 5.5) solutions which maintain an acidic interior within the liposome. Doxorubicin within the acidic interior becomes protonated at the tertiary amine functional group. Doxorubicin encapsulation methods that rely on the use of trapped metal ions have evolved from the use of $MnSO_4$, which can be used (C) to create a pH gradient by addition of A23187 an ionophore, which exchanges protons for Mn^{2+} ions. (D) Direct metal complexation can also be used to encapsulate doxorubicin in liposomes. The drug–metal complex forms in the liposome and the metal-complexed drug is less membrane permeable and trapped.

1.10.3 Use of A23187 (Divalent Metal Ionophores) to Facilitate Drug Loading in Liposomal Formulations Prepared with Metal Ion Gradients

The use and success of divalent metal ionophores such as A23187 (calcimycin) to create and maintain pH gradients in liposomes with encapsulated divalent metals such as manganese and copper is well established and characterized [242-244]. The ionophore facilitates the movement of two protons from the external buffer inside the liposome in exchange for one divalent cation. In addition to Mn^{2+} and Cu^{2+} other metals such as Mg^{2+} and Zn^{2+} can be used [17, 245]. When using this approach, the liposomes are prepared in the metal containing solutions which are typically acidic (pH <4.5). Thus the initial driving force for encapsulation of a compound added to the outside of liposomes prepared with a metal ion gradient is the pH gradient even in the absence of the ionophore. The addition of the ionophore facilitates maintenance of the pH gradient. Thus this method has been consistently applied to candidate drugs that have protonizable amine functions, albeit some of those compounds also carry metal binding motifs such as the camptothecins and anthracyclines.

It should be noted that published reports indicate that A23187 addition to liposomes with encapsulated copper can result in a candidate drug loaded liposome that exhibits improved drug retention when compared to other metal ions [246]. Although, this may be a result of copper-candidate drug interactions, it is unlikely that this contributes significantly to improved retention since the encapsulated copper concentration is reduced substantially when adding the ionophore to the liposomes. The remaining encapsulated copper, however, may interact with the phospholipids in the inner leaflet of the liposomal membrane and this copper-lipid interaction may improve drug retention [247, 248]. Notably, this is not observed for all metals as the improved retention is not observed for Mg^{2+}, Zn^{2+} or Mn^{2+} solutions.

1.11 Summary

The development of copper-based therapeutics has been hindered due to their very poor aqueous solubility. As an example, diethyldithiocarbamate (DDC) is the primary metabolite of disulfiram, an approved drug for alcoholism that is being repurposed for cancer. The

anti-cancer activity of DDC is dependent on complexation with copper to form copper bis-diethyldithiocarbamate ($Cu(DDC)_2$), a highly insoluble complex that has not been possible to develop for indications requiring parenteral administration. I have resolved this issue by synthesizing $Cu(DDC)_2$ inside liposomes (see Chapters 3 and 4). DDC crosses the liposomal lipid bilayer, reacting with the entrapped copper; a reaction that can be observed through a colour change as the solution goes from a light blue to dark brown. This method was successfully applied to other CDCs including the anti-parasitic drug clioquinol, the natural product quercetin and the novel targeted agent CX-5461 (see Chapters 3 and 5). The studies described in Chapter 6 supports the potential use of copper-based therapeutics to treat cancers that are insensitive to platinum drugs. The method, whereby CBTs are synthesized inside liposomes, provides a simple, transformative solution enabling, for the first time, the development of CDCs as viable candidate anti-cancer drugs; drugs that would represent a brand new class of therapeutics for cancer patients.

2. Materials and Methods

2.1 Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) , cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[carboxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}), Sphingomyelin (SM), 1,2-distearoyl-***sn***-glycero-3-phospho-(1'-***rac-***glycerol) (DSPG) and 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) was purchased from Avanti Polar Lipids (Alabaster, AL). Sephadex G-50 beads were purchased from GE healthcare (Boston, MA). ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Plumbagin (Plum) was obtained from Plumbago Indica, pyrithione (Pyr;2-mercaptopyridine N-oxide sodium salt), 8-Hydroxyquinoline, Sodium diethyldithiocarbamate trihydrate, clioquinol (5-Chloro-7-iodo-8-quinolinol), quercetin (Qu), DSF, Nigericin sodium salt, copper sulfate ($CuSO_4$), HEPES, and all other chemicals (Reagent grade) were purchased from Sigma Aldrich (Oakville, ON, Canada). CBDCA and CDDP were obtained from Hospira (Lake Forest, IL). CX-5461 was purchased from Selleck Chemicals (Houston, TX).

2.2 Cell Lines

The A549, FaDu, Cal-27, SCC-25, U87, U251MG, F98 and MV-4-11 cell lines were purchased from ATCC. A2780-S and A2780-CP cell lines were obtained from Dr. Mark W. Nachtigal at the University of Manitoba (Winnipeg, Canada) and the H1933 cells were provided by Dr. William Lockwood at the BC Cancer Agency's Research Centre (British Columbia, Canada). HBEpC (Human Bronchial Epithelial Cells) was obtained from Cell Applications (San Deigo, California) and MDA-231-BR was kindly donated by Patricia Steeg, NIH/NCI. All cell lines were used for 15-18 passages. A549 and H1933 cells were maintained in RPMI (Gibco). A2780-S, A2780-CP and SCC-25 were maintained in DMEM/F12 (Gibco). MV-4-11 and FaDu cells were maintained in IMDM (Gibco), and MEM (Gibco), respectively. U-87, U251MG, Cal-27 and MDA-231-BR were maintained in DMEM (Gibco). Media for all cell lines was supplemented with 2mM L-glutamine (Gibco) and 10% fetal bovine serum

(Gibco) and maintained at 37°C and 5% CO_2 in a humidified incubator. Media for the SCC-25 cell line was also supplemented with 400 ng/mL hydrocortisone. All cell lines tested negative for mycoplasma when obtained by the laboratory.

2.3 *In Vitro* Cell-Based Assays

2.3.1 Cytotoxicity Assay of Pt Drugs, Disulfiram, Ligands and Copper Complexes

For *in vitro* testing, copper complexes were synthesized prior to cell treatment by mixing $CuSO_4$ and the ligand at a fixed 2:1 ratio in DMSO (50%). Disulfiram and/or $CuSO_4$/$Cu(CQ)_2$ were mixed at a 1:1 ratio in DMSO (50%). Disulfiram or the ligands DDC, CQ, 8-HQ, Plum were solubilized in DMSO and Pyr was dissolved in sterile water. CBDCA and CDDP were dissolved in sterile saline (0.9%).

Prior to drug treatment, the cells were seeded into 384 well plates and incubated 24 hrs in media prior to treatment. The adherent cell lines (A549, A2780-S, A2780-CP, FaDu, Cal-27, SCC-25, H1933, U-87, U251, F98, MDA-231-BR and HBEpC) were exposed to the indicated compounds in triplicate wells for 72 hrs (unless indicated). Following treatment, cells were stained *in situ* with Hoechst 33342 and ethidium homodimer-I to differentiate between viable (Hoechst-positive/ethidium homodimer-negative) and dead cells that had lost membrane integrity (Hoechst-positive/ethidium-positive). Cells were imaged with the IN Cell Analyzer 2200 (GE Healthcare Life Sciences) and 4 images/well were collected. Images were analyzed with the ToolBox Developer 1.9 software (GE Healthcare Life Sciences) to obtain viable and dead cell counts based on differential staining of cell nuclei. The suspension cell line (MV-4-11) was treated for 72 hrs and viability was assessed using the Presto Blue™ assay (Life Technologies) following the manufacturer's instructions. The viability data were normalized to vehicle control (0.5% DMSO in media) and expressed as fraction affected where value of 1 corresponded to 100% loss of cell viability relative to vehicle controls and 0 corresponds to a viability comparable to control cells in culture.

2.3.2 Western Blot Analysis for Ubiquitinylated Protein

Cells were seeded in 6-well plates (400,000 cells per well) and treated with the IC_{30} of the indicated test compound for 24 hrs. Cell lysates were prepared using lysis buffer

comprising 50 mM Tris-HCl (pH 7.4), 150mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1mM EDTA, and Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics) for 1 hr on a shaker at 4°C. Cell lysates were centrifuged at 14000 x *g* for 10 min to collect total protein. A BCA Protein Assay Kit (Pierce) was used to determine protein concentrations and 10 µg of lysate protein were run on a 4-12% Bis-Tris gel (Life Technologies) at 170V for 1 hr before being transferred to a 0.2 µm nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in TBST (20mM Tris-base, 140mM NaCl, 0.1% Tween 20) and then probed for Ubiquitin (Cell Signaling Technology, 1:1000) and β-Actin (Sigma-Aldrich, 1:50,000) overnight at 4°C. Blots were then washed with TBST (3 x 5 min) and incubated with horseradish peroxidase-conjugated secondary antibody (Promega) for 1 hr at room temperature (1:10,000 for β-actin, 1:5000 for Ubiquitin). After washing with TBST (3 x 5 min), the blots were developed using Clarity Western ECL Substrate (Bio-Rad) for 5 min before imaging with the ChemiDoc MP Imaging System and ImageLab software (Bio-Rad).

2.3.3 Flow Cytommetric Analysis of $Cu(DDC)_2$ Treated Cells

MV-4-11 cells were seeded in 6-well plates for 24 hrs and then treated with vehicle, DDC, $CuSO_4$ or $Cu(DDC)_2$ (5µM final concentration). At 24 hrs post-treatment cells were washed 3 times with cold HBSS and fixed in 70% ethanol. The final concentration of cells was adjusted to 10^6 cells/mL. The samples were left for 1 hr on ice followed by an overnight incubation at -20˚C. Cells were centrifuged and the pellet was stained using phosphate buffered saline (PBS, pH 7.4) buffer containing 50 µg/mL propium iodide (Invitrogen), 1 mg/mL RNase A (Sigma), and 0.1% Triton X-100 (Bio-Rad) for 15 mins at 37˚C followed by an incubation for 1 hr on ice. Data were acquired and analyzed using a FACS Calibur flow cytometer and WINMDI 2.9 software, respectively.

2.3.4 Reactive Oxygen Species Assay

The reactive oxygen species (ROS) assay was performed using the ROS-Glo™ H_2O_2 Assay Kit (Promega) as per manufacturer instructions. Briefly, MV-4-11 cells were seeded at 14,000 cells/well in 96-well, white, clear-bottom plates. Treatment was added subsequently along with 20 µL substrate per well as per the supplier's protocol. Cells were

treated with either menadione (positive control, 50μM) or $Cu(DDC)_2$ (200μM) for 4 hrs. All treatments were completed with and without cells to account for ROS generation as a result of interaction with vehicle and medium components. Finally, the H_2O_2 detection solution was added at 100 μL/well and incubated at room temperature for 20 mins. ROS generation was measured based on luminescence signal using a FluorStar Optima plate reader.

2.3.5 Phen Green™ FL Copper Ionophore Testing

Phen Green™ FL was used to assess the amount of copper in the presence and absence of CQ in A2870-CP cells. Cu reduces the fluorescence intensity of Phen Green™ and thus allows for the identification of Cu entering the cell. Cells were grown to 80-90% confluency and treated with vehicle (0.01% DMSO), CQ (25μM), $CuSO_4$ (100μM) and CQ/$CuSO_4$ (25/100μM) for 1 hr. The cells were washed three times with HBS prior to media replacement with fresh medium containing 5μM Phen Green™ FL for 0.5 hr. Cells were then washed three times with Hanks Buffered Saline Solution and imaged using InCELL Analyzer 2200 (excitation 420 nm and emission 538 nm).

2.4 Liposome Preparation and Characterization

2.4.1 Liposome Preparation

The extrusion method for liposome preparation has been well documented by others [240]. Briefly, for the cholesterol containing formulations (DSPC/Chol (55:45), SM/Chol (55:45), DSPC/DSPG/Chol/ (70:20:10),DSPC/DC-Chol/DSPE-PEG_{2000} (30:50:20) or , DSPC/Chol/DSPE-PEG_{2000} (45 to 50: 45: 0 to 5 mole ratio) the lipids were removed from the freezer (-80^{o}C) and placed in a desiccator for 2 hrs before being weighed and dissolved in chloroform at the appropriate ratios. A non-exchangeable and non-metabolizable lipid marker, ^{3}H-CHE, was incorporated into the chloroform mixture to achieve a specific activity of approximately 0.025 μCi/mmol total lipid. The solution was dried from chloroform using nitrogen gas and the thin film generated was further dried under high vacuum for 3 hrs. The lipid film was then rehydrated at 65^{o}C with unbuffered 300mM $CuSO_4$ (pH 3.5) or 300mM Cu-Gluconate (pH 3.5) for at least 2 hrs. To prepare $Cu(DDC)_2$-liposomes with final

$Cu(DDC)_2$ to lipid ratios of 0.1 or 0.05, copper salt solutions of 150mM and 75mM were used. The resulting multilamellar vesicles underwent 5 freeze (in liquid nitrogen) and thaw (65°C water bath) cycles [249]. These were then placed in an extruder (Evonik Transffera Nanosciences, Vancouver) and extruded through stacked 0.1 μm polycarbonate filters at least 10 times at 65°C. All formulations were characterized for size and polydispersity (ZetaPals, Brookhaven). Samples were diluted to 1-5 mM in filtered 0.9% NaCl or SH buffer for size and polydispersity analysis.

For cholesterol-free formulations (DSPC/DSPE-PEG_{2000} or SM/DSPE-PEG_{2000}, 95:5), the indicated lipids were removed from the freezer and placed in a desiccator for at least 2 hrs before being weighed and dissolved in ethanol. ^{3}H-CHE was incorporated in the dissolved lipids in ethanol. The final ethanol solution with dissolved lipids was added dropwise to a solution of 300mM unbuffered $CuSO_4$ (pH 3.5) in a 65°C water bath with constant mixing. The final ethanol concentration of the aqueous ethanol mixture was approximately 15% (v/v). This solution was then extruded using the same process as written above.

For all liposomal preparations the unencapsulated copper was removed by first exchanging the sample into a sucrose (300mM), HEPES (20mM) and EDTA (15mM) buffer (SHE buffer, pH 7.4) by passing the sample through a Sephadex G-50 column equilibrated with the buffer. The resulting solution was then dialyzed against a sucrose (300mM) and HEPES (20mM) buffer (SH buffer, pH 7.4) and concentrated to the desired concentration for experimental studies using tangential flow. Liposomal lipid concentration was determined by measuring ^{3}H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer).

2.4.2 Copper Complexation Synthesis:

Copper loaded-liposomes were mixed with DDC (4 or 25°C), CQ (40°C), Qu (50°C) or CX-5461 (60°C) at the indicated compound to liposomal lipid ratio in the SH buffer (pH 7.5) and incubated over a 60-min time course. Liposome and associated compound were separated from unassociated (free) compound using a Sephadex G-50 column equilibrated with SH buffer. The eluted liposome fractions (collected with the excluded volume of the

column) were analyzed for copper, compound (as the copper complex or after dissociation of the bound copper) and liposomal lipid concentrations. Lipid concentrations were measured by assaying for [^{3}H]-CHE by liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer) where 20 µL of eluted liposome sample was mixed into 5 mLs Pico-Fluor Plus (Perkin Elmer). For the spectrophotometric assay samples were diluted into 1 mL methanol for $Cu(DDC)_2$ and $Cu(CQ)_2$ and absorbance was measured at 435nm (1-10 µg/mL) or 275nm (0.25-2.5 µg/mL), respectively. CuQu and CuCX-5461 were dissolved in 1 mL of 3% acetic acid in methanol and Qu and CX-5461 were measured by assessing absorbance at 372nm (1-10 µg/mL) or 288nm (1-10 µg/mL), respectively. Copper was measured using atomic absorption spectrophotomer (AAnalyst600, Perkin Elmer). The Cu-containing liposomes were diluted in 10 mLs of 0.1% HNO_3. A copper (Cu^{2+}) standard curve was generated using Cu^{2+} (0- 100 ng/mL) in 2% nitric acid (Sigma Aldrich).

2.4.3 Cryo-Electron Microscopy:

Further analysis of the $Cu(DDC)_2$ formulations was done by cryo-electron microscopy (CEM). CEM analysis was performed using a Zeiss Libra 120 transmission electron microscope at the University of Uppsala, Sweden. Briefly, liposomes were prepared as described above containing either $CuSO_4$ or $Cu(DDC)_2$ with SH buffer at pH 7.4. In a controlled chamber for humidity and temperature (25^{o}C) samples of 1-2 µL 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 108 K, which minimizes formation of ice crystals. Images were taken in a zero-loss bright-field mode and an accelerating voltage = 80 kV.

2.4.4 $Cu(DDC)_2$ and $Cu(CQ)_2$ Release From Liposomes

$Cu(DDC)_2$ or $Cu(CQ)_2$ containing liposomes (final liposomal lipid concentration 5 mM) were suspended in SH buffer with 50% (v/v) FBS and incubated with constant mixing at 37^{o}C in a water bath. At the indicated time points, an aliquot (100 µL) of the liposome solution was passed through a 1 mL Sephadex G-50 spin column equilibrated with SH buffer. The columns were centrifuged at 680 x *g* for 3 min at 25˚C. The eluate was assayed for

$Cu(DDC)_2$ or CQ using HPLC and lipid concentration was determined by measuring ^{3}H-CHE using scintillation counting. An aliquot (50 µL) of eluate was mixed with 950 µL methanol and the sample was then centrifuged at 10000 x g for 10 mins at 4^{o}C to pellet precipitated proteins. The supernatant was assayed for $Cu(DDC)_2$ or CQ using HPLC.

2.5 Analytical Assays:

2.5.1 $Cu(DDC)_2$ Quantification

For the copper complexation experiments an aliquot of liposomal $Cu(DDC)_2$ was diluted into 1 mL methanol and absorbance was measured 435 nm (1-10 µg/mL) on an Agilent 8453 UV-Vis spectrophotometer (Waldbronn, Germany).

For *in vivo* and release studies, $Cu(DDC)_2$ was measured on a Waters Alliance HPLC Module 2695 with a photodiode array detector (model 996, Milford, MA) and the resulting chromatograms were analyzed by Empower 2 software. A Pronto SIL 120-3-C18ace-EPS (3.0 µm, 4.6 x 150 mm) column was used with a mobile phase composed of 90% methanol and 10% water. A 100 µL sample volume was injected, the flow rate was 1 mL/min and column temperature was set to 40^{o}C. Only samples with >90 ng/mL could be detected. Thus, HPLC assay developed for $Cu(DDC)_2$ was not sensitive enough for the *in vivo* studies completed; therefore Cu was used as a surrogate marker for $Cu(DDC)_2$. Samples were diluted in 0.1% HNO_3 and subsequently the Cu concentration was measured using atomic absorption spectrophotometer (AAS; AAnalyst600, Perkin Elmer) see below.

2.5.2 $Cu(CQ)_2$ Quantification

For the copper complexation experiments an aliquot of liposomal $Cu(CQ)_2$ was diluted into 1 mL methanol and absorbance was measured at 275 nm (0.25-2.5 µg/mL) on an Agilent 8453 UV-Vis spectrophotometer (Waldbronn, Germany).

For *in vivo* and release studies, $Cu(CQ)_2$ was measured on a Waters Alliance HPLC Module 2695 and Empower 2 Software. A 30 µL sample volume was injected, and an isocratic mobile phase of water (pH 3 phosphoric acid) and acetonitrile (60:40) at a flow rate of 1 mL/min was used over a Luna C18 column (5 µm, 4.6 x 150 mm) heated to 55°C. CQ was

detected post column with a Waters 996 photodiode array detector (Milford, MA) at 254 nm. Pyrrolidine diethyldithiocarbamate was added to samples and standards at an excess of 3 mol equivalents prior to injection to ensure dissociation of CQ from Cu.

2.5.3 CuQu and CuCX-5461 Quantification

For copper complexation synthesis experiments, liposomal CuQu and CuCX-5461 were dissolved in 1 mL of 3% acetic acid in methanol and Qu and CX-5461 were measured by assessing absorbance at 372 nm (1-10 μg/mL) or 288 nm (1-10 μg/mL) on an Agilent 8453 UV-Vis spectrophotometer (Waldbronn, Germany), respectively.

The quantification of drugs in plasma was performed using HPLC. Qu was measured at 368 nm following separation on a symmetry C18 column (3.5 μm, 3.0 x 150 mm) using a mobile phase of 0.1% TFA in water and acetonitrile (2.3:1). A 25 μL sample volume was injected, the flow rate was set at 1 mL/min and the column temperature was 30^{o}C. Samples and standards were prepared in acidified methanol so as to dissociate the CuQu complex prior to HPLC analysis. Similarly, the quantification of CX-5461 was performed in acidified methanol to dissociate the complex and CX-5461 was measured at 300 nm following separation on a Luna C18 column (5 μm, 4.6 x 150 mm). The mobile phase contained a 1:1.2 mixture of 0.1% TFA in water and 0.1% TFA in methanol. A 5 μL sample volume was injected, the flow rate was set at 1 mL/min and the column temperature was 35^{o}C.

2.5.4 Copper Quantification

Liposomal and plasma copper quantification was performed using AAS. The Cu-containing liposomes were diluted in 0.1% HNO_3. A Cu^{2+} standard curve was generated using Cu^{2+} (0-100 ng/mL) in 2% nitric acid (Sigma Aldrich).

2.5.5 Liposomal Lipid Quantification

Lipid concentrations were measured by assaying for [^{3}H]-CHE by liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer) where 20 μL of eluted liposome sample was mixed into 5 mLs Pico-Fluor Plus (Perkin Elmer).

2.6 In Vivo Characterization of Liposomal Formulations:

2.6.1 Dose Range Finding Studies

2.6.1.1 Single Dose

Female CD-1 mice were given a single bolus tail vein i.v.(intravenous) injections of $Cu(DDC)_2$ (15 mg/kg, drug-to-lipid ratio 0.2 mol:mol), $Cu(CQ)_2$ (30 mg/kg, drug-to-lipid ratio 0.2 mol:mol), CuQu (70 mg/kg, drug-to-lipid ratio 0.2 mol:mol), or CuCX-5461 (50 mg/kg, drug-to-lipid ratio 0.2 mol:mol). All formulations were prepared using DSPC:Chol (55:45 mole ratio) liposomes with encapsulated 300 mM copper sulfate as described above. To define tolerability of the CBT formulations, mice (n=3) were given an intravenous injection (lateral tail vein) of the indicated formulation at the specified dose. The health of the animals was measured over a 14 day period after administration and a full necropsy was performed at that time to assess changes in tissue/organ appearance.

2.6.1.2 Multi-Dose

To define the maximum tolerated dose of $Cu(DDC)_2$ formulations, mice (n=3) were given an i.v. injection (lateral tail vein) of $Cu(DDC)_2$ using a Monday, Wednesday and Friday x 2 dosing schedule.

To define a dose of the $Cu(CQ)_2$ formulation that was well tolerated, mice (n=3) were given an i.v. injection (lateral tail vein) of $Cu(CQ)_2$ using a Monday, Wednesday and Friday x 2 dosing schedule. These studies also assessed the tolerability of $Cu(CQ)_2$ when combined with DSF; where DSF was dosed orally at 100 mg/kg, once daily Monday through Friday for 2 weeks). In these studies $Cu(CQ)_2$ was dosed at 30 mg/kg, Monday, Wednesday and Friday x 2 weeks.

For all dose range finding studies 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 surviving animals

were monitored for two weeks (14 days) after administration of the last dose and full necropsies were completed on all treated mice at that time to assess whether there were gross changes in tissue/organ appearance.

2.6.2 Pharmacokinetic Studies

DSPC/Chol (55:45) liposomes containing $Cu(DDC)_2$ (15 mg/kg, drug-to-lipid ratio 0.2 mol:mol), $Cu(CQ)_2$ (30 mg/kg, drug-to-lipid ratio 0.2 mol:mol), CuQu (70 mg/kg, drug-to-lipid ratio 0.2 mol:mol), CuCX-5461 (50 mg/kg, drug-to-lipid ratio 0.2 mol:mol) or DSPC/DSPE-PEG_{2000} (95:5) $Cu(DDC)_2$ (15 mg/kg, drug-to-lipid ratio 0.25 mol:mol) were injected into CD-1 mice. At selected time points mice (n=4 per time point) were terminated by isoflurane followed by CO_2 asphyxiation and blood was collected by cardiac puncture directly into EDTA coated tubes kept on ice. Blood samples were centrifuged (Beckman Coulter Allegra X-15R) at 1500 x *g* for 15 min at 4˚C. Plasma was collected and placed into a separate tube prior to assaying for copper, drug and liposomal associated lipid. The copper was measured using AAS by diluting plasma into 0.1% HNO_3 and drugs were measured using the HPLC method described above. Plasma (30 µL) was added to Pico-Fluor 40 scintillation cocktail and the amount of [^{3}H]-CHE was determined as described above.

2.7 In Vivo Efficacy Studies

2.7.1 Convection Enhanced Delivery in F98 Glioma Model

Male Fischer rats were purchased from Charles River Laboratories International Inc. (Wilmington, MA). The implantation method was described previously [250]. F98 cells (10,000 cells in 5 µL) were prepared and implanted into the right caudate nucleus (1 mm anterior, 3 mm right of the bregma, and 6 mm deep) of the brain in 5 min. The CED procedure [251] was performed 10 days after implantation of F98 cells, at the same injection site using a 33 gauge Hamilton syringe. Before infusion, the burr was filled with bone wax and the needle was inserted 6.5 mm deep, retained their for 5 min and then, withdrawn to 6 mm. Test articles and a control vehicle to be injected were vehicle (SH Buffer), $CuSO_4$ (300mM)- DSPC/Chol liposomes (copper 0.1 mg/mL, lipid 3.5 mg/mL) lipid or $Cu(DDC)_2$ formulated in DSPC/Chol liposomes (0.5 mg/mL, lipid 3.5 mg/mL). A

total volume of 10 µL was infused at an infusion rate of 0.5 µL/min for 20 minutes (5µg $Cu(DDC)_2$ per rat). After the infusion, the needle was left in, to reduce backflow and increase convection volume, for 5 min prior to being withdrawn.

2.7.2 $Cu(DDC)_2$ Efficacy Studies in the MV-4-11 and A2780-CP Subcutaneous Tumour Models

Animals were monitored at least three times weekly for body weight and tumour growth which was measured with calipers and tumour volumes were calculated based on a formula of (tumour length x tumour width^2)/2. Animals were terminated by isoflorane followed by CO_2 asphyxiation when animals reached a humane endpoint for these studies was defined when tumours exceeded 800 mm^3 or when tumours ulcerated.

2.7.2.1 MV-4-11 Subcutaneous Tumour Model

As indicated above MV-4-11 cells used for sc implantation were between passages 3-10 and were always collected when maintained at a confluence of 80-90% at the time of harvesting for implantation. RAG-2M mice (up to 9 per group) were inoculated with 1 x 10^6 cells in an injection volume of 50 µL per animal using a 28-gauge needle. Treatment was initiated on day 12 and treatment included the vehicle control (SH buffer), copper liposomes or $Cu(DDC)_2$ prepared in liposomes composed of either DSPC/Chol (55:45) or DSPC/DSPE-PEG_{2000} (95:5). .

2.7.2.2 A2780-CP Subcutaneous Tumour Model

A2780-CP cells that were used for subcutaneous (sc) implantation were between passages 3-10 and maintained in DMEM/F12 (Gibco) at a confluence of 80-90%. NRG mice (7 per group) were inoculated sc with 1 x 10^6 cells in a volume of 50 µL using a 28-gauge needle. Treatment was initiated on day four and treatment groups included the vehicle control (SH buffer), $CuSO_4$-liposomes (1.7 mg/kg copper, 50 mg/kg lipid), and $Cu(DDC)_2$ liposomes (8 mg/kg $Cu(DDC)_2$ and 50 mg/kg lipid) on a Monday, Wednesday and Friday x2 weeks dosing schedule. The copper dose of the $CuSO_4$ liposome control was equivalent to that of $Cu(DDC)_2$.

2.7.5 $Cu(CQ)_2$ Efficacy Studies in the U251 and A2780-CP Subcutaneous Tumour Models

Animals were monitored at least three times weekly for body weight and tumour growth which was measured with calipers and tumour volumes were calculated based on a formula of (tumour length x tumour width^2)/2. Animals were terminated by isoflorane followed by CO_2 asphyxiation when animals reached a humane endpoint for these studies was defined when tumours exceeded 800 mm^3 or when tumours ulcerated.

2.7.5.1 U-251 Subcutaneous Tumour Model

U-251 cells were grown in culture for 4-8 passages prior to inoculation. Rag2M mice (n=6 per group) were inoculated subcutaneously using a 28-gauge needle into the right flank of the mouse with 5 x 10^6 cells in a total volume of 50 µL. When the tumours reached 50-100 mm^3,as measured using digital calipers, they were treated with vehicle (SH buffer), $CuSO_4$-liposomes (Cu = 3.2 mg/kg) or $Cu(CQ)_2$-liposomes (CQ = 30 mg/kg) via iv injection on a Monday, Wednesday and Friday x 2 weeks treatment schedule. The copper dose of 3.2 mg/kg was selected as it is the equivalent amount of copper found in the $Cu(CQ)_2$ formulation.

2.7.5.2 A2780-CP Subcutaneous Tumour Model

A2780-CP cells that were used for subcutaneous (sc) implantation were between passages 4-8 and maintained in DMEM/F12 (Gibco) at a confluence of 80-90%. NRG mice (7 per group) were inoculated sc with 1 x 10^6 cells in a volume of 50 µL using a 28-gauge needle. Treatment was initiated four days after cell inoculation with either vehicle (SH buffer), $CuSO_4$-liposomes (Cu = 3.2 mg/kg) or $Cu(CQ)_2$-liposomes (CQ = 30 mg/kg) via iv injection on a Monday, Wednesday and Friday x 2 weeks treatment schedule. The copper dose of 3.2 mg/kg was selected as it is the equivalent amount of copper found in the $Cu(CQ)_2$ formulation.

2.7.6 $Cu(CQ)_2$ and DSF Combination Efficacy Studies in A2780-CP Subcutaneous Tumour Models

For combination studies, A2780-CP cells were grown and inoculated in NRG mice (n=13 per group) as outlined above. On day 4 mice were treated iv with vehicle (SH buffer),

$CuSO_4$-liposomes (Cu = 3.2 mg/kg) or $Cu(CQ)_2$-liposomes (CQ = 30 mg/kg) on a Monday, Wednesday and Friday x 2 weeks schedule. Additionally, DSF (100 mg/kg) was dosed by oral gavage Monday through Friday for 2 weeks alone and in combination with $CuSO_4$-liposomes or $Cu(CQ)_2$-liposomes given intravenously. Tumor size and body weight were measured three times weekly throughout the study. The health status of the animals was monitored daily following an established standard operating procedure as described above. Animals were terminated by CO_2 asphyxiation following isoflurane anaesthesia when tumors reached a maximum size of 800 mm^3 or underwent ulceration.

2.8 Statistical Analysis

All data were plotted using the Prism 6.0 software (GraphPad) as mean ± SEM or mean ± SD as described in the figure legends. The IC_{50} values and 95% confidence intervals (CI) were extrapolated from nonlinear regression (curve fit) of the cytotoxicity curves using Prism 6.0 (GraphPad software). To determine whether the cytotoxic effects of copper complexes are associated with platinum sensitivity, the IC_{50} values of each copper complex was plotted against the CDDP IC_{50} for each cell line. Each data point represents one cell line. The Pearson Correlation coefficient and corresponding two-tail p-values were then determined using Prism 6.0 (GraphPad software). A P-value < 0.05 was considered statistically significant. Survival study statistical analysis was performed using the log rank test; a P-value < 0.05 was considered statistically significant. Statistical analyses comparing $Cu(DDC)_2$ plasma levels, PhenGreen Cu ionophore comparison or tumour growth studies were performed using one-way ANOVA followed by Tukey adjustments to correct for multiple comparisons.

3. Nanoscale Reaction Vessels Designed for Synthesis of Copper-Drug Complexes Suitable for Preclinical Development*

3.1 Introduction

Over the past decade, the number of copper complexes with anti-cancer activity has been increasing and a number of reviews have detailed their synthesis and development [25, 48, 62]. However, CBTs have not been approved for use in patients. A significant challenge associated with the development of CBTs is their extremely low aqueous solubility, thus making it difficult to establish the utility of these copper-complexes in preclinical models or patients. Further, development of intravenous dosage formulations requires the use of solubilising agents to create products suitable for use. For example, the therapeutic promise of CBTs has largely been based on data obtained with compounds solubilized in DMSO [252-255]. Here we describe, for the first time, a novel method to prepare formulations of CBTs, formulations that are suitable for intravenous administration.

Methods that rely on copper complexation to encapsulate water-soluble drugs including anthracyclines and camptothecins have already been described; however, optimal drug loading and drug retention in these formulations relied on use of both the encapsulated metal and a transmembrane pH gradient [14, 242, 256, 257]. In fact, it is established that drugs with protonizable amine functions, can readily be encapsulated in liposomes using a transmembrane pH gradients [238]. Here, liposomes prepared with encapsulated copper were mixed with compounds that have copper-binding moieties. The compounds selected exhibit common attributes of extremely low (< 1 mg/mL) water solubility prior to or after complexation with copper.

The method was characterized using DDC (see Figure 1) as a model compound, which is known to be the active metabolite generated following administration of DSF

*Adapted from : M. Wehbe, M. Anantha, I. Backstrom, A. Leung, K. Chen, A. Malhotra, K. Edwards, M.B. Bally, Nanoscale Reaction Vessels Designed for Synthesis of Copper-Drug Complexes Suitable for Preclinical Development, *Plos One*, 11 (2016) e0153416

[118]. It has become a drug of interest for use in the treatment of human immunodeficiency virus (HIV) and cancer [116, 258-261]. DSF is metabolized to DDC, a well-known copper chelator [262-264]. DDC forms a copper complex at a 2:1 mole ratio (DDC:Cu^{2+}), a reaction that is detected by eye as a brown precipitate forms (Figure 3.1E). Unlike DDC, $Cu(DDC)_2$ is highly insoluble in water. Thus, pursuing opportunities related to repurposing DSF for oncology indications should focus on $Cu(DDC)_2$. The formulation relies on the fact that DDC would be membrane permeable and upon addition to preformed liposomes with entrapped copper salts, complexation would occur within the interior of the liposome. The $Cu(DDC)_2$ complex would not be membrane permeable and hence trapped. The resulting formulation was characterized and defined as suitable for intravenous administration.

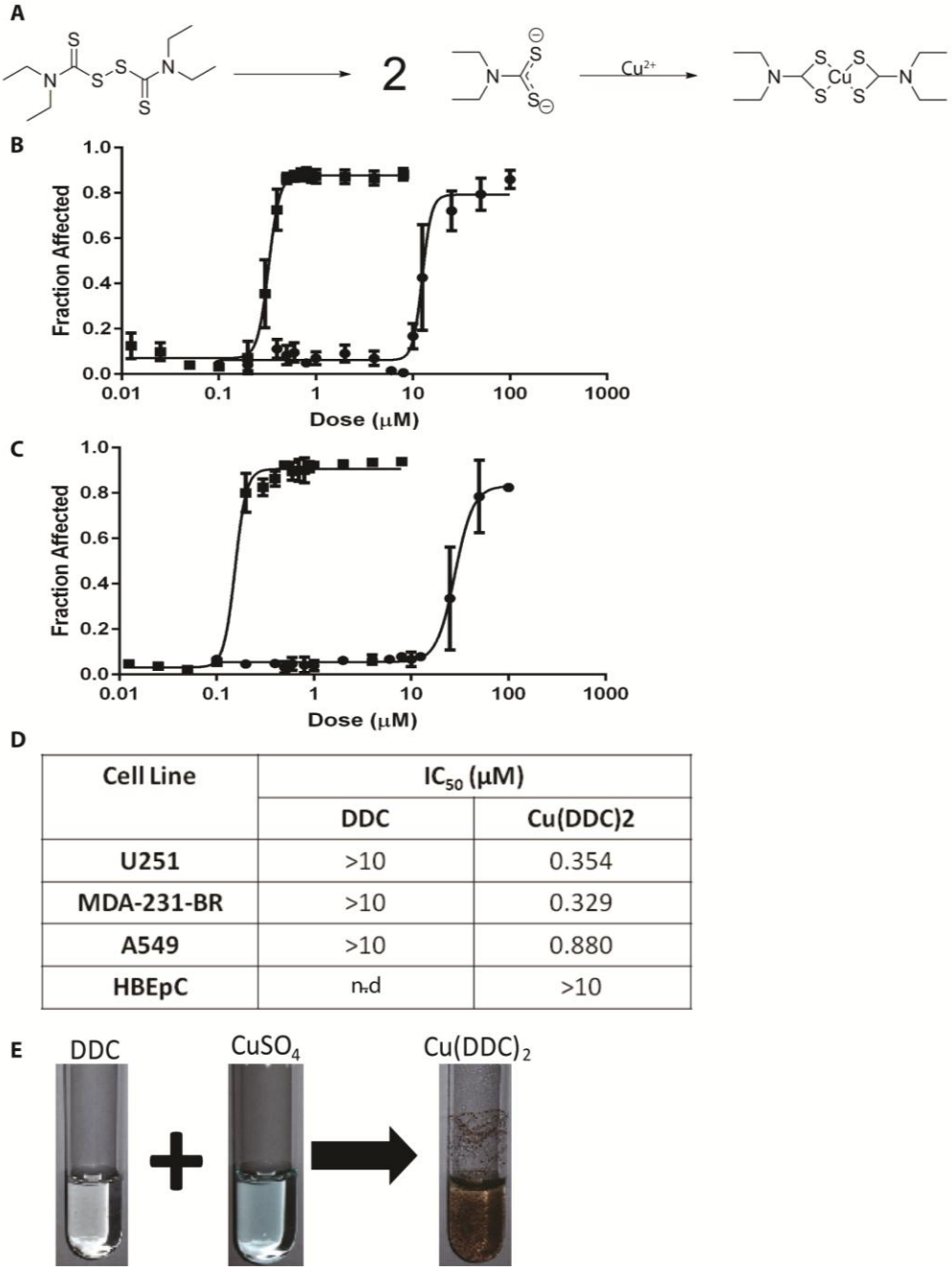
Importantly, the method for preparing $Cu(DDC)_2$ in liposomes proved suitable for other copper complexing compounds. Three types of copper-binding moieties were evaluated including S-Donor, O-Donor and N,O-Donor systems. Examples of drugs that are described here, in addition to DDC (an S-Donor), include Qu (an O-Donor), CQ (an N, O donor) as well as a compound, CX-5461, previously not identified as a copper complexing agent. CQ is an analogue of 8-hydroxyquinole and an approved antibacterial agent. It forms a Cu(II) complex which inhibits proteosome function and is known as a copper ionophore [50, 191]. Qu is a natural product belonging to the class of compounds known as flavanoids. In recent work it was shown to exhibit anti-cancer effects through generation of reactive oxygen species [265]. CX-5461 is a RNA polymerase inhibitor being evaluated in clinical trials [266, 267] and its use exemplifies the versatility of this method as CX-5461 is a high molecular weight compound with many functional groups capable of binding copper. With the existence of other donor systems not described here, it is likely that this method can be applied to a broad range of drugs and drug candidates with a variety of structures, sizes and functional copper-binding moieties [48, 62].

3.2 Results

3.2.1 DSF, DDC and $Cu(DDC)_2$ Cytotoxicity

DSF is metabolized to DDC (Figure 3.1A) and DDC is a well-established copper chelator [42, 268]. The cytotoxic activity of DSF when added to cancer cells is copper

dependent. As shown in Figure 3.1B, the IC_{50} of DSF against U87 glioblastoma cells is >10 μM in the absence of copper. In the presence of copper there is a substantial shift (2-orders of magnitude) in cytotoxicity when copper is added with DSF at a 1:1 molar ratio. DSF is unable to interact with copper; thus, activity of DSF depends on its degradation to DDC. As shown in Figure 3.1C, the activity of DDC in the absence of copper is also >10 μM and in the presence of copper (2:1 molar ratio of DDC to copper) is approximately 220 nM. Similar results were obtained in 3 other cell lines where the IC_{50} of copper + DDC was 345, 329 and 880 nM when used against U251 (glioblastoma line), MDA-231BR (a triple negative breast cancer line selected for its propensity to metastasize to the brain) and A549 (lung cancer line) cells, respectively. DDC as well as $Cu(DDC)_2$ exhibited little activity when added to normal human bronchial epithelial cells (HBEpC), suggesting specificity of $Cu(DDC)_2$ against cancer cells. It is argued that attempts to repurpose DSF as an anti-cancer drug should focus on $Cu(DDC)_2$; however, $Cu(DDC)_2$ is almost completely insoluble in aqueous solution (Figure 3.1E).



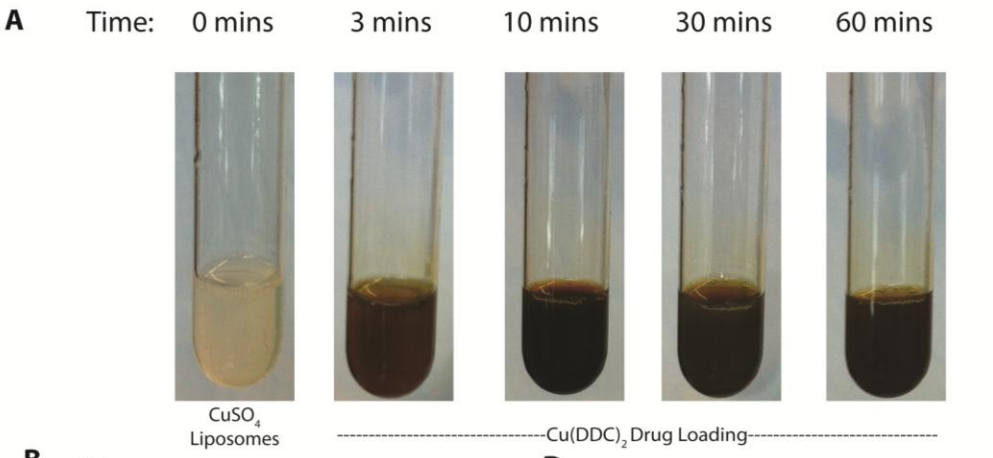
Cell Line	IC_{50} (μM)	
	DDC	Cu(DDC)2
U251	>10	0.354
MDA-231-BR	>10	0.329
A549	>10	0.880
HBEpC	n.d	>10

Figure 3.1: The anti-cancer activity of diethyldithiocarbmamate (DDC) is dependent on copper. (A) Disulfiram is metabolized to diethyldithiocarbamate (DDC) and DDC complexes with Copper (Cu) (II). **(B)** Cytotoxicity curves for DSF (●) and DSF + $CuSO_4$ (■) were obtained with the IN CELL Analyzer using U87 glioblastoma cells where cell viability was assessed based on loss of plasma membrane integrity 72 hours following treatment; i.e. total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively. **(C)** Cytotoxicity curves for DDC (●) and DDC + $CuSO_4$ (■); where cytotoxicity was measured as described above. (D) DDC and $Cu(DDC)_2$ IC_{50} for U251, MDA-231-BR, and A549 cancer cell lines as well as HBEcP a normal cell line; averages (±SEM) are reported from three separate experiments each done in triplicate. (E) Photograph of DDC, $CuSO_4$ and $Cu(DDC)_2$ solutions in water.

3.2.2 Formation of $Cu(DDC)_2$ by Addition of DDC to Pre-formed Copper Containing Liposomes

As illustrated in Figure 3.2, within minutes of DDC addition to copper containing lipid vesicles a color change indicative of $Cu(DDC)_2$ formation is visible (Fig. 3.2A). The rate of $Cu(DDC)_2$ formation inside the liposome can be quantified by separating liposome-associated $Cu(DDC)_2$ from unassociated DDC and then assaying for $Cu(DDC)_2$ spectrophotometrically. As shown in Fig. 3.2B, the amount of liposome-associated $Cu(DDC)_2$, measured as the $Cu(DDC)_2$ to liposomal lipid ratio, is temperature-dependent. $Cu(DDC)_2$ association is rapid when DDC is added to copper-containing liposomes at 25°C (RT) and 40°C, where the maximum $Cu(DDC)_2$ to lipid ratio of 0.2 (mol ratio) is achieved within 3 minutes. If the temperature is decreased to 4°C, the $Cu(DDC)_2$ to lipid ratio of 0.2 (mol ratio) is achieved at 60 minutes. The movement of DDC from the external media into the copper-containing liposomal core is not affected by external pH. As shown in Fig. 3.2C, when the external pH is adjusted to 3.5 the loading rate is comparable to that observed at pH 7.4. To determine the maximum $Cu(DDC)_2$ to liposomal lipid ratio that can be achieved when using liposomes prepared in 300 mM copper sulfate, the amount of external DDC was titrated from 0.04 to 0.40 (moles DDC to moles liposomal lipid) and the results suggest that the maximum $Cu(DDC)_2$ to lipid ratio achievable under these condition was 0.2 (mol:mol). This was achieved when the initial DDC to liposomal lipid ratio was 0.4 (mol:mol). As indicated, $Cu(DDC)_2$ forms an insoluble precipitate and it was possible that the synthesis of $Cu(DDC)_2$ inside the liposomes may have engendered formation of a precipitate within the liposomes. To evaluate this, the liposomes were visualized by CEM (Fig. 3.2E). The results illustrate two points: (1) the $Cu(DDC)_2$ liposomes exhibited a mean particle size that was comparable to that observed with the copper containing liposomes before addition of DDC, and (2) the formation of $Cu(DDC)_2$ inside the liposomes did not result in the formation of an electron dense core. It should be noted that while a precipitate was not seen in the cryo-TEM images it does not indicate that $Cu(DDC)_2$ has not precipitated in the liposome core. It is possible that a $Cu(DDC)_2$ precipitate is present but does not exist in a physical state which could effectively absorb electrons such that it could be seen using cryo-TEM. These points do suggest that there is a homogenous distribution

of CDCs across all liposomes in solution. It should be noted that the liposome size estimated by CEM analysis was comparable to that determined by QELS (Fig. 3.2F).



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Lipid Nanoparticles	Quasi-Electric Light Scattering			Cryo-Electron Microscopy	
	Size (nm)	Std. Dev (nm)	PDI	Size (nm)	Std. Dev (nm)
$CuSO_4$	103.9	10.71	0.070	80.90	21.76
$Cu(DDC)_2$	96.08	12.21	0.100	88.4	16.93

Figure 3.2: Diethyldithiocarbamate (DDC) loading into DSPC/Chol (55:45) liposomes prepared with encapsulated 300 mM $CuSO_4$. (A) Photograph of solutions consisting of DDC (5mg/mL) and added to $CuSO_4$-containing DSPC/Chol (55:45) liposomes (20 mM liposomal lipid) over a 1 hour at 25°C. (B) Formation of $Cu(DDC)_2$ inside DSPC/Chol liposomes (20 mM) as a function of time over 1 hour at 4(●), 25(■) and 40(▲)°C following addition of DDC at a final DDC concentration of (5 mM); $Cu(DDC)_2$ was measured using a UV-Vis spectrophotometer and lipid was measured using scintillation counting. (C) $Cu(DDC)_2$ formation inside DSPC/Chol (55:45) liposomes over time where the external pH was 7.4 (▲) and 3.5 (▼). (D) Measured $Cu(DDC)_2$ as a function of increasing DDC added, represented as the theoretical $Cu(DDC)_2$ to total liposomal

lipid ratio; where the lipid concentration was fixed at 20 mM and final DDC concentration was varied. (E) Cryo-electron microscopy photomicrograph of $CuSO_4$- containing DSPC/Chol (55:45) liposomes and the same liposomes after formation of encapsulated $Cu(DDC)_2$. (F) Size of the $CuSO_4$- containing liposomes and liposomes with encapsulated $Cu(DDC)_2$ as determined by quasi-electric light scattering and cryo-electron microscopy; data points are given as mean ± SD.

The only change in liposomal lipid composition considered in these studies was incorporation of polyethylene glycol (PEG_{2000}) modified DSPE. This was considered for two reasons: (1) DSPE- PEG_{2000} is a negatively charged lipid and its use should increase the amount of encapsulated copper when preparing the liposomes; and (2) DSPE- PEG_{2000} is known to prevent surface-surface associations that can influence liposome-liposome aggregation and liposome-cell interactions which, in turn, can effect elimination rates *in vivo* [269]. When DSPE- PEG_{2000} was added to our base lipid formulation of DSPC:CHOL (55:45) ranging from 0.5 to 5% (based on reductions of DSPC content) the maximum amount of liposome-associated $Cu(DDC)_2$ as measured by the $Cu(DDC)_2$ to liposomal lipid ratio increased from 0.2 to 0.4 (Figure 3.3A, gray bars). When analyzing the amount of copper associated with these liposomes (black bars) it was clear that the $Cu(DDC)_2$ to liposomal lipid ratio was related to the amount of copper encapsulated in the liposomes. The addition of DSPE- PEG_{2000} increased copper encapsulation, likely due to the introduction of an anionic change that is known to enhance liposome trapped volume [240]. The DSPC/CHOL/DSPE-PEG_{2000} (50/45/5 mol ratio) was selected to establish the relationship between amount of encapsulated copper and final $Cu(DDC)_2$ to liposomal lipid ratio. These liposomes were prepared using copper sulfate solutions with copper concentrations ranging from 0 to 300 mM. The osmolarity (~300 mOs/kg) of these solutions was balanced with $MgSO_4$. These liposomes were analyzed for copper content prior to DDC addition and after addition of DDC in excess (>2-fold molar excess to the measured liposome associated copper for liposomes prepared in the 300 mM copper sulfate solution). The results (Fig. 3.3B and 3.3C) are consistent with the data in Fig. 3A; the $Cu(DDC)_2$ to liposomal lipid ratio achieved was directly proportional to the amount of copper retained in the liposomes. A plot of encapsulated copper vs encapsulated $Cu(DDC)_2$ demonstrated a linear regression fit of R^2=0.9754. This is consistent with a 1:2 mol ratio between copper and added DDC.

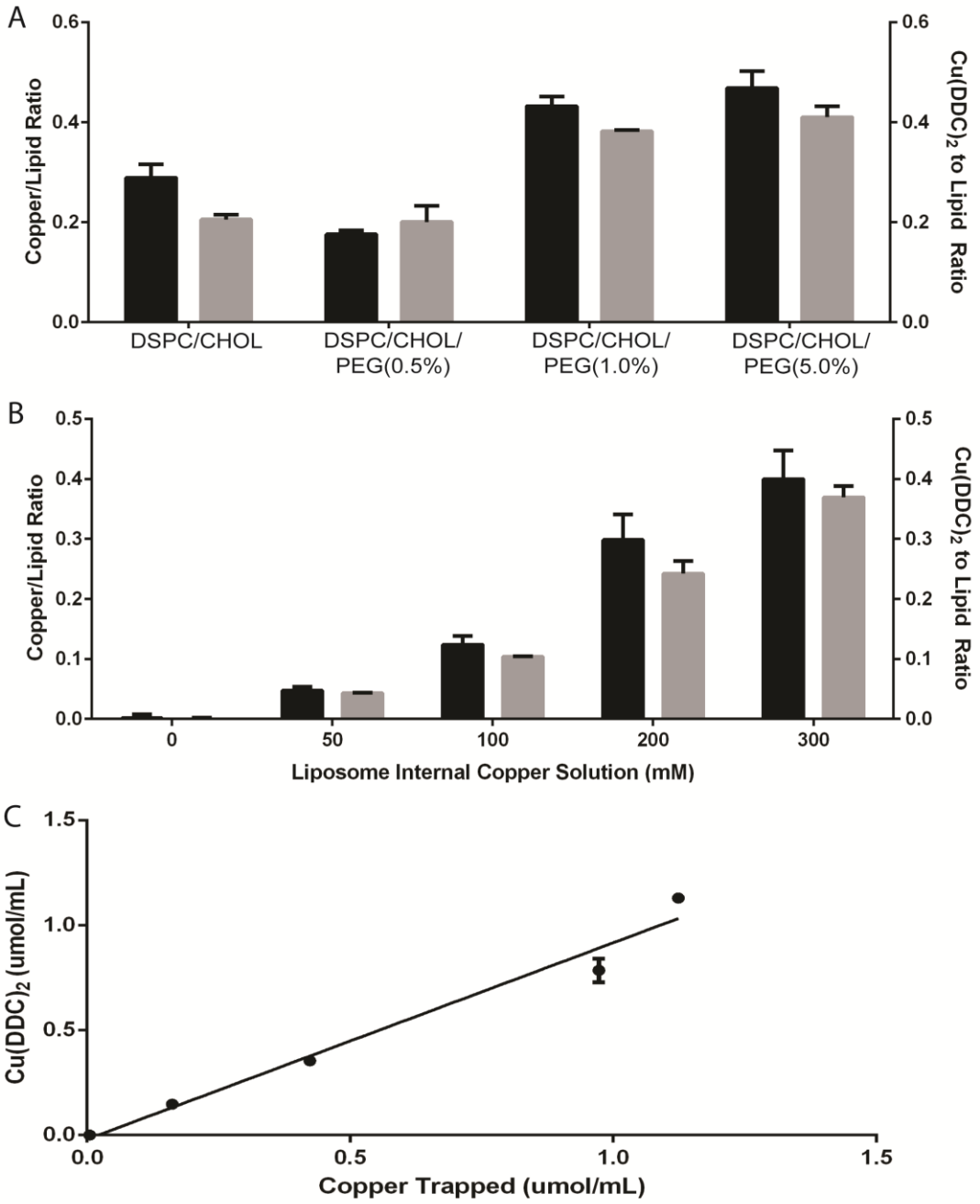


Figure 3.3: Characterization of copper-complex loading method. (A) Measured (AAS) copper to liposomal lipid ratio (black bars) compared to measured $Cu(DDC)_2$ (UV-Vis spectrophotometer) to liposomal lipid ratio (grey bars) after DDC was added to $CuSO_4$-containing DSPC/Chol liposomes prepared with different amounts of DSPE-PEG_{2000} (ranging from 0 to 5 mole%). (B) Formation of $Cu(DDC)_2$ inside $CuSO_4$-containing DSPC/ Chol/DSPE-PEG_{2000} (50:45:5) liposomes as a function of the $CuSO_4$ concentration used to prepare the liposomes (ranging from 0 to 300 mM); where the measured copper (AAS) to liposomal lipid ratio (black bar) is compared to the measured $Cu(DDC)_2$ (UV-Vis spectrophotometer) to liposomal lipid ratio (grey bar). (C) Linear regression analysis comparing measured (AAS) copper concentration (assuming encapsulated copper was free in solution) to measured $Cu(DDC)_2$ (UV-Vis spectrophotometer) concentration (assuming encapsulated $Cu(DDC)_2$ was free in solution); R^2= 0.9754; each data point represents a mean ± SEM determined from at least three separate experiments done in duplicate.

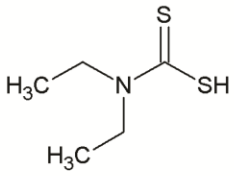
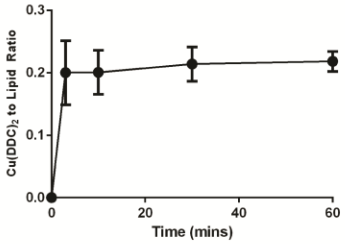
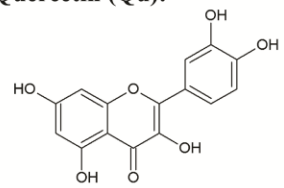
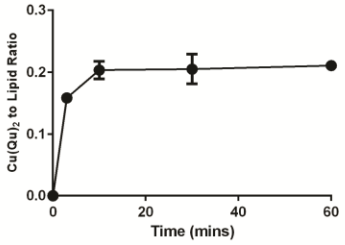
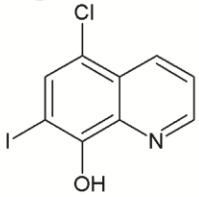
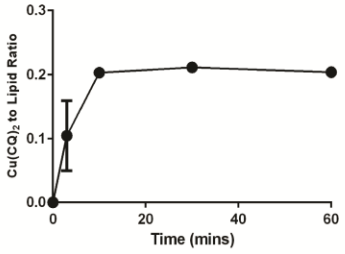
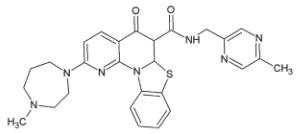
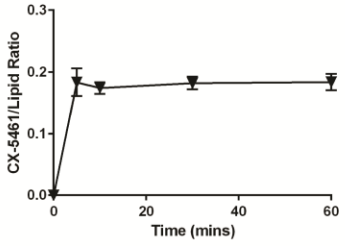
3.2.3 Use of Copper Containing Liposomes to Prepare CBTs Suitable for Intravenous Injection

The approach is compatible to other copper-binding drugs and candidates. As a platform approach, this method would address one of the most important problems limiting the development of CBTs. To assess the breadth of this approach, other compounds that contain functional groups known to bind copper were evaluated. These are summarized in Table 3.1 and include, but are not limited to, S-Donor, O-Donor and N,O-Donor systems. Examples tested, in addition to DDC (an S-Donor), include Qu (an O-Donor), CQ (an N,O donor) as well as a compound, CX-5461, previously not identified as a copper complexing agent. The indicated drugs are all sparingly soluble in aqueous solutions at pH 7.4 and all can be encapsulated when added to lipid vesicles (DSPC/CHOL (55:45 mole ratio)) prepared with encapsulated copper. It should be noted that due to their very low aqueous solubility Qu and CQ were added in a solid/powdered form. CX-5461 is also poorly soluble in aqueous solutions but could be prepared as a metastable solution in low pH (4.5) phosphate buffer as described elsewhere [270]. As noted in Table 3.1 (far right column) all formulations could be designed to achieve a final Cu-complexed drug to liposomal lipid ratio of 0.2 (mol:mol). In every example formation of the CDC was rapid, but the temperature needed for CDC was empirically determined. $Cu(DDC)_2$ formation was optimal at 25^{o}C, $Cu(CQ)_2$ formation was optimal at 40^{o}C, the Cu(Qu) and Cu(CX-5461) formations were optimal at 50^{o}C and 60^{o}C, respectively.

The goal of this method was to facilitate the ability to test copper complex compounds as therapeutics following intravenous administration. Preliminary data to support this is provided in Figure 3.4. The formulations described in Table 3.1, were prepared for single dose safety studies in mice and once a safe dose was defined, the elimination of the copper complex compound was determined as described in the Methods. Figure 3.4A summarizes the change in body weight of mice injected with the indicated formulation at the determined maximum tolerated dose. The formulations caused <15% body weight loss and other health status indicators suggested mild and reversible changes in animal health status. The elimination behaviors of the intravenously injected compounds were dependent on the compound tested. $Cu(DDC)_2$ was eliminated the most rapidly with

<10% of the injected dose remaining after 1 hr. Cu(CX-5461) exhibited the longest circulation longevity with > 30% of the injected dose remaining in circulation after 8 hr.

Table 3. 1: Donor systems that can be used in Copper(II)-complex loading. Copper is able to form complexes with compounds containing S-Donor, O-Donor and N,O-Donor systems as well as other mixed donor systems. Examples of drugs that are described here, in addition to DDC (an S-Donor), include quercetin (Qu) (an O-Donor), clioquinol (CQ) (an N,O donor) as well as CX-5461, previously not identified as a copper complexing agent. Each was loaded into DSPC/Chol (55:45 mol ratio) liposomes prepared with 300 mM $CuSO_4$. The loading temperature used in these examples was 25, 50, 40 and 60°C, respectively.

System	Examples	Compound Selected	Synthesis Rate
S-Donor	- Thiosemicarbazones - Dithiocarbamates - Thioureas - Dithiolates	Diethyldithiocarbamate (DDC): 	
O-Donor	- Pyridine N-Oxides	**Quercetin (Qu):** 	
N,O-Donor	- Phenol analogues of 8-Hydroxyquinoline - Napthoquinones - Carboxylates - Triethanolamines	**Clioquinol (CQ):** 	
Mixed/ Unknown	- Systems containing multiple or competing Cu-binding sites - Systems with unresolved Cu-complex structure	**CX-5461:** 	
Others: Schiff Base Systems, Hydrazones, P-donor Phosphine systems, N-donor systems (including: pyrazoles, imidazoles, triazoles and indoles)			

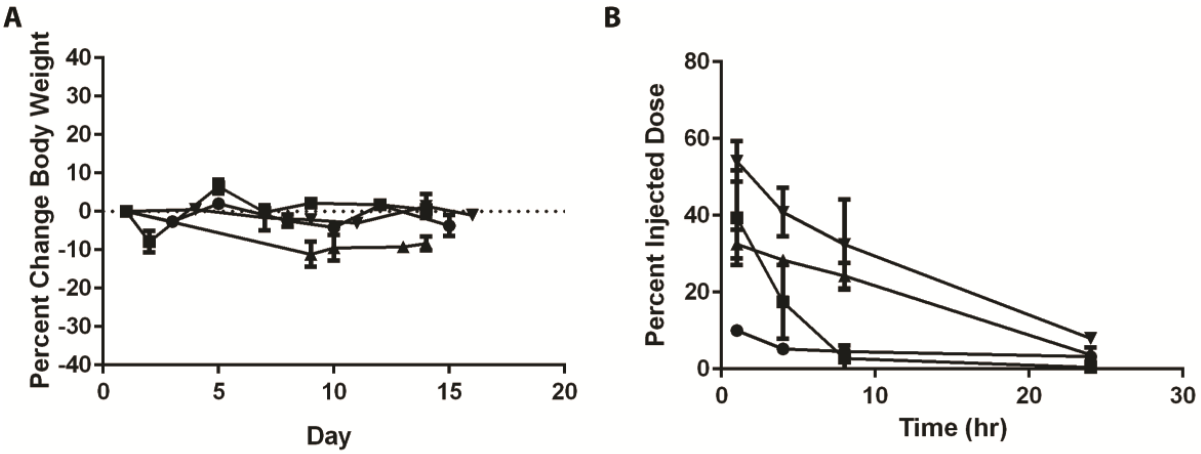


Figure 3. 4: Preliminary tolerability and plasma elimination profiles for liposomal formulations of $Cu(DDC)_2$, $Cu(CQ)_2$, CuQu and CuCX-5461 after intravenous injection into CD-1 mice. Mice were injected with a single dose of 15 mg/kg $Cu(DDC)_2$ (-●-), 30mg/kg $Cu(CQ)_2$ (-■-), 70mg/kg CuQu (-▲-) and 50 mg/kg Cu-CX-5461 (-▼-). (A) Changes in body weight following administration of the indicated liposomal formulation where body weights were measured over 14 days after injection (n=3). (B) Preliminary plasma elimination profiles of the indicated liposomal formulations where the copper-complexed compound was measured at 1, 4, 8 and 24 hrs after administration (n=4); concentrations were measured using HPLC or AAS as described in the Methods.

3.3 Discussion

The therapeutic (anti-cancer) activity of DDC (a metabolite generated after administration of DSF) is inactive in the absence of copper; further, DDC is not stable in acidic solutions leading to rapid degradation [226]. The complex formed when mixing $CuSO_4$ and DDC is charge neutral and forms as a dark brown precipitate [271]. It was hypothesized that lipid vesicles (liposomes) could behave as "nano-scaled reaction vessels" for $Cu(DDC)_2$-complex formation. Provided that the liposome structure remains intact, synthesized $Cu(DDC)_2$ would remain in suspension and would be suitable for intravenous administration. This proved to be the case as illustrated by the results in Figure 3.2 and 3.4. While the cryo-TEM images do not confirm the formation of an electron dense precipitate inside the liposomes it does not rule out the possibility that such a precipitate exists. To confirm that $Cu(DDC)_2$ was being formed in the liposome and not as a nano-precipitate, fractionated column chromatography (data not shown) was performed and shows that $Cu(DDC)_2$ does elute with the liposome fraction. In aggregate, these data confirm the formation of $Cu(DDC)_2$ inside liposomes.

The process described here is distinct from other liposomal formulation methods which rely on compounds considered somewhat soluble in aqueous solution (>1mg/mL). Comparisons may be drawn to pH gradient loading methods [13, 14] or comparable methods relying on use of encapsulated ammonium sulfate [19, 244] or encapsulated ions and an added ionophore designed to generate a pH directly [242] or indirectly through formation of a transmembrane potential [272]. In these previous examples, the agents formulated into the liposomes have protonizable amine functions and encapsulation and/or drug retention is influenced by the presence of the transmembrane pH gradient (acidic interior) and/or the presence of residual liposome associated metal. The method described here works for compounds that do not have protonizable amine functions and are sparingly soluble in water. In fact, changing the pH from 7.4 to 3.5 showed no effect on the ability for DDC to complex copper trapped in the lipid vesicles. For the $Cu(DDC)_2$ formulation, evidence shows that the amount of $Cu(DDC)_2$ formed is directly related to the amount of encapsulated copper (see Fig. 3.3). For DDC, one copper molecule binds two DDC molecules to form $Cu(DDC)_2$. This appears to be the case of CQ as well. Whereas, Qu or CX-5461 complexation appears to be defined at a 1 to 1 ratio. Future studies will further characterize what factors influence the nature of the copper complex formed within the liposome.

The rate of copper-complex formation is likely dependent on the rate at which the externally added compound crosses the liposomal lipid bilayer, which in the examples here were prepared from DSPC/Chol. These formulations are generally considered stable and exhibit reduced permeability at physiological temperatures. Formation of $Cu(DDC)_2$ is rapid, occurring in minutes, while the reaction between CX-5461 and lipid vesicle entrapped copper is more gradual. The rate of loading may, however, be dependent on several factors, including temperature, divalent metal ion, the counter ion, the ionic strength, pH etc. Using Qu as an example, this compound is added as a powder to the pre-formed copper containing liposomes. The amount of Qu in free solution, albeit low, will increase with increasing temperature. Solubilized Qu will be free to move across the liposomal lipid bilayer (from the outside to the inside), and the permeability of Qu across the membrane will be lipid composition and temperature dependent. Once inside the

liposome, Qu will complex with copper, creating a complex that has reduced solubility and reduce membrane permeability.

The preliminary data provided in Figure 3.4 demonstrate that the rate of release of the CDCs is dependent on the agent used. Each CDC formulation is unique and will be examined more specifically in ensuing publications focusing on *in vitro* and *in vivo* behaviour, including stability of the CDC as well as the retention of the active agents. $Cu(DDC)_2$ is released rapidly from the liposomes following i.v. administration of the formulations as evidenced by the rapid elimination of $Cu(DDC)_2$. The rate of elimination of Cu-complexed CX-5461 is considerably slower. Although not shown, it is important to note that both copper and complexed agent are released when administering the $Cu(DDC)_2$ and $Cu(CQ)_2$ formulations. In contrast, it appears that only Qu and CX-5461 are released from the CuQu and CuCX-5461 formulations in a form that is not complexed to copper. This has only been established in lipid vesicles prepared from DSPC/Chol (55:45, mole ratio) and further study into the effects of lipid composition and copper salt will be performed.

By formulating complexes through an inorganic synthesis reaction occurring within the liposomal core, one can obtain high drug-to-lipid ratios that are dependent on the number of copper ions inside and the nature of the complex formed. The formulations prepared this way appear stable; all formulations described here were stable with respect to particle size, polydispersity and complex to liposomal lipid ratio for at least 21 days at 4^oC (results not shown). The method is scalable and suitable for manufacturing a pharmaceutical product. The inability to test poorly soluble complexes is a problem that has limited the ability of promising copper-based therapeutics to transition from the bench to the clinic. There are studies that show efficacy in tumour models using solubilising agents that have been formulated with other strategies like very low pH or Cremphor/DMSO/Ethanol mixtures [273-275]; solutions that are not particularly suitable for human use.

3.4 Conclusion

This method provides a simple, transformative solution enabling, for the first time, the development of CDCs as viable candidate anti-cancer drugs; drugs that would represent a brand new class of therapeutics for cancer patients. The focus of this method

development was on the synthesis of $Cu(DDC)_2$ in lipid vesicles and the resulting product is the first injectable $Cu(DDC)_2$ formulation described in the literature. The process has been extended to other compounds including CQ, Qu and CX-5461. In each case the candidate CDC formulation appears stable and addresses problems related to the solubility for each agent. This allowed for the preclinical assessment of these CDCs and it will be expanded to other agents.

4. Development and Optimization of an Injectable Formulation of Copper Diethyldithiocarbamate, an Active Anti-cancer Agent*

4.1 Introduction

DSF is an FDA approved drug for the treatment of alcoholism. It functions to inhibit acetaldehyde dehydrogenase 1 (ALDH1) which in turn produces an acute sensitivity to alcohol via the uninhibited production of acetaldehyde [118]. DSF has recently become of interest in the treatment of HIV and cancer through independent mechanisms [116, 258-261]. This has sparked an interest in repurposing DSF for indications other than alcoholism and this chapter focuses on the potential repurposing of this drug or its metabolites for treating cancer.

A high-throughput chemical screen demonstrated that DSF was active against glioma stem cells; an activity that was substantially increased in the presence of Cu(II) [277]. These results have been reported by others with a series of *in vitro* experiments highlighting this combinatorial effect [116, 118, 277, 278]. Although some publications have speculated that DSF and Cu form a complex [116, 279, 280], studies *in vitro* and *in vivo* show DSF is reduced to form a Cu binding ligand [120, 281]. Since DSF has been used clinically for over 60 years, the pharmacokinetic properties of the drug have been explored in depth [268]. There have been reports of the formation of a copper complex in the stomach as a result of DSF conversion to DDC, a well-known copper chelator [262-264, 282, 283]. Interestingly, $Cu(DDC)_2$ shows the same cytotoxic activity against gliomablastoma cells as when disulfiram and copper are administered concurrently. This suggests clearly that the copper complex of DDC is the therapeutically active agent [66, 115]. Cvek *et al.* have shown that $Cu(DDC)_2$ acts as a proteosome inhibitor [42], specifically through

* Adapted from: M. Wehbe, M. Anantha, M. Shi, A. Leung, W.H. Dragowska, L. Sanche, M.B. Bally, Development and optimization of an injectable formulation of copper diethyldithiocarbamate, an active anticancer agent, *Int J Nanomed*, (2017) In Press[276] M. Wehbe, M. Anantha, M. Shi, A. Leung, W.H. Dragowska, L. Sanche, M.B. Bally, Development and Optimization of an Injectable Formulation of Copper Diethyldithiocarbamate, an Active Anticancer Agent, International Journal of Nanomedicine, (2017) In Press.

binding to the 19S lid of the proteosome rather than the 20S subunit, which is targeted by bortezomib [184].

Prior to developing any therapeutic application for $Cu(DDC)_2$, there are formulation issues that need to be addressed if its use *in vivo* is to be evaluated. Specifically, the complex is a slightly soluble precipitate (<0.1 mg/mL)[66] and, to date, there has not been any pharmaceutically appropriate formulations of $Cu(DDC)_2$ suitable for *in vivo* use. In Chapter 3, a method wherein the copper-complexation reaction occurs inside copper-containing liposomes was described which solves the solubility challenges [66]. This Chapter examines, for the first time, the anti-cancer activity of the resultant $Cu(DDC)_2$ formulation. This method allows for the development of a parenterally suitable $Cu(DDC)_2$ formulation and can take advantage of the well-established potential for liposomes to modulate the pharmacokinetic characteristics of an associated drug candidate. These $Cu(DDC)_2$ formulations are the first of their kind to be used to assess the therapeutic potential of this interesting Cu complex.

4.2 Results

4.2.1 $Cu(DDC)_2$ Characterization

A general method for preparing metal complexed drug candidates has been described in Chapter 3 [66]. One of the examples included the use of DSPC/Chol (55:45) liposomes, prepared in 300mM copper sulfate, as nano-scale reaction vessels for the synthesis of $Cu(DDC)_2$. This process solved solubility issues because the highly insoluble complex was formed when the ligand was added to the outside of copper-containing liposomes. This synthesis reaction (Figure 4.1A) was completed at 25^{o}C within 5 mins and was easily detected by eye as the absorbance of the solution changed to exhibit a broad peak around 435nm. The resultant product appeared stable, as the reaction could be carried out for 1 hr with no significant change in the $Cu(DDC)_2$ to lipid ratio (Figure 4.1B). The $Cu(DDC)_2$ formulation showed negligible dissociation from the liposomes *in vitro* when the samples were incubated at 37^{o}C over 72 hrs in a solution containing 50% FBS in SH buffer (pH 7.4) (Figure 4.1C). The *in vitro* activity of this novel formulation of $Cu(DDC)_2$ was compared to the complex prepared in the absence of liposomes and dissolved in DMSO by

generating dose response curves against M-V-411 leukemia cells (Figure 4.1D). The 72 hour IC_{50} determined for both formulations were identical (~0.11 μM), however in contrast to the DMSO solubilized $Cu(DDC)_2$ there was no evidence of precipitates in the incubation wells when $Cu(DDC)_2$ was added as the liposomal formulation.

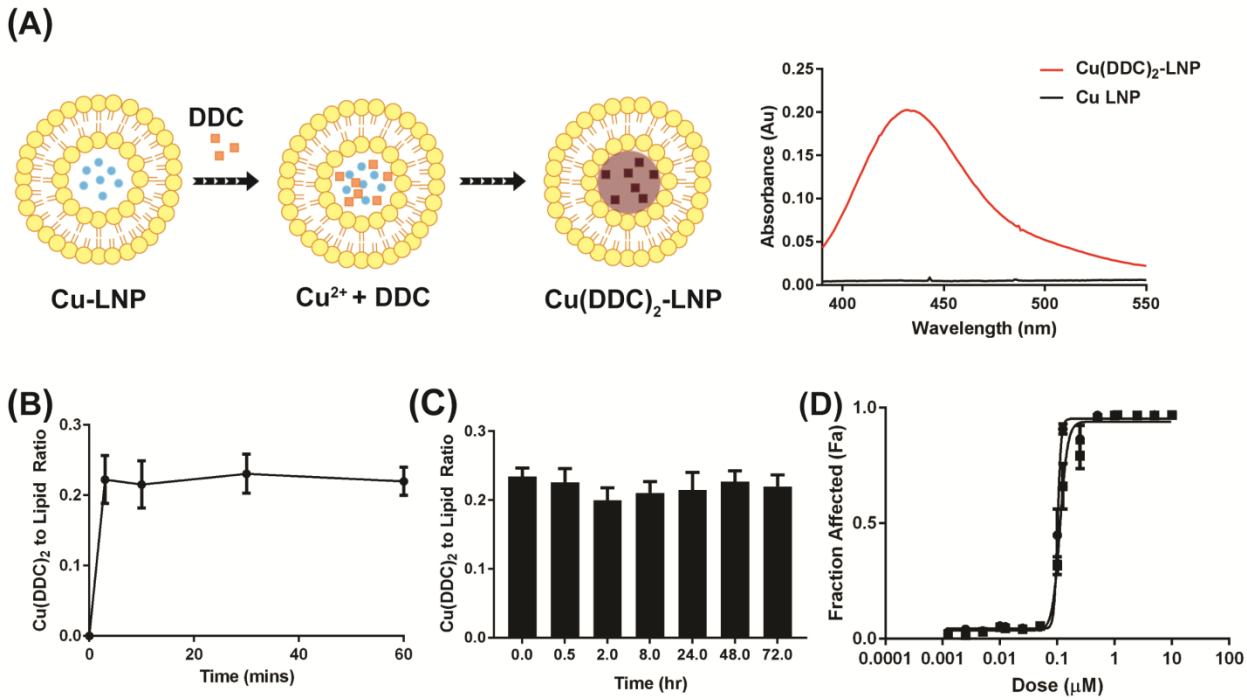


Figure 4. 1: Characterization of $Cu(DDC)_2$ prepared in a liposomal formulation (DSPC/Chol (55:45)) wherein the $Cu(DDC)_2$ was synthesized inside the copper containing liposomes. (A) Schematic representation of $Cu(DDC)_2$ complex formation inside copper liposomes upon addition of DDC. Cu-liposomes and $Cu(DDC)_2$-liposomes were scanned on a UV-VIS, the formation of $Cu(DDC)_2$ results in a peak at 435 nm indicative of complex formation. (B) Formation of $Cu(DDC)_2$ inside DSPC/Chol liposomes (final liposomal lipid concentration was 20 mM) as a function of time over 1 hr at 25°C following addition of DDC to the liposomes at a final DDC to lipid ratio of 0.4. (C) *in vitro* $Cu(DDC)_2$ release from DSPC/Chol (55:45) liposomes over a 72 hr time course in the presence of 50% FBS at 37°C. $Cu(DDC)_2$ was measured using a UV-Vis spectrophotometric assay (see Methods) and lipid was measured through use of a radioactive lipid marker (^{3}H-CHE). (D) M-V-411 cytotoxicity curves for $Cu(DDC)_2$ dissolved in DMSO (■) and the $Cu(DDC)_2$ liposomal preparation (●) where cell viability was measured (using PrestoBlue®) following a 72 hr exposure to the added $Cu(DDC)_2$. Data are presented as mean ± SEM of 3 experiments. If error bars are not seen then they are within the size of the symbol used.

4.2.2 Studies to Assess the Mechanism of $Cu(DDC)_2$ Activity

Previous publications [42, 284], indicated that the mechanism of DDC cytotoxicity was mediated, at least in part, through proteosome inhibition. Proteosome inhibitors, such as the FDA approved Bortezomib, have been used for the treatment of acute myeloid leukemia [285]. For this reason, an acute myeloid leukemia M-V-411 cell line was used to characterize the cytotoxicity of $Cu(DDC)_2$ prepared within DSPC/Chol liposomes. The IC_{50} determined using PrestoBlue (see Chapter 2) of $Cu(DDC)_2$ at 24 and 72 hrs was 0.42 and 0.11 μM, respectively (Figure 4.2A - B). To determine whether $Cu(DDC)_2$ inhibits the proteosome, MV-4-11 cells were treated with vehicle, $CuSO_4$ or $Cu(DDC)_2$ and the accumulation of ubiquitinylated proteins, a marker of proteosome inhibition, was subsequently measured via western blotting. Only the $Cu(DDC)_2$ treated groups showed marked accumulation of ubiquinylated protein relative to the controls (Figure 4.2C). To further investigate mechanisms involved in $Cu(DDC)_2$ cytotoxicity, cell cycle analysis was performed using flow cytometry (see Chapter 2). The results showed no significant changes in the cell cycle after 24 hours of $Cu(DDC)_2$ exposure. However, $Cu(DDC)_2$ caused an increase in the sub G_0/G_1 phase indicative of cell death (Figure 4.2D). Since some published reports have suggested that $Cu(DDC)_2$ treatment leads to production of reactive oxygen species (ROS) [184], $Cu(DDC)_2$ was evaluated to examine if cell death was a result of this mechanism. Cells were treated for 4 hrs with $Cu(DDC)_2$ or menadione (positive control). The results showed that $Cu(DDC)_2$ treatment did not result in ROS generation (Figure 4.2E), suggesting that it is unlikely to be an important mechanism of cytotoxicity for the formulation described here.

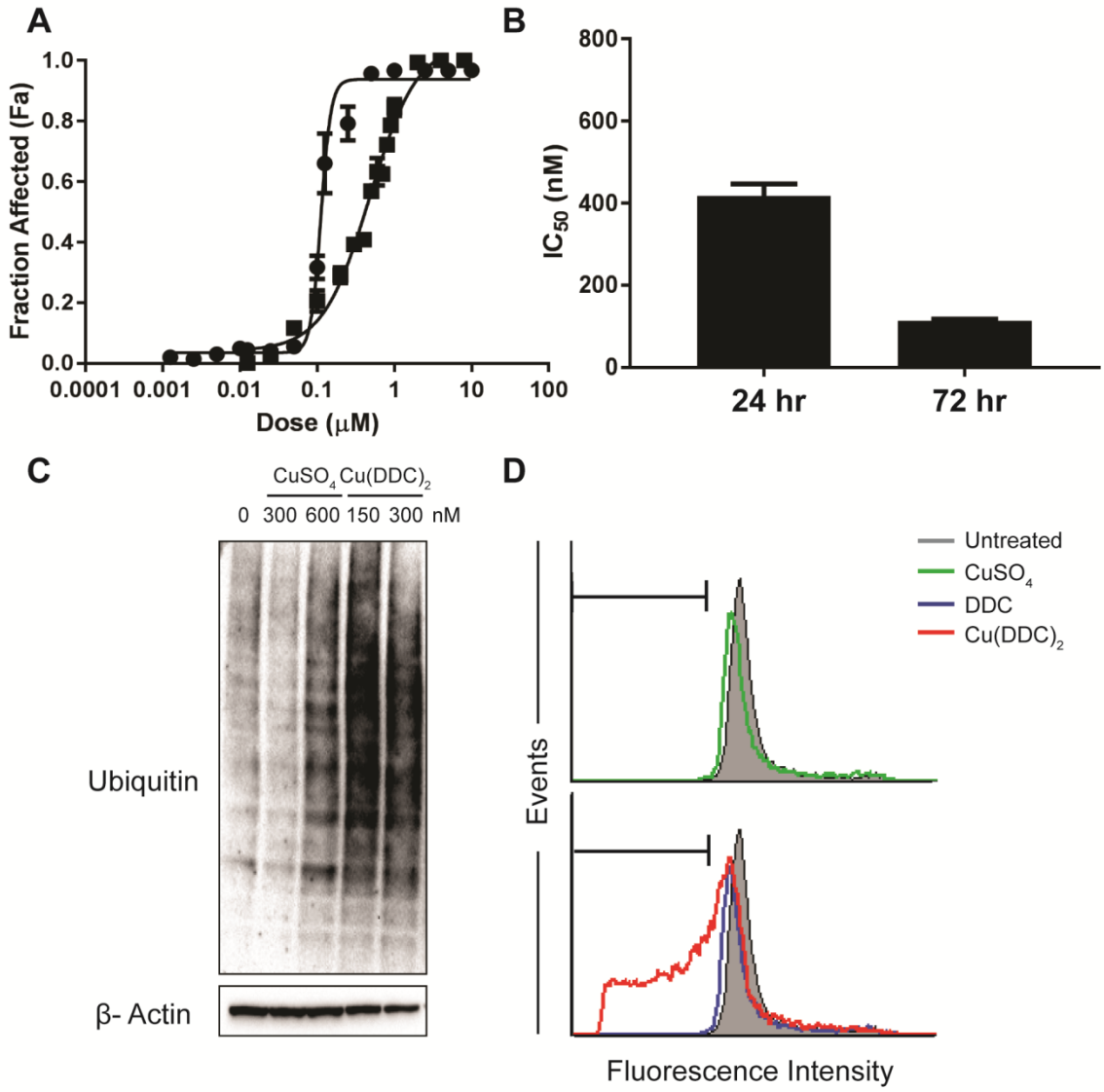

A
Fraction Affected (Fa)
1.0
0.8
0.6
0.4
0.2
0.0
0.0001 0.001 0.01 0.1 1 10 100
Dose (μM)
B
IC50 (nM)
800
600
400
200
0
24 hr
72 hr
C
CuSO4 Cu(DDC)2
0 300 600 150 300 nM
Ubiquitin
β- Actin
D
Untreated
CuSO4
DDC
Cu(DDC)2
Events
Fluorescence Intensity
E
Average Luminescence (RLU)
60000
40000
20000
0
Untreated
Menadione (50 μM)
Cu(DDC)2 (200 μM)

Figure 4. 2: $Cu(DDC)_2$ acts primarily as a proteosome inhibitor and induces cell death in MV-4-11 cells. (A) Cytotoxicity curves generated when MV-4-11 cells are exposed to $Cu(DDC)_2$ (prepared inside DSPC/Chol Liposomes) for either 24 (■) or 72 (●) hrs where viability was measured using PrestoBlue®. (B) The IC_{50} values of MV-4-11 cells treated with $Cu(DDC)_2$ for 24 and 72 hrs. The proteasome inhibition activity was determined as described in the Methods using MV-4-11 cells treated with the indicated doses of $CuSO_4$ or $Cu(DDC)_2$ (prepared inside DSPC/Chol Liposomes) for 24 h. (C) Proteasome inhibition resulted in accumulation of ubiquitinated proteins presented as long dark bands on the Western blot following $Cu(DDC)_2$ treatment (150 and 300nM) but not for vehicle or $CuSO_4$ (300-600nM). (D) Cell cycle analyses was completed using MV-4-11 cells treated with $CuSO_4$, DDC, or $Cu(DDC)_2$ for 24 hrs and the results indicated no significant change in the cell cycle upon $Cu(DDC)_2$ exposure. There was an increase in the subG_0/G_1 fraction (marked with horizontal bar) when cells were treated with $Cu(DDC)_2$, indicative of cell death as evident by DNA fragmentation. (E) ROS formation was tested in MV-4-11 cells treated with $Cu(DDC)_2$ (prepared inside DSPC/Chol Liposomes; where ROS formation was measured 4 hrs following initiation of treatment. $Cu(DDC)_2$ treatment did not induce ROS formation. Menadione was used as a positive control and ROS formation in the cells was evident by a statistically significant difference in luminescence relative to the corresponding cell-free condition. Data are presented as mean ± SEM of 3 experiments.

4.2.3 Plasma Elimination of $Cu(DDC)_2$ Prepared in DSPC/Chol (55:45) Liposomes

The $Cu(DDC)_2$ formulation prepared as described above was suitable for i.v. administration. To assess $Cu(DDC)_2$ elimination from plasma, mice were given a single i.v. dose (15 mg/kg) of $Cu(DDC)_2$ and plasma samples were collected as described in Chapter 2. Attempts to measure $Cu(DDC)_2$ in the plasma compartment were, however, unsuccessful even when using a 15min. time point. The $Cu(DDC)_2$ levels were below the detection limits of the HPLC assay (0.09 μg/mL). Plasma copper levels were measurable by AAS and in $Cu(DDC)_2$ treated mice these levels were above the level of copper determined in plasma collected from untreated mice. For this reason, plasma copper levels (after subtraction of control plasma copper levels) were used as a surrogate marker of $Cu(DDC)_2$. The results, summarized in Figure 4.3, indicate that the percent of the injected copper dose remaining in plasma was 5.2% at 15 min., indicative of 95% eliminations of the injected $Cu(DDC)_2$ (Fig. 3A; filled circles). This value decreased to 0.25% of the injected dose 1 hr after administration. In contrast, 15 minutes after administration of the formulation, 93% of the injected liposomal lipid dose was still in the plasma compartment; a value that decreased to 80% 1hr following administration (Fig. 4.3A, open circles). The liposomal lipid elimination rate was comparable to that described previously for DSPC/Chol liposomes [286].

These results suggest that $Cu(DDC)_2$ prepared inside DSPC/Chol liposomes does not remain inside the liposomes following i.v. dosing. This result was surprising given the

stability of the formulation as determined using *in vitro* methods (see Figure 4.1). Loss of associated $Cu(DDC)_2$ from the liposomes after administration is highlighted by the copper to lipid ratio data (Fig. 4.2B). Prior to injection, this ratio was 0.25 (time 0), but it drops to 0.02 after 15 minutes. This is indicative of 92% loss of $Cu(DDC)_2$ from the liposome within 15 minutes after injection. In contrast, 1 hr after administration of uncomplexed $CuSO_4$ containing DSPC/Chol liposomes, the copper to lipid ratio suggested >90% retention of the encapsulated copper. This is indicative of $Cu(DDC)_2$ being released from the liposome as the copper complex whereas uncomplexed Cu^{2+} ions are, as expected, retained in the liposome owing to their charge. It can be suggested that the DSPC/Chol liposomal formulation of $Cu(DDC)_2$ addresses the solubility challenges of this compound, but because the complex is released rapidly from the liposomes following administration it should be noted that the liposomes are not behaving as drug delivery vehicles.

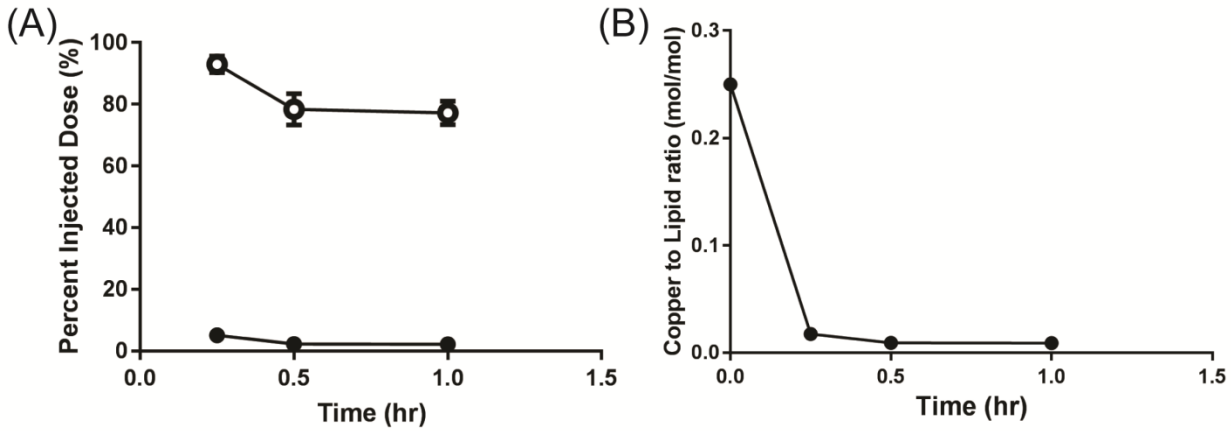


Figure 4. 3: **$Cu(DDC)_2$ (prepared in DSPC/Chol (55/45) liposomes) elimination after intravenous injection in CD-1 mice.** (A) The percent injected dose of copper (-●-) was measured using AAS as a surrogate for $Cu(DDC)_2$. The percent injected dose of liposomal lipid (-○-) was measured by assessing ^{3}H-CHE as a non-exchangeable, non-metabolizable liposomal lipid marker (see Methods). (B) The decrease in copper-to-lipid ratio suggests that the $Cu(DDC)_2$ prepared inside the liposome dissociated from the liposomes within 15 mins. All data are plotted as mean ± SEM (n=4 per time point), if the error bars are not visible then the error bars are within the size of the symbol used.

4.2.4 $Cu(DDC)_2$ Efficacy in Models of Cancer

The results thus far demonstrate that. $Cu(DDC)_2$ synthesized in liposomes remain in solution in a form that could be safely administered i.v. The potential for using the resultant product as an anti-cancer drug was measured in two different rodent models of cancer and

the results are summarized in Figure 4.4. The activity of $Cu(DDC)_2$ (prepared in DSPC/Chol liposomes) was measured in mice with established sc tumours models generated following sc injection of the MV-4-11 cells (Fig. 4.4A). Prior to assessing activity, the MTD of $Cu(DDC)_2$ was determine in tumour free CD-1 mice. Given the rapid elimination of $Cu(DDC)_2$ following i.v. injection (Fig. 4.3) a dose intensive schedule was used where the animals were injected iv on Monday, Wednesday and Friday for 2 weeks (M,W,F x 2). Using this schedule the MTD of $Cu(DDC)_2$ was determined to be 8 mg/kg. At doses greater than 8 mg/kg (e.g. 15 mg/kg) the animals were terminated at humane endpoints (5 days following initial treatment). On necropsy, these animals showed no obvious changes in tissue or organ appearance other than the occasional enlarged spleen and discolouration of the liver and kidney. It should be noted that extravasation injuries (at the site of injection) were observed with some mice. If the extravasation injury was considered severe, then the mouse was terminated for ethical reasons and that animal was excluded from the treatment group. For animals with established MV-4-11 sc tumours, dosing began on day 12 when mice were injected (i.v.) with vehicle (SH buffer), DSPC/Chol (55:45)-copper sulfate liposomes (1.3 mg copper/kg) or $Cu(DDC)_2$ prepared in DSPC/Chol liposomes (8 mg $Cu(DDC)_2$ /kg, copper dose 1.3 mg/kg), respectively. The liposomal lipid dose was ~50 mg/kg. The results (Fig. 4.4A) suggest that tumour growth was comparable in animals treated with the vehicle and the copper containing liposome control. The $Cu(DDC)_2$ (prepared in DSPC/Chol liposomes) treated animals exhibited a delay in tumour growth rate. Animals in this group showed a 45% reduction in tumour volume when compared to the vehicle and copper treated animals on day 41. Although there was a treatment engendered delay in tumour growth when using the MV-4-11 sc tumour model, the results suggested that $Cu(DDC)_2$ activity was unremarkable on the basis of RECIST criteria [287] where notable activity is reflected by stable disease, a partial response or a complete response. For this reason, $Cu(DDC)_2$ (prepared in DSPC/Chol liposomes) was tested in an orthotopic F98 (rat glioblastoma) model, where treatment was administered directly into the site of tumour inoculation by CED [251] The glioblastoma line was of interest because previous publications suggested that DSF and its metabolite DDC in the presence of copper were particularly active against glioblastoma cells [116, 288]. The *in vivo* efficacy of $Cu(DDC)_2$ (prepared in DSPC/Chol liposomes) was evaluated in the F89 rat glioma model

where each rat was injected at the site of tumour cell inoculation with a 0.5 mg/mL solution of $Cu(DDC)_2$, the vehicle or copper containing liposomes (0.08 mg/mL) (see Methods). This was defined as the MTD of the $Cu(DDC)_2$ formulation when administered by CED methods. In these studies, control animals exhibited a median survival time (MST) of 20.5 and 19.5 days, respectively (Fig. 4.4B). In animals treated with $Cu(DDC)_2$, the MST increased to 25 days (a 25% increase in MST). The difference between the MST of those animals treated with the controls and $Cu(DDC)_2$ was statistically significant ($p< 0.05$) and comparable to previous studies demonstrating a 11.3% increase in MST when the F98 glioblastoma model was treated with lipoplatin (a liposomal formulation of cisplatin) [251].

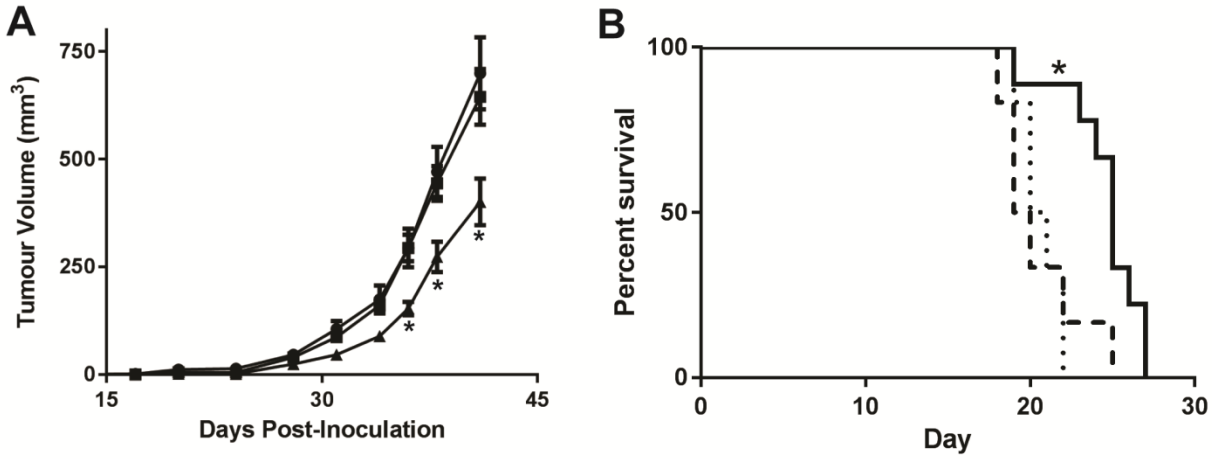


Figure 4. 4: $Cu(DDC)_2$ efficacy studies. (A) MV-4-11 cells (1×10^6) were inoculated subcutaneously (sc) into RAG-2M mice and the tumours were treated intravenously with vehicle (SH buffer, ●), copper containing liposomes (1.3 mg/kg, ■) or $Cu(DDC)_2$ (8 mg/kg, ▲) formulated in DSPC/Chol liposomes using a M,W,F x 2 dosing schedule. Tumour volumes were measured three times a week and are plotted as mean ± SEM (up to 9 mice per group). As shown, there was a significant delay in tumour growth when comparing tumour sizes in animals that were treated with $Cu(DDC)_2$ to those treated with the vehicle control. "*" indicates statistically significant difference ($p<0.05$) upon statistical analysis using a 2-way ANOVA followed by Tukey adjustments to correct for multiple comparisons. (B) The activity of $Cu(DDC)_2$ (prepared in DSPC/Chol liposomes) was evaluated following CED of $Cu(DDC)_2$ in a rat glioma model wherein 10,000 F98 cells were implanted intracranially into the right caudate nucleus (see Methods). A single 10 µL injection of vehicle (SH buffer, ·····), copper liposomes (0.08 mg/mL, – –) or $Cu(DDC)_2$ (0.5 mg/mL, ——) was injected 10 days after F98 implantation (n=9). Survival study statistical analysis was performed using the log rank test with GraphPad Prism 6. A P-value under 0.05 was considered statistically significant.

4.2.5 Enhancing the Circulation Longevity of $Cu(DDC)_2$

Although the results presented thus far suggest that the $Cu(DDC)_2$ formulation prepared in DSPC/Chol liposomes is therapeutically active, a significant advantage of the technology used here to create the first injectable $Cu(DDC)_2$ formulation is that the synthesis reaction to form $Cu(DDC)_2$ can be completed in liposomes of different lipid compositions and the environment within the liposome can be controlled. Since it is possible that the efficacy of $Cu(DDC)_2$ may be enhanced through strategies that enhance its circulation lifetime, a number of strategies were pursued to gain an understanding of the factors influencing $Cu(DDC)_2$ retention in the liposomes. Since the *in vitro* $Cu(DDC)_2$ assay measuring the stability of the $Cu(DDC)_2$ formulation proved to be a poor predictor of *in vivo* stability, thus, an *in vivo* assay was utilized where plasma levels of copper or $Cu(DDC)_2$ were measured 30 minutes after i.v. administration into mice. This single time point assay allowed us to assess how changes in the formulation impacted the stability of the injected $Cu(DDC)_2$ formulation. These results are summarized in Figures 4.5 and 4.6. First $Cu(DDC)_2$ was synthesized in DSPC/Chol (55:45) liposomes which were prepared to contain copper sulfate or copper gluconate and, in addition, the $Cu(DDC)_2$ to lipid ratios were varied in these formulations to determine if the amount of entrapped $Cu(DDC)_2$ affected drug retention. As noted in Fig. 4.5A, the synthesis of $Cu(DDC)_2$ inside the DSPC/Chol liposomes was similar when the liposomes were prepared with copper gluconate or copper sulfate. When evaluating the effect of copper salt on the plasma levels of $Cu(DDC)_2$ 30 minutes after injection (Fig.4.5B, first and fourth bar) it can be concluded that use of copper gluconate had no impact on $Cu(DDC)_2$ elimination. These $Cu(DDC)_2$ formulations were also prepared such that selected formulations had lower $Cu(DDC)_2$to lipid ratios (Fig. 5B, 2nd, 3rd, and 5th column). These formulations exhibited plasma copper levels comparable to the formulations with the higher $Cu(DDC)_2$ to lipid ratios. The elimination of liposomal lipid was unaffected by the formulation changes described above.

When preparing the $Cu(DDC)_2$ formulation using 300mM copper sulfate, it is assumed that the internal pH of the liposome is 3.5 (the pH of the copper solution used when making the liposomes). To determine whether the pH within the liposome effects $Cu(DDC)_2$ elimination the monvalent K^+ ion ionophore nigericin was added prior to

$Cu(DDC)_2$ synthesis inside the liposomes. Nigericin facilitates the exchange of K^+ ions in exchange for H^+. Thus, $CuSO_4$-liposomes were exchanged into a KCl-histidine containing buffer (see Methods) and subsequently nigericin was added. As noted in Fig. 4.5C, the formation of $Cu(DDC)_2$ inside the DSPC/Chol liposomes was not affected by the change in external buffer or the addition of nigericin. Following i.v. administration into mice, the plasma copper levels 30 minutes after administration were comparable for the nigericin formulation and the formulation prepared without nigericin (Fig. 4.5D). This suggests that an increase in pH within the liposomes has no impact on the stability of the injected formulation.

To assess how liposomal lipid composition influences the *in vivo* elimination of i.v. injected $Cu(DDC)_2$, formulations prepared in: (i) cholesterol containing liposomes DSPC/Chol (55:45) and SM/Chol (55:45)), (ii) in cholesterol-free liposomes (DSPC/DSPE-PEG_{2000} (95:5) and SM/DSPE-PEG_{2000} (95:5)) as well as (iii) charged liposomes (anionic (DSPC/DSPG/Chol (70:20:10) and cationic (DSPC/DC-Chol/DSPE-PEG_{2000} (30:50:20)) were evaluated. These studies (summarized in Fig. 4.6) used plasma $Cu(DDC)_2$ levels determined 30 minutes following i.v. administration as a measure of whether changes in lipid composition could engender decreases in $Cu(DDC)_2$ elimination. Although not shown, the ability to synthesize $Cu(DDC)_2$ in the different liposomal formulations was not affected by liposomal lipid composition. As indicated in Fig. 6 (3^{rd} and 6^{th} bar) the only formulations which exhibited significantly higher levels of $Cu(DDC)_2$ in the plasma when compared to $Cu(DDC)_2$ formulated in DSPC/Chol liposomes were the DSPC/DSPE-PEG_{2000} and DSPC/DSPG/Chol formulations. These retained 20.8% and 11.3% of the injected copper dose in the plasma compartment at 30 min, respectively. The DSPC/DSPE-PEG_{2000} formulation showed the highest plasma copper levels ; a nearly 7-fold increase over that measured for animals given $Cu(DDC)_2$ formulated in DSPC/Chol liposomes. For this reason, the remaining studies focus on $Cu(DDC)_2$ formulations prepared in the DSPC/DSPE-PEG_{2000} liposomes.

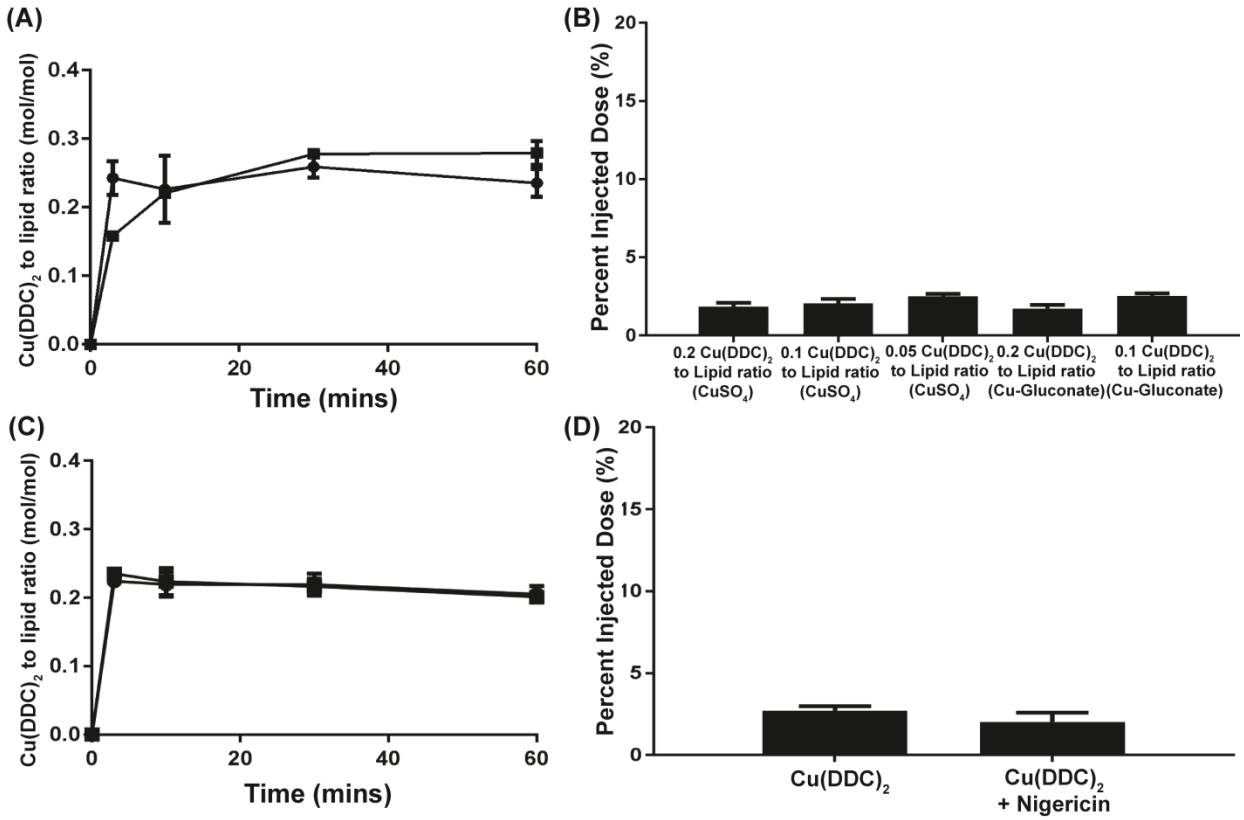


Figure 4. 5: Examining the role of factors within the DSPC/Chol liposomes that may affect $Cu(DDC)_2$ levels in the plasma compartment 30 min after administration. (A) Preparation of $Cu(DDC)_2$ inside DSPC/Chol (55:45) liposomes containing either $CuSO_4$ (●) or Cu-Gluconate (■) as a function of time at 25°C in SH Buffer. (B) The percent of injected $Cu(DDC)_2$ dose administered i.v. to CD-1 mice (n=4) remaining in the plasma 30 minutes after injection. The formulations were prepared at different $Cu(DDC)_2$ to lipid ratios (0.2, 0.1 and 0.05, mole: mole) and prepared using liposomes with encapsulated $CuSO_4$ or Cu-Gluconate buffers (see Methods). Copper levels were measured by AAS and, after subtraction of background plasma copper levels, these levels were used as a surrogate for $Cu(DDC)_2$. (C) Formation of $Cu(DDC)_2$ inside DSPC/Chol (55:45) liposomes containing $CuSO_4$ with (■) or without (●) Nigericin as a function of time at 25°C. The external buffer for these liposomes was KCl (150mM) and histidine (20mM) (see Methods). (D) The percent injected dose of $Cu(DDC)_2$ injected into CD-1 mice (n=4) remaining after 30 minutes following i.v. administration of $Cu(DDC)_2$ prepared in DSPC/Chol liposomes in the presence and absence of Nigericin. Copper levels were measured by AAS and, after subtraction of background plasma copper levels, these levels were used as a surrogate for $Cu(DDC)_2$. For panels A and C, n= 3 replicate experiments. In panels A and C if the error bars are not visible then the error is within the size of the symbol used. For Panels B and D, n= 4 mice per group. All data are plotted as mean ± SEM

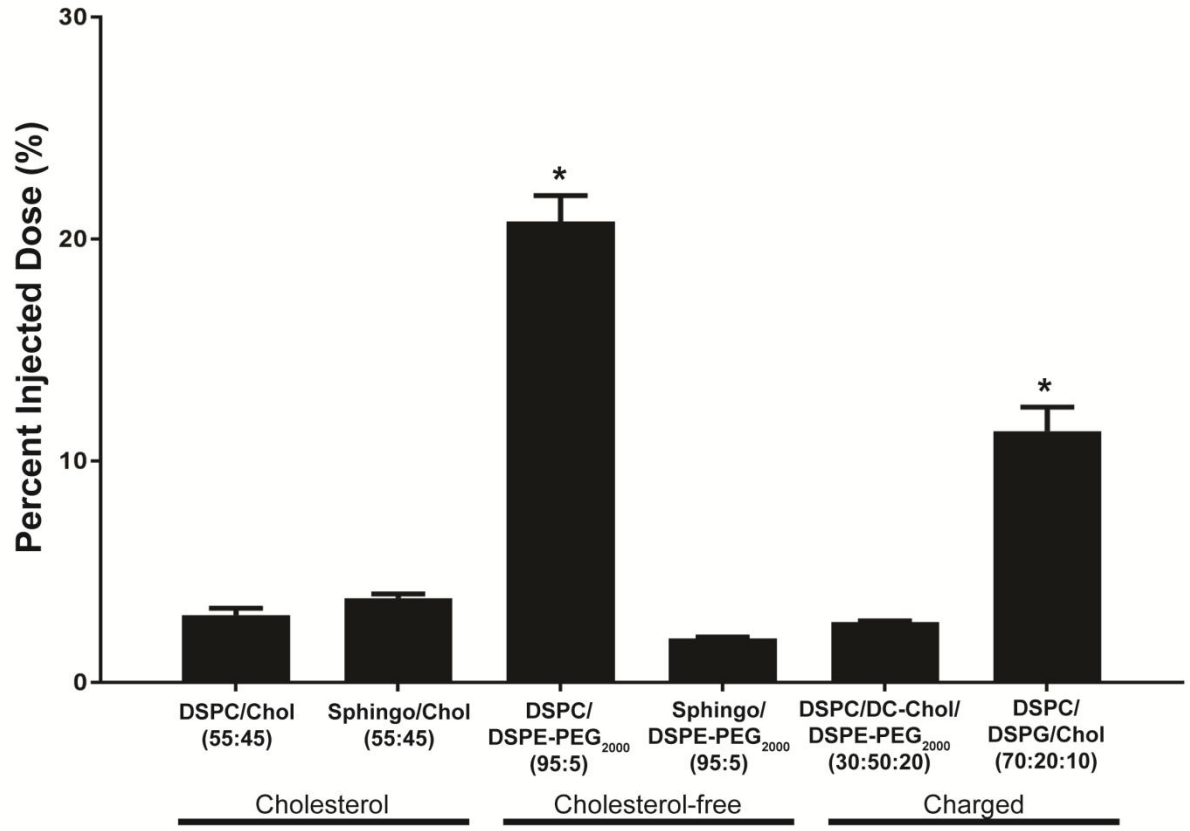


Figure 4. 6: Examining the role of lipid composition on $Cu(DDC)_2$ levels in the plasma compartment 30 min after administration. Liposomes composed of DSPC/Chol (55:45), SM/Chol (55:45), DSPC/DSPE-PEG_{2000} (95:5), SM/DSPE-PEG_{2000} (95:5), DSPC/DC-Chol/DSPE-PEG_{2000} (30:50:20) and DSPC/DSPG/Chol (70:20:10) were prepared in unbuffered 300mM $CuSO_4$. $Cu(DDC)_2$ was prepared in these liposomes to achieve a final $Cu(DDC)_2$ to lipid ratios of 0.2 (mol:mol) (see Methods) and subsequently injected i.v. into CD-1 mice (n=4) at 15 mg/kg. The plasma levels of $Cu(DDC)_2$ were measured 30 minutes after administration. Copper levels were measured by AAS and, after subtraction of background plasma copper levels, these levels were used as a surrogate for $Cu(DDC)_2$. "*" indicates statistically significant difference ($p>0.05$) when $Cu(DDC)_2$ levels in the plasma were compared to the levels obtained after injecting $Cu(DDC)_2$ in DSPC/Chol liposomes (first column) . Significance was determined by a one-way ANOVA followed by Tukey's post-hoc test. For each lipid composition, n= 4 mice per group. All data are plotted as mean ± SEM.

4.2.6 Characterizing $Cu(DDC)_2$ Prepared in DSPC/DSPE-PEG_{2000} (95:5) Liposomes

To generate the data shown in Fig. 4.6, the ability to create $Cu(DDC)_2$ in liposomes prepared of different lipid compositions needed to be determined. The formation of $Cu(DDC)_2$ in DSPC/DSPE-PEG_{2000} (95:5) liposomes is illustrated by the data shown in Fig. 4.7. When incubated at 25°C, a $Cu(DDC)_2$ to lipid ratio of 0.2 (molar ratio) is achieved within 10 minutes following addition of DDC to the copper-containing liposomes (Fig.4.7A, filled squares). DDC enters the liposome through passive diffusion across the liposomal

lipid bilayer. Since DDC is a relatively small molecule (171 g/mol) it can cross the lipid bilayer rapidly, even when the incubation temperature was reduced to 4°C. It should be noted that the rate of $Cu(DDC)_2$ formation at 4°C in the DSPC/DSPE-PEG_{2000} liposomes was slower than that observed in DSPC/Chol liposomes. This is reflected by the data summarized in Fig. 4.7B where the $Cu(DDC)_2$ to lipid ratio for DSPC/Chol and DSPC/DSPE-PEG_{2000} liposomes measure after a 1 hr incubation at 4°C (filled bars) suggests that 80% and 50% of the encapsulated Cu is becoming complexed with DDC over the 60 minute incubation time frame, respectively. Cryo-electron microscopy was performed on the DSPC/DSPE-PEG_{2000} liposomes before and after $Cu(DDC)_2$ synthesis. As illustrated in Fig. 4.7C, there is no visible difference between the liposomes with encapsulated copper sulfate and those with encapsulated $Cu(DDC)_2$.

Following i.v. administration of $Cu(DDC)_2$ formulated in the DSPC/DSPE-PEG_{2000} liposomes, there was about 70% of the injected lipid dose in the plasma compartment at 4 hrs. In contrast, only 5% of the injected $Cu(DDC)_2$ was detected (Fig. 4.7D). This is reflected in the change in the $Cu(DDC)_2$ to lipid ratio (molar) which indicates that there was 90% loss of the $Cu(DDC)_2$ from the DSPC/DSPE-PEG_{2000} liposomes within 4 hrs after injection (see Fig. 4.7E). Although $Cu(DDC)_2$ was still eliminated rapidly from the plasma compartment following injection of the formulation prepared in DSPC/DSPE-PEG_{2000} liposomes, this formulation retained $Cu(DDC)_2$ significantly better than the DSPC/Chol liposomes. The plasma $AUC_{(0-\infty)}$ for $Cu(DDC)_2$ inside DSPC/Chol (55:45) liposomes was found to be 4.6 μg•hr/mL while that of $Cu(DDC)_2$ inside of DSPC/DSPE-PEG_{2000} (95:5) liposomes was found to 19.3 ug•hr/mL, representing a 4.2 fold increase in $AUC_{(0-\infty)}$.

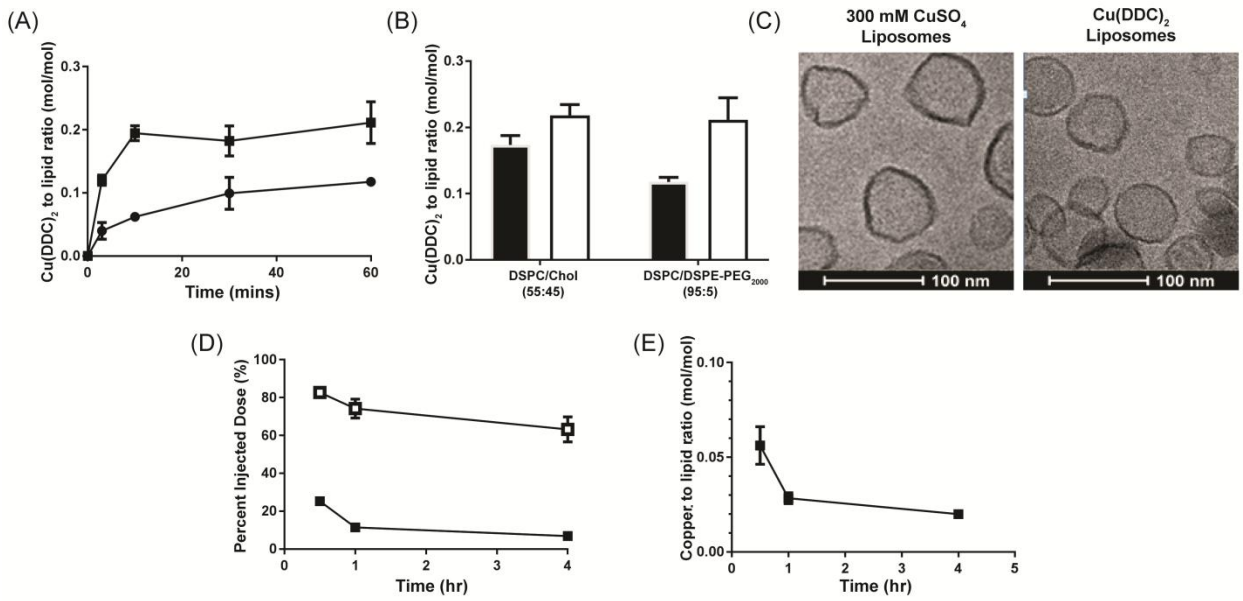


Figure 4. 7: Characterizing $Cu(DDC)_2$ prepared inside DSPC/DSPE-PEG_{2000} (95:5) liposomes. (A) DSPC/DSPE-PEG_{2000} liposomes prepared in 300mM unbuffered $CuSO_4$ were incubated at 4°C (●) or 25°C (■) with DDC (see Methods) and the amount of $Cu(DDC)_2$ formed as a function of time was measured. (B) The $Cu(DDC)_2$ to lipid ratio measured after a 1 hr incubation following addition of DDC to $CuSO_4$ containing DSPC/Chol or DSPC/DSPE-PEG_{2000} liposomes after an incubation at 4°C (filled bar) or 25°C (empty bar). (C) Cryo-electron microscopy photomicrograph of $CuSO_4$- containing DSPC/DSPE-PEG_{2000} liposomes and $Cu(DDC)_2$ prepared in the same liposomes. (D) $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes were injected into CD-1 mice (n=4) at 15 mg/kg and mice were sacrificed at 0.5, 1 and 4 hrs. Plasma concentration of $Cu(DDC)_2$ and liposomal lipid were determined (see Methods) and the % injected dose of $Cu(DDC)_2$ (■) and liposomal lipid (□) was calculated at the indicated time points. (E) $Cu(DDC)_2$ to lipid ratios calculated based on the data provided in panel D. All data are plotted as mean ± SEM; n= 3 replicate experiments for panel A/B and n= 4 mice per group in panel D and E.

4.2.7 Therapeutic Activity of $Cu(DDC)_2$ Prepared in DSPC/DSPE-PEG_{2000} (95:5) Liposomes

To determine if the 4.2-fold increase in $AUC_{(0-\infty)}$ achieved when using $Cu(DDC)_2$ formulated in DSPC/DSPE-PEG_{2000} affected therapeutic activity, mice with established MV-4-11 were treated with this $Cu(DDC)_2$ formulation and the activity was compared to animals treated with $Cu(DDC)_2$ prepared in DSPC/Chol liposomes. As indicated above, the MTD of $Cu(DDC)_2$ prepared in DSPC/Chol liposomes was determined to be 8 mg/kg when using the M,W,F x 2 schedule. $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes was better tolerated and the MTD of this formulation was determined to be 12.5 mg/kg when given via the M, W, Fx2 schedule. To assess efficacy, $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes was administered (M, W, F x2 schedule) at 8 mg/kg and 12.5 mg/kg. The

injected liposomal lipid doses were 46 mg/kg and 63 mg/kg, respectively. When $Cu(DDC)_2$ was prepared in DSPC/Chol liposomes, the dose of $Cu(DDC)_2$ was 8 mg/kg and the liposomal lipid dose was 50mg/kg. Mice bearing MV-4-11 tumours were treated 12 days after tumour cell inoculation and tumour growth was monitored over time. The results, summarized in Figure 4.8, indicated that all $Cu(DDC)_2$ treated animals showed a statistically significant decrease in tumour volume when compared to vehicle treated animals (Fig.4.8A). When comparing tumour size on day 33, the day when some control mice were euthanized due to large tumour sizes (the animal care protocol specifies the humane endpoint for animals with sc tumours is when the tumour volume is greater than 800 mm^3), animals treated with $Cu(DDC)_2$ prepared in DSPC/Chol liposomes had tumours that were 50% the size of control animals. At the same dose of $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes the tumour size was only 28% smaller than the tumours from vehicle treated mice; suggesting that at equivalent doses, the DSPC/DSPE-PEG_{2000} formulation was less effective. However, when efficacy was compared at the MTD of each formulation, the antitumour activity was equivalent (Fig. 4.8B). Both formulations were able to delay progression of tumour growth in this model, but further formulation changes will be required to optimize $Cu(DDC)_2$ activity.

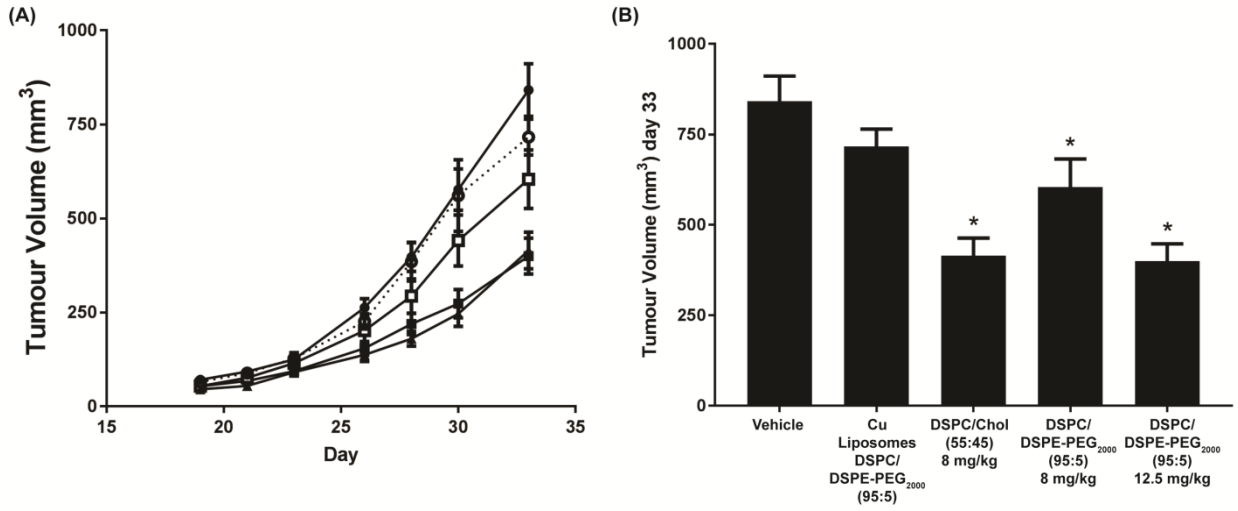


Figure 4. 8: $Cu(DDC)_2$ anti-tumour activity determined in mice bearing established sc MV-4-11 tumours. 1 x 10^6 MV-4-11 cells were inoculated sc into RAG-2M mice (see Methods) and were treated intravenously with vehicle (-●-), copper containing DSPC/DSPE-PEG_{2000} (95:5) liposomes (-○-,1.7 mg/kg copper, 63 mg/kg lipid), 8 mg/kg $Cu(DDC)_2$ prepared in DSPC/Chol liposomes (-▲-, 8 mg/kg $Cu(DDC)_2$ and 50 mg/kg lipid), 8 mg/kg $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes (-□-, 8 mg/kg $Cu(DDC)_2$, 40 mg/kg lipid) or 12.5 mg/kg $Cu(DDC)_2$ prepared in DSPC/DSPE-PEG_{2000} liposomes (-■-,12.5 mg/kg $Cu(DDC)_2$,

63 mg/kg lipid) using a M,W,F x 2 schedule (see Methods). (A) Tumour volumes were measured three times a week (see Methods) and were plotted as mean ± SEM. (B) Mean tumour volume ± SEM determined on day 33 (the humane endpoint for vehicle treated animals). "*" indicates statistically significant difference p>0.05; one-way ANOVA followed by Tukey's post hoc test.

4.3 Discussion

DSF and Cu have been the focus of many oncology focused research programs and clinical trials because of the surprising anti-cancer activity observed when the two components are given together *in vitro* [116, 118, 289]. This discovery exemplified an opportunity to repurpose DSF, an agent with over 60 years of clinical use in the context of alcohol addiction [268, 290, 291]. It has been previously shown by others and in Chapter 3 that the cytotoxicity of DSF and Cu is attributed to the formation of a $Cu(DDC)_2$ complex [66, 120]. Attempts have been made to prepare the complex *in vivo* by administering DSF and Cu (usually given as Cu-Gluconate) separately. However, the pharmacokinetic and distribution behavior of copper and DSF are remarkably different and this has contributed to the lack of success using this approach. Thus, while the DSF/Cu combination shows remarkable activity *in vitro* this has not translated *in vivo*. To date, the therapeutic activity of the $Cu(DDC)_2$ complex has not been directly evaluated *in vivo* owing to the insolubility of the complex in aqueous solutions. This challenge was solved by synthesizing $Cu(DDC)_2$ inside liposomes [66]. This methods relies on liposomes serving as nano-scale reaction vessels to support the synthesis of $Cu(DDC)_2$ when DDC is added to the outside of copper sulfate-containing liposomes. This is the first characterization of the anti-cancer activity of $Cu(DDC)_2$ following i.v. administration.

$Cu(DDC)_2$ was cytotoxic to cancer cells but did not have any effect in healthy bronchial epithelial cells *in vitro*.[66] While no precipitation was visible when cells were examined under a microscope it is possible that $Cu(DDC)_2$ could exist as a micro-precipitate. Consistent with other *in vitro* studies [42, 292], the primary mechanism of action for $Cu(DDC)_2$ is through proteosome inhibition (see Figure 2). Flow cytometric studies suggested that cell cycle arrest does not contribute to $Cu(DDC)_2$ cytotoxicity. Interestingly, DSF and copper have been reported to be effective against brain tumour initiating cells (BTICs) which are senescent [116, 288, 293]. Thus, the ability of $Cu(DDC)_2$ to

be effective regardless of cell cycle stage may contribute to the drug's ability to kill BTICs. The *in vitro* studies (see Figure 4.2) indicate that the activity of $Cu(DDC)_2$ is not mediated by the generation of ROS, i.e. no increase in ROS production was observed when cells were incubated with $Cu(DDC)_2$. Previously Tawari *et al.* noted an increase in ROS when DSF and copper were added to cell media but not when DDC and copper were added [115]. The authors suggested that ROS generation was a by-product produced which occured when DSF and Cu were mixed *in vitro*. However ROS would not be generated if using pre-formed $Cu(DDC)_2$ complex in liposomes, as done in the studies reported here. This was done to help avoid precipitation of $Cu(DDC)_2$ under conditions where the complex is added as a DMSO solubilised form. The *in vitro* results suggested that $Cu(DDC)_2$ prepared in DSPC/Chol liposomes was available to the cells in culture, a preliminary indication that $Cu(DDC)_2$ dissociated from the liposomes.

The *in vivo* studies completed with $Cu(DDC)_2$ produced in DSPC/Chol liposomes indicated that the drug was rapidly eliminated from plasma after i.v. administration (>90% of the injected dose was eliminated with 15 minutes, see Figure 4.3). This was surprising, in part because the *in vitro* studies shown in Figure 4.1C suggested that the $Cu(DDC)_2$ formulations were stable when prepared in the DSPC/Chol liposomes. The inability for *in vitro* release assays to predict drug release from liposomes is a widely discussed challenge in liposomology. The result seen herein could be attributed to an insufficient sink to drive $Cu(DDC)_2$ release. Methods such as the use of "acceptor" multilamellar vesicles could be employed to better accurately predict drug release [294]. $Cu(DDC)_2$ prepared in DSPC/Chol liposomes was therapeutically active. When administered at its MTD, the effects resulted in a delay in tumour progression. This was determined in a sc model as well as an intracranial model where $Cu(DDC)_2$ was given by CED directly to the site of tumour cell inoculation (Figure 4.4). The therapeutic activity of $Cu(DDC)_2$ may be limited because of its rapid elimination from the plasma compartment and this would be addressed in this model. An advantage of the technology used here is that the composition of the liposome in which the $Cu(DDC)_2$ is formed can be changed to improve the *in vivo* stability of the resulting formulation. When $Cu(DDC)_2$ was prepared in DSPC/DSPE-PEG_{2000} (chol-free) liposomes there was an improvement in plasma $Cu(DDC)_2$ levels which likely equated to an improvement in $Cu(DDC)_2$ retention within the liposome. $Cu(DDC)_2$ prepared in

DSPC/DSPG/Chol liposomes also showed some improved stability *in vivo.* In both of these examples, it could be argued that the anionic surface charge played a role in improved stability of the formulation[269]. Alternatively, because the DSPC/DSPG/Chol liposomes contained only 10% cholesterol (a low cholesterol formulation), improved $Cu(DDC)_2$ retention could be due to the removal of cholesterol[256]. It is important to note that an increase in drug retention was not seen in the chol free SM/DSPE-PEG_{2000} formulation. For $Cu(DDC)_2$ prepared in DSPC/Chol liposomes, factors such as $Cu(DDC)_2$ to lipid ratio, choice of entrapped copper salt and the internal liposomal pH did not appear to affect the stability of the formulation (see Figure 4.5). The $Cu(DDC)_2$ plasma $AUC_{(0-\infty)}$ was 4.2 fold higher for the DSPC/DSPE-PEG_{2000} formulation when compared to $Cu(DDC)_2$ prepared in DSPC/Chol liposomes. Although the resulting DSPC/DSPE-PEG_{2000} formulation was better tolerated, its therapeutic activity was not better than the DSPC/Chol formulation (Figure 4.8). It can be concluded that the decreased plasma elimination rate affects the safety profile of the resultant formulation, but more significant improvements in the stability of the formulation will be needed to enhance the activity of $Cu(DDC)_2$ *in vivo*.

The $Cu(DDC)_2$ formulations described here do provide some therapeutic benefit, but the full therapeutic potential of $Cu(DDC)_2$ may only be achieved through careful selection of the appropriate cancer indication and/or by using it in combination with another drug. For example, Lun *et al.* have demonstrated that temozolomide (TMZ) can be used in combination with DSF and copper [288]. It would be a natural transition to examine if $Cu(DDC)_2$ could be used in combination with TMZ. Also, given a mechanism that involves proteosome inhibition, $Cu(DDC)_2$ may be useful when combined with other drugs known to enhance the activity of known proteasome inhibitors such as BTZ. In this context, it has been shown that BTZ acts synergistically with CDK9 kinase inhibitors [295]. Cvek *at al*. have examined the mechanism by which $Cu(DDC)_2$ inhibits the cellular 26S proteosome and have described that this occurs through inhibition of the JAMM domain in the 19S proteasome lid [42], a site distinct to where BTZ acts. For this reason $Cu(DDC)_2$ may be effective when used in combination with drugs that work well with BTZ, or alternatively, $Cu(DDC)_2$ may prove active against BTZ resistant cancers [296].

4.4 Conclusion

This Chapter disclosed the first studies ever to evaluate the anti-cancer activity of $Cu(DDC)_2$ prepared using a technique which synthesizes $Cu(DDC)_2$ in the core of liposomes. This method solves problems associated with $Cu(DDC)_2$ insolubility and allows for its direct administration. Two formulations were tested in an *in vivo* MV-4-11 leukemia model and both produced a ~50% reduction in tumour volume at their respective MTDs when compared to control groups. Additional studies are needed to optimize the therapeutic potential of the $Cu(DDC)_2$ formulations described here. Additionally, it would be of particular interest to establish the activity of $Cu(DDC)_2$ in combination with other drugs.

5. Development of a Copper-Clioquinol Formulation Suitable for Intravenous Use [*]

5.1 Introduction

Clioquinol (CQ) was commonly used as an oral antimicrobial agent for treating diarrhea and skin infections [297, 298]. However, in the 1960s its use in Japan was associated with a debilitating neurological disorder referred to as subacute myelo-optic neuropathy (SMON). This eventually led to CQ being withdrawn from the market [297, 299]. Interestingly, epidemiologic reports suggest that CQ was not responsible for SMON and no other population showed a similar adverse response [297, 298]. Today, CQ is commonly used as a topical antibiotic under the trade name Vioform®[298] and more recently this drug has been the focus of repurposing efforts for the treatment of Alzheimer's disease[136, 300] and cancer [191, 274].

This Chapter focuses on the potential use of CQ as an anti-cancer agent. It has been noted that the anti-cancer effects of CQ are enhanced when it is administered as a copper CQ ($Cu(CQ)_2$) complex[50, 191, 217, 274, 301]. The structure of $Cu(CQ)_2$ has been characterized [190], however the mechanism(s) responsible for its activity have not been fully elucidated. Ding *et al.* have suggested that CQ may act as a copper ionophore [50, 191]. Alternatively, others have suggested that $Cu(CQ)_2$ may act as a proteasome inhibitor, similar to the postulated mechanism of copper diethyldithiocarbamate [302, 303]. Regardless, there have been no reports assessing the activity of $Cu(CQ)_2$ *in vivo.* The low aqueous solubility of this complex has hindered its development as an anti-cancer drug candidate. CQ has been tested as a single agent and its use required a mixed solvent system containing DMSO, cremophor and ethanol [274]. Due to the toxicities associated with such formulations, it has not been possible to fully assess the anti-cancer potential of $Cu(CQ)_2$ [64].

[*] Adapted from: M. Wehbe, A. Malhotra, M. Anantha, C. Lo, N. Dos Santos and M.B. Bally. Repurposing the topical antifungal clioquinol for the treatment of cancer. (2017) *Submitted to Drug delivery and translational research*

The goals of the current study were: (i) to develop and characterize a $Cu(CQ)_2$ formulation suitable for parenteral administration without the use of solvents, (ii) to evaluate the efficacy of $Cu(CQ)_2$ as an anti-cancer agent, (iii) to assess the use of $Cu(CQ)_2$ as a copper ionophore to boost the anti-cancer activity ascribed to disulfiram (DSF). In Chapter 3, it was demonstrated that copper complexes can be synthesized inside liposomes. The resultant formulation remains in solution and is suitable for intravenous administration. The studies presented here are the first to assess the anti-cancer activity of $Cu(CQ)_2$. The results suggest that $Cu(CQ)_2$ is not potent enough to exert meaningful anti-cancer activity *in vivo*, even when used in combination with DSF.

5.2 Results

5.2.1 Cytotoxicity of CQ and its Copper Complex

The activity of CQ and $Cu(CQ)_2$ against A2780-S, A2780-CP, A549, U251 and MV-4-11 cells were determined and the results are summarized in Figure 5.1. Both compounds were solubilised in a final DMSO concentration of 0.5% and viability was measured 72 hrs post-treatment. It should be noted that a visual precipitate was observed when the CQ or $Cu(CQ)_2$ concentration added was >100 μM. The results with A2780-S, A2780-CP and A549 cells suggest that the activity of CQ is enhanced significantly when added as the copper complex (Fig. 5.1A, B, C). The IC_{50} of $Cu(CQ)_2$ was between 20-60 μM, while CQ alone showed very little toxicity even at concentrations >100 μM. In contrast, the activity of CQ and $Cu(CQ)_2$ were equivalent when added to U251 and MV-4-11 cell lines (Fig. 5.1D and E). The IC_{50} of CQ was 32 and 46 μM while it was 27 and 32 μM for $Cu(CQ)_2$ in U251 and MV-4-11 cells, respectively.

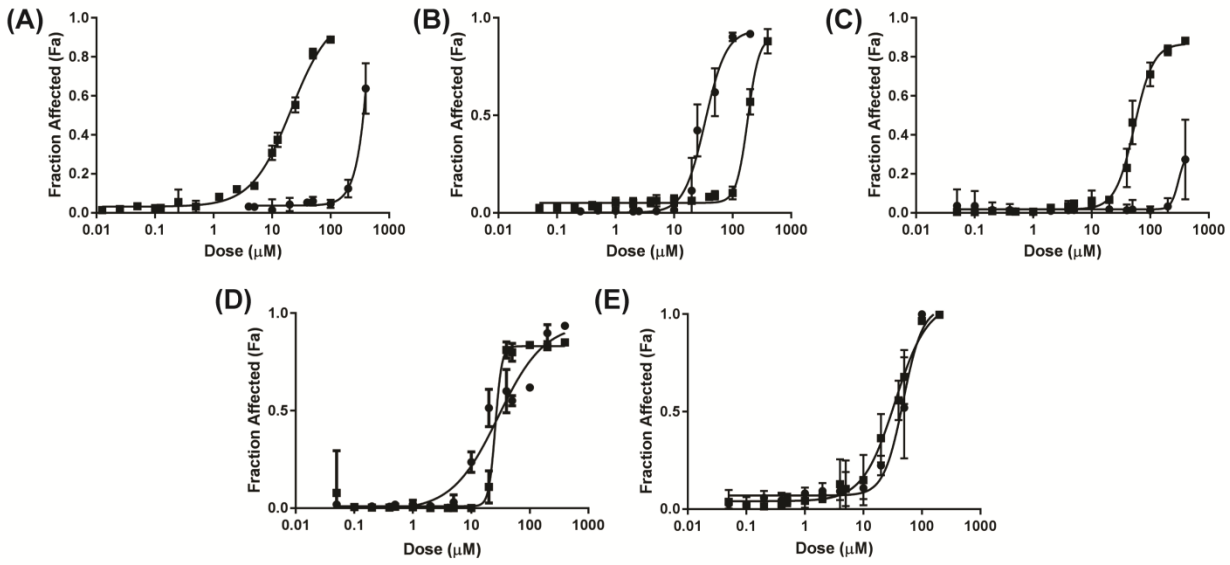


Figure 5. 1: The cytotoxicity of CQ and $Cu(CQ)_2$ against 5 different cancer cell lines. Cytotoxicity curves for CQ (-●-) and Cu(CQ)2 (-■-) were obtained for (A) A2780-S, (B) A2780-CP (C) A549, (D) U251 and (E) MV-4-11 cells. Cells were seeded for 24 hrs and then treated with CQ or $Cu(CQ)_2$ at doses ranging from 0.05 – 400 μM for 72 hrs. Cell viability for the adherent cell lines (A2780-S, A2780-CP, A549, U251) was determined using an INCell analyzer 2200 where viability was assessed based on loss of plasma membrane integrity 72 hours following treatment; i.e. total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively. The viability of the non-adherent cell line (MV-4-11) was measured using PrestoBlue™. Data are presented as mean ± SEM, where the values were determined in triplicate three times.

5.2.2 Copper CQ Liposome Characterization

The results summarized above demonstrate that $Cu(CQ)_2$ is active against a number of cancer cell lines *in vitro*; with an IC_{50} ranging from 20-60 μM. It is a challenge to develop a drug formulation when the selected drug exhibits activity in the micromolar range and this challenge becomes even greater when the drug is sparingly soluble in aqueous solution. To address this challenge, a formulation method is available where the copper complex is synthesized inside liposomes, as described in Chapter 3. This strategy is illustrated by the data summarized in Figure 5.2, wherein $Cu(CQ)_2$ was synthesized inside DSPC/Chol (55:45, mol ratio) liposomes prepared to contain $CuSO_4$. The liposomes (20 mM final liposomal lipid concentration) were added to 5 mg CQ (as powder) prior to incubating at 40°C. The colour of the solution changed from white to yellow/green within 3 minutes indicative of $Cu(CQ)_2$ formation (Fig. 5.2A). Formation of $Cu(CQ)_2$ was found to be temperature dependant (Fig. 5.2B). When samples were incubated at 4°C there was no

observable color change and the measured $Cu(CQ)_2$ to liposomal lipid ratio was less than 0.02 after 60 minutes. The rate of $Cu(CQ)_2$ synthesis was faster as the incubation temperature increased to 25°C, where the measured $Cu(CQ)_2$ to liposomal lipid ratio was 0.1 after 30 minutes. The optimal temperature for complex formation was 40°C, where the measured $Cu(CQ)_2$ to liposomal lipid ratio was 0.2 after 3 minutes. In these studies, CQ was added in excess but as noted in [13], the amount of $Cu(CQ)_2$ formed inside the liposomes is completely dependent on the amount of copper trapped in the liposome. This is illustrated in Fig. 5.2C. Increasing the initial theoretical $Cu(CQ)_2$ to liposomal lipid ratio beyond 0.15 produced no further increase in the measured $Cu(CQ)_2$ to liposomal lipid ratio when using the optimal temperature of 40°C. In this context, the initial theoretical $Cu(CQ)_2$ to liposomal lipid ratio was estimated on the assumption that each mole of copper would complex 2 moles of CQ [190]. When the initial theoretical $Cu(CQ)_2$ to liposomal lipid ratio was 0.2, the measured $Cu(CQ)_2$ to liposomal lipid ratio was 0.17, which was similar to what was measured for formulations prepared with a large excess of CQ. Preliminary studies assessing the stability of the resulting $Cu(CQ)_2$ suggested less than 10% of the associated $Cu(CQ)_2$ was released from the liposomes when incubated in serum (80%) over 24 hr (Fig. 5.2D).

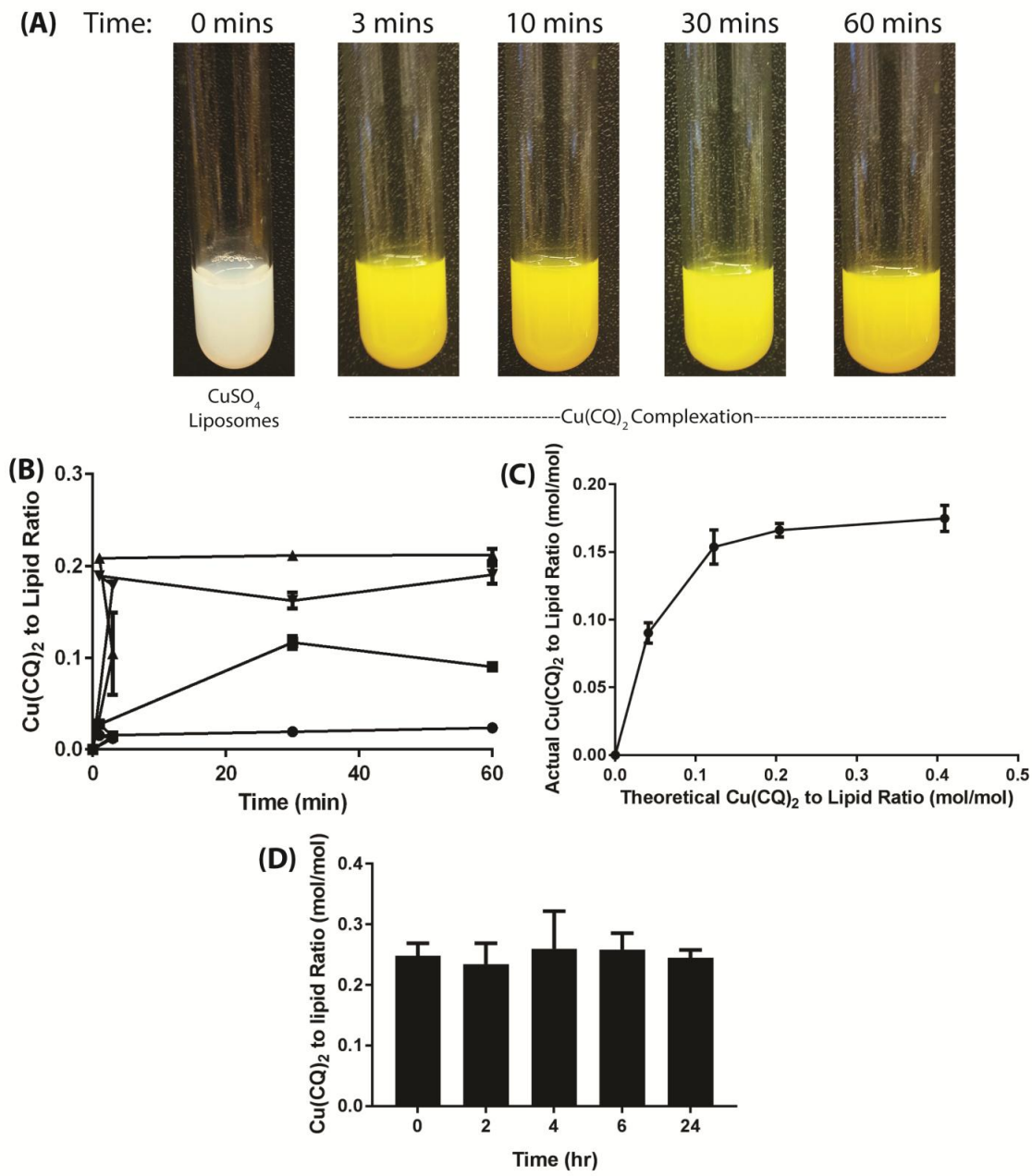


Figure 5. 2: Synthesis of $Cu(CQ)_2$ in liposomes prepared with encapsulated 300 mM $CuSO_4$. (A) Photograph of solutions consisting of CQ (5 mg/mL) added to $CuSO_4$-containing liposomes (20 mM liposomal lipid) over a 1 hr time course at 40°C. (B) Formation of $Cu(CQ)_2$ inside DSPC/Chol liposomes (20 mM) as a function of time over 1 hr at 4°C (●), 25 °C (■) 40°C (▲) and 60°C (▼) following addition of CQ (5 mg/mL). (C) Measured $Cu(CQ)_2$ to liposomal lipid as a function of theoretical $Cu(CQ)_2$ to total liposomal lipid ratio estimated based on the amount of CQ added to the liposomes. For these studies the liposomal lipid concentration was fixed at 20 mM and the added CQ amount was varied. (D) *In vitro* stability of the $Cu(CQ)_2$ formulation over 24 hrs in 80% fetal bovine serum. $Cu(CQ)_2$ was measured using a spectrophotometric assay (B-C) or HPLC (D) and liposomal lipid was measured through use of a radiolabeled lipid (^{3}H-CHE). All data are plotted as mean ± SEM.

5.2.3 Tolerability and Pharmacokinetics Following Intravenous Administration of the $Cu(CQ)_2$ Formulation

The maximum tolerated dose of $Cu(CQ)_2$ was found to be 30 mg/kg i.v once daily Monday, Wednesday, Friday for two weeks. The formulation was well tolerated at this dose, no weight loss greater than 5% (data not shown) and no notable changes in health status were observed. Following necropsy (14 days after last treatment) there were no gross morphological changes noted. This dose (30mg/kg) and route of administration was used for the pharmacokinetic studies. The $Cu(CQ)_2$ elimination profile was characterized and compared to control liposomes (prepared in 300 mM copper sulfate and exchanged into SH buffer pH 7.4) and the results have been summarized in Figure 5.3. For the analysis of plasma samples, an HPLC assay designed to measure CQ was used (see Methods) as the measurement of $Cu(CQ)_2$ was not possible. At 24 hrs post-injection of $Cu(CQ)_2$, the amount of CQ in the plasma falls below the limit of detection (Fig. 5.3A). Based on these data, approximately 25% of the injected $Cu(CQ)_2$ dose was eliminated within one hour and greater than 90% was eliminated within 8 hrs. As illustrated in Fig. 5.3B, the CQ to liposomal lipid ratio decreases as a function of time after administration. This is indicative of CQ release from the liposomes and into plasma compartment. For example, the measured CQ to liposomal lipid ratio at 4 hrs is 50% less than that of the injected formulation. It is not clear from this data that CQ is being released from the liposomes as CQ or $Cu(CQ)_2$. For this reason, the plasma copper concentrations were also determined (see Methods). The results, shown in Fig. 5.3C and 5.3D, are based on plasma copper levels determined after subtraction of background copper levels determined in plasma obtained from untreated mice. It is assumed, therefore, that the copper being measured is due to the injection of the $Cu(CQ)_2$ formulation. As shown in Fig. 5.3C (filled circles), animals injected with $Cu(CQ)_2$ have plasma copper levels that decrease over time, where >90% of the injected copper dose was eliminated after 8 hrs. The results shown in Fig. 5.3D (filled circles), also suggest that the copper to liposomal lipid ratio is decreasing as a function of time after administration. To gain a better understanding of whether the liposomal formulation using (DSPC/Chol, 55:45 mol ratio) was releasing $Cu(CQ)_2$ as the complex, the results obtained in animals treated with the $Cu(CQ)_2$ formulation were compared to animals treated with control liposomes prepared to contain just copper (see Methods). This

data (filled squares in Fig. 5.3C and 5.3D) indicates that copper elimination is significantly reduced following administration of the copper containing liposomes. This is best illustrated by the results in Fig. 5.3D where it appears that the initial copper to liposomal lipid ratio decreases by less the 50% for the copper liposomes, but more than 85% for the $Cu(CQ)_2$ formulation at 24 hrs. As noted in Fig. 5.3E, the elimination of liposomal lipid following administration of $Cu(CQ)_2$ and the copper containing liposomes were comparable. In aggregate, the results suggest that following administration of the $Cu(CQ)_2$ formulation, both copper and CQ are released. The release rates are comparable which suggest that $Cu(CQ)_2$ is being released from the liposomes as a function of time after administration. Since the assays used here were unable to directly measure $Cu(CQ)_2$, it was not possible to assess whether the $Cu(CQ)_2$ complex is stable following release from the liposomes. It can be concluded that the estimated $Cu(CQ)_2$ (assuming CQ is complexed to copper) are potentially sufficient to engender therapeutic effects based on the IC_{50} of $Cu(CQ)_2$ shown above.

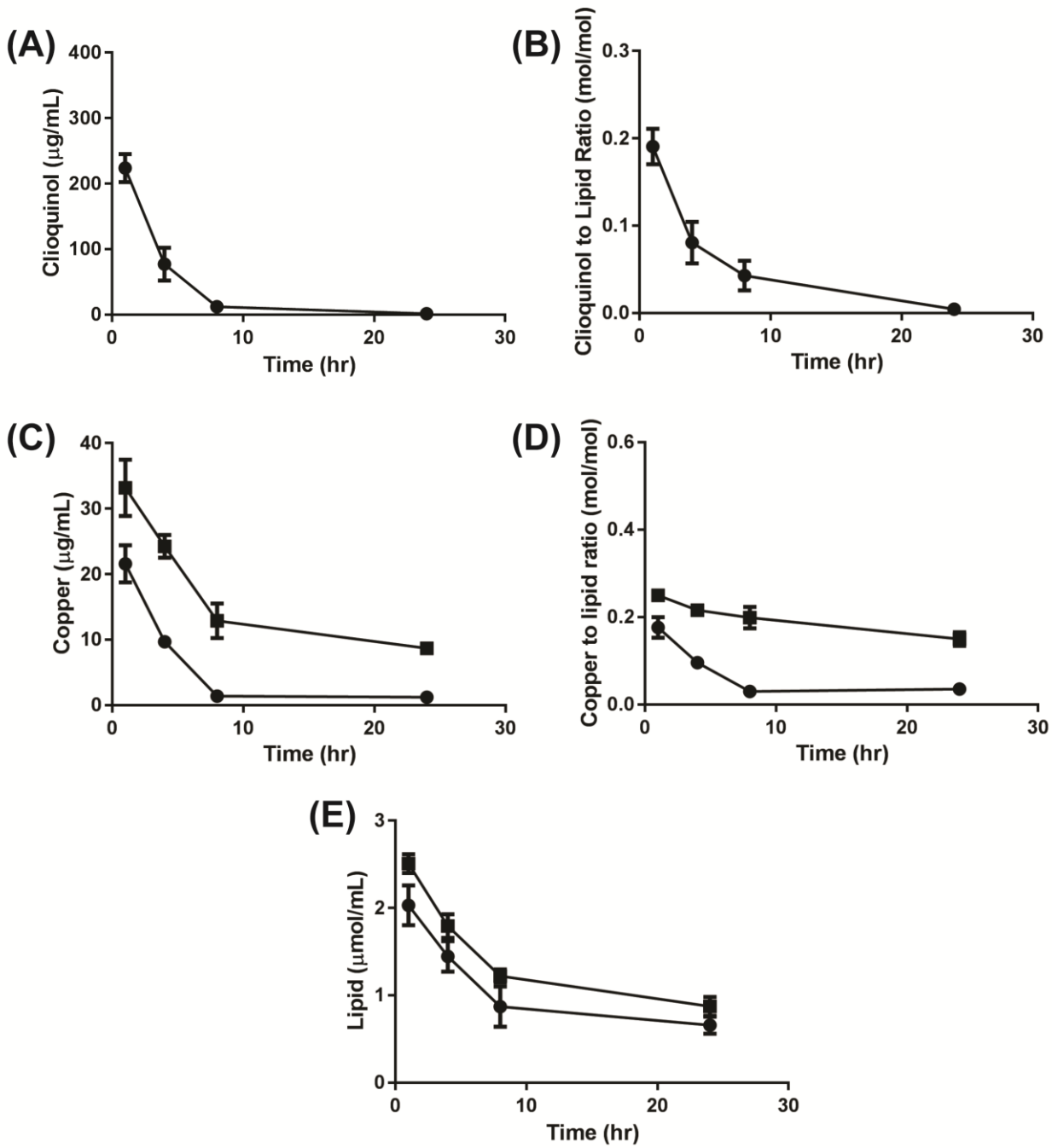


Figure 5. 3: $Cu(CQ)_2$ and copper liposome plasma elimination following intravenous injection in CD-1 mice. The $Cu(CQ)_2$ liposomes (30 mg/kg CQ, 3.2 mg/kg Cu, 115.6 mg/kg lipid) were dosed in CD-1 mice. Copper liposomes (liposomes prepared in 300mM $CuSO_4$) were injected at the same copper and liposomal lipid dose of 3.2 and 115.6 mg/kg, respectively. (A) CQ plasma concentration over 24 hrs; where CQ was measured by HPLC methods. (B) CQ to liposomal lipid ratio over 24 hrs following administration of the $Cu(CQ)_2$ formulation. (C) Plasma copper levels following injection of $Cu(CQ)_2$ (●) and copper liposomes (■) over 24 hrs, where Cu^{2+} was measured using AAS (see Methods). (D) Copper to liposomal lipid ratio measured over 24 hrs following injection of copper liposomes or the $Cu(CQ)_2$ formulation. (E) The liposomal lipid concentration was measured using scintillation counting of ^{3}H-CHE. All data are plotted as mean ± SEM, (n=5).

5.2.4 Anti-tumor Efficacy Following Intravenous Administration of $Cu(CQ)_2$

A sc tumor model of A2780-CP (a platinum resistant ovarian cancer cell line) was developed, in these cells the IC_{50} of $Cu(CQ)_2$ was approximately 20 µM and the IC_{50} of CQ was >100 µM. The A2780-CP model is fast growing, where control animals must be terminated due to tumor progression (tumors reach a size >800 mm^3, see Methods) within 18-22 days following cell injection. For these studies, $Cu(CQ)_2$ treatment was initiated 4 days post cell inoculation. The results, summarized in Fig. 5.4A, indicate that treatment with copper liposomes and $Cu(CQ)_2$ caused a slight, but not significant change in A2780-CP tumor growth rate.

The therapeutic activity of $Cu(CQ)_2$ was then evaluated in NRG mice bearing sc U251 tumours. This cell line was selected because it was sensitive to both CQ and $Cu(CQ)_2$ (Figure 5.1); the IC_{50} of $Cu(CQ)_2$ and CQ in this glioblastoma cell line was approximately 30 µM. Dosing (30 mg/kg i.v once daily Monday, Wednesday, Friday for two weeks) began when the average tumour size reached 50-100 mm^3.The results, summarized in Figure 5.4B, suggest that treatment with $Cu(CQ)_2$ had no impact on the growth rate of the U251 tumors when compared to the growth rate in animals treated with the vehicle (SH buffer) or control copper liposomes. Based on these studies it was concluded that $Cu(CQ)_2$, when administered as a single agent, was not efficacious.

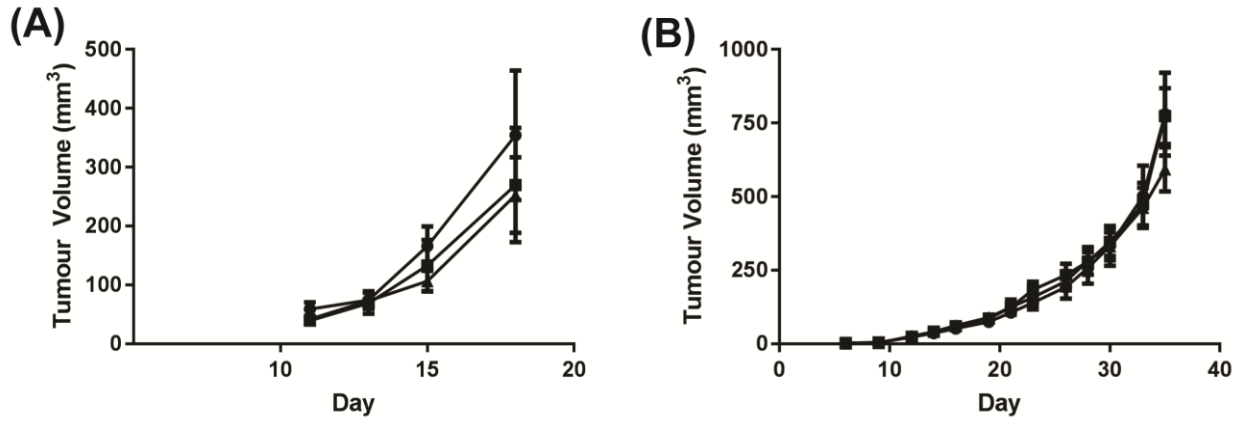


Figure 5.4: Efficacy of $Cu(CQ)_2$ in animals bearing subcutaneous A2780-CP and U251 tumour xenographs. NRG mice with sc injected cell lines (see Methods) where treatment with vehicle (SH buffer, ●), copper liposomes (liposomes prepared in 300 mM $CuSO_4$) (Copper dose of – 3.2 mg/kg, ■) or $Cu(CQ)_2$ (30 mg/kg, ▲). The liposomal lipid dose was 115.6 mg/kg. Treatments were given iv on Monday, Wednesday and Friday for 2 weeks. (A) A2780-CP tumour growth in NRG mice (n=8), dosing began on day 4 post cell

inoculation. (B) U251 tumour growth in NRG mice (n=6), treatment began when tumours reached 50-100 mm^3. Data are reported as mean ± SEM.

5.2.5 Efficacy of $Cu(CQ)_2$ in Combination with Disulfiram

The studies summarized above suggest that the $Cu(CQ)_2$ formulation was not efficacious when administered as a single agent. $Cu(CQ)_2$ acts as a copper ionophore [50, 217], thus its potential to act in combination with DSF was explored. The anti-cancer effects of DSF are dependent on having high intracellular copper levels which could be achieved using a copper ionophore [259]. These studies were completed using the A2780-CP cell line which exhibits sensitivity to $Cu(CQ)_2$ but not CQ. The results summarized in Fig. 5.3D, are consistent with the published literature suggesting that $Cu(CQ)_2$ can cross lipid bilayers [50]. To illustrate this in a cell model, an assay based on copper dependent quenching of Phen Green™ fluorescence was used [304, 305]. The results, summarized in Figure 5.5, show that the fluorescent intensity of cells incubated with Phen Green™ decreases following addition of $Cu(CQ)_2$. This decrease in A2780-CP cell associated Phen Green™ was not observed when cells were treated with copper alone. A decrease, albeit not significant, in fluorescence was noted when the cells were treated CQ alone, but this is likely due to CQ binding copper in the serum containing cell culture media.

DSF is known to metabolize to form DDC which can complex with copper to form $Cu(DDC)_2$, a cytotoxic agent [66, 120]. To test whether combinations of $Cu(CQ)_2$ and DSF were cytotoxic, the agents were added alone and in combination to A2780-CP cells. The results, summarized in Figure 5.5B, indicate that cells exposed to $Cu(CQ)_2$ (inverted filled triangles) or DSF (filled triangles) alone exhibited compound IC_{50} values of 19μM and 1.7μM, respectively. When DSF was combined with cells with $Cu(CQ)_2$ (1:1 ratio) the IC_{50} of DSF decreased to 110nM. The IC_{50} of DSF in cells treated with $Cu(CQ)_2$ is essentially equivalent to the IC_{50} of DSF and $CuSO_4$ (90 nM) which indicates that $Cu(CQ)_2$ does not inhibit the formation of $Cu(DDC)_2$.

The *in vivo* activity of $Cu(CQ)_2$ when combined with DSF was evaluated in animals bearing sc A2780-CP tumors. For these studies DSF was dosed orally (100 mg/kg) as described elsewhere [288]. DSF treated animals were dosed concurrently with the $Cu(CQ)_2$ formulation or copper liposomes (liposomes prepared in 300 mM $CuSO_4$). The results,

summarized in Figure 5.6, suggest that Combinations of DSF with $Cu(CQ)_2$-liposomes resulted in a modest, but not significant, reduction in tumor growth which could not be differentiated from animals treated with combinations of DSF with copper liposomes.

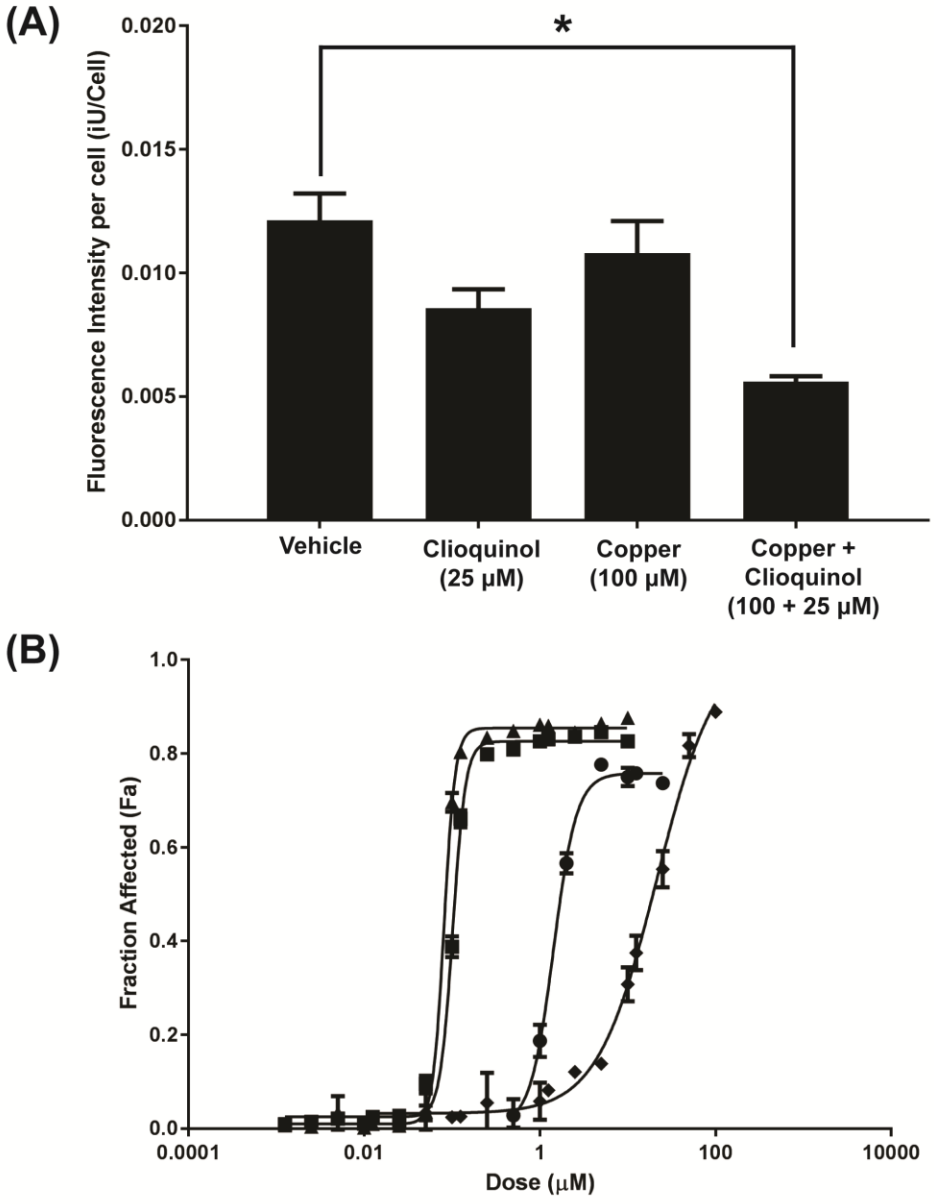


Figure 5. 5: $Cu(CQ)_2$ mediated increase in copper delivery to cells and its *in vitro* activity when combined with disulfiram (DSF). (A) A2780-CP intracellular copper levels were assessed using the cell permeable dye Phen Green™. Cell associated Phen Green™ fluorescence was measured 1 hr treatment with the vehicle (0.01% DMSO), CQ, copper, or $Cu(CQ)_2$. The cells were then incubated with Phen Green™ for 30 minutes (see Methods). The fluorescence of the probe is quenched in the presence of Cu and thus a decrease in cell associated fluorescence is indicative of higher intracellular copper levels. Cell associated fluorescence

was measured using an INCell Analyzer 2200. Results shown are an average of 3 studies done in triplicate (mean ± SEM). (B) Cytotoxicity curves were generated in A2780-CP cells after 72 hr treatment with DSF (-●-), $Cu(CQ)_2$ (-◆-) or DSF in combination with $Cu(CQ)_2$ (-■-) or $CuSO_4$ (-▲-). Cell viability was determined using an INCell analyzer 2200 where viability was assessed based on loss of plasma membrane integrity 72 hours following treatment; i.e. total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively.

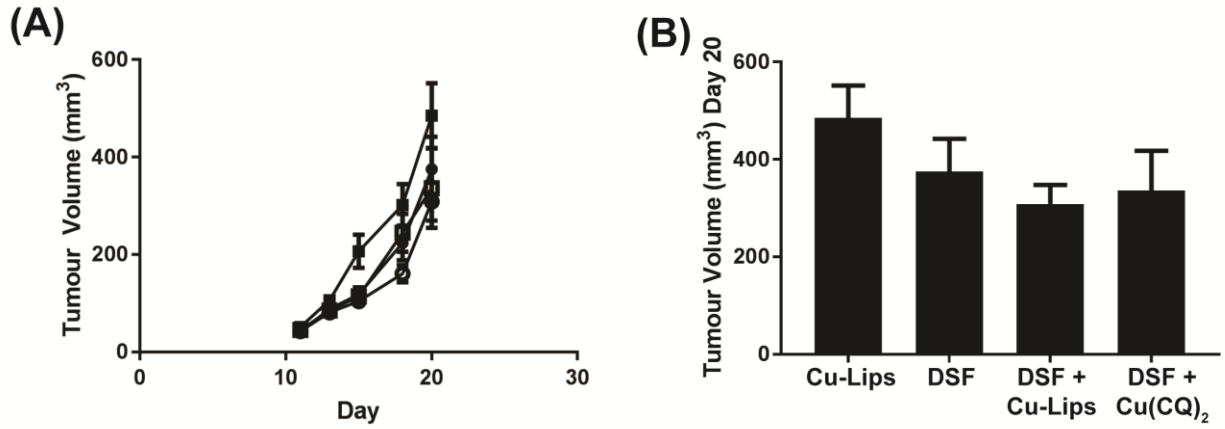


Figure 5.6: Efficacy of DSF in combination with $Cu(CQ)_2$ and copper liposomes (liposomes prepared in 300mM $CuSO_4$) determined in NRG mice with sc A2780-CP tumors. Treatment with $CuSO_4$-liposomes (Cu-lips) (copper does of 3.2 mg/kg ■), DSF (100 mg/kg,●), DSF and Cu-liposomes (100 mg/kg DSF and 3.2 mg/kg copper, ○) or DSF and $Cu(CQ)_2$ (100 mg/kg DSF and 30 mg/kg $Cu(CQ)_2$, □) was initiated 4 days after sc inoculation of the A2780-CP cells. $Cu(CQ)_2$ and copper liposomes were dosed iv Monday, Wednesday and Friday for two weeks and DSF was dosed orally (see Methods) Monday to Friday for 2 weeks. (A) A2780-CP tumour growth in NRG mice (n=13) and (B) tumour size on day 20 was determined as described in the Methods Data are reported as mean ± SEM.

5.3 Discussion

Recently, efforts have been directed towards repurposing CQ as an anti-cancer drug [303]. The activity of CQ and $Cu(CQ)_2$ against a range of cancer cell lines suggests that $Cu(CQ)_2$ is only effective at concentrations ranging for 20-60 μM (see Figure 5.1). The anti-cancer activity of CQ alone is worse, with IC_{50} values greater than 100 μM. Interestingly, the activity of CQ is enhanced when administered as a copper complex, although some cell lines show copper independent activity. This may be a consequence of the cellular context in which CQ is presented [299, 306], or it may be due to higher intracellular copper levels in some cell lines when compared to others [307]. Regardless, the preclinical data suggesting CQ anti-cancer effects was compelling enough to lead to a clinical trial wherein CQ was given to 11 patients with hematologic malignancies [17]. This study was designed to test whether the metal ionophore activity of CQ and its associated inhibition of the proteasome could engender therapeutic effects in patients with refractory hematologic malignancies.

CQ was given oral in a classic dose escalation phase 1 study. The maximum tolerated dose was determined, however there was minimal activity and no evidence of proteasome inhibition. These authors concluded that the poor activity was due to poor intracellular delivery of CQ [303].

CQ can be administered orally but does suffer from extensive first pass metabolism [298]. This did not affect its utility as an anti-microbial drug but did pose a challenge when attempting to repurpose this drug for cancer, where high plasma concentrations are required. One method to overcome first pass metabolism is through iv injection; owing to the solubility challenges of both CQ and $Cu(CQ)_2$ this was not possible. Herein, a novel formulation of CQ, where the $Cu(CQ)_2$ complex is synthesized inside the core of liposomes that is suitable for development as a pharmaceutical was investigated.

The aqueous core of the liposome is used to carry out a synthesis reaction between copper and CQ, the complex is left in solution (suspended inside the liposome). The amount of Cu inside the liposome is the limiting reagent when forming $Cu(CQ)_2$ (see Figure 5.2) and the complex formed inside the liposome showed no release *in vitro* over a time course of 24 hrs. The formulation appears stable with respect to particle size, polydispersity and $Cu(CQ)_2$/liposomal lipid ratio. Pharmacokinetic studies completed with the resultant $Cu(CQ)_2$ formulation indicate that blood levels of CQ can be maintained at concentrations well above the $Cu(CQ)_2$ IC_{50} for at least 8 hrs after iv administration at a dose of 30 mg/kg (see Figure 3). Analysis of the plasma samples strongly suggest that $Cu(CQ)_2$ dissociates from the $Cu(CQ)_2$ formulation following administration (see Figure 5.3D). However because of an inability to measure $Cu(CQ)_2$ in plasma, it was unclear whether the $Cu(CQ)_2$ released from the liposomes remained in a complexed form. The CQ concentration in the plasma was more than sufficient to exert therapeutic effects for at least 8 hours and the formulation addresses the limitation encountered by investigators interested in evaluating CQ activity in patients.

Having overcome the formulation challenges of $Cu(CQ)_2$, it was reasonable to ask whether the resulting formulation was efficacious *in vivo*. Our results suggest that the $Cu(CQ)_2$ formulation is not effective, even when administered in combination with DSF, an agent that is significant more potent when combined with a copper [66, 116]. The studies

with $Cu(CQ)_2$ alone were completed in two subcutaneous tumour models (A2780-CP and U251) representing cell lines in which CQ toxicity was copper dependant (A2780-CP) and copper independent (U251). These studies used a dose intensive schedule (Monday, Wednesday and Friday x 2 weeks) because $Cu(CQ)_2$ is active only when present at μM levels. Despite a multi-dosing schedule and evidence to suggest that the CQ levels in the plasma compartment were above 100 μM for at least 8 hrs, the $Cu(CQ)_2$ formulation did not show any activity. The original studies with CQ were based on its potential to act as a copper ionophore [50] and for this reason it was explored in combination with DSF. The *in vitro* results (see Figure 5.5) support the fact that DSF/ $Cu(CQ)_2$ combinations are effective and that nanomolar levels of DSF (in the presence of $Cu(CQ)_2$) are sufficient to exert significant cytotoxicity, however the activity of the combination *in vivo* (see Figure 5.6) indicated otherwise. While the combination did show good activity *in vitro*, there is an inability to coordinate the pharmacokinetics of both DSF and $Cu(CQ)_2$ formulation such that the two agents reach the tumour site at sufficient levels to achieve effective therapy. It is possible that this may be overcome by assessing sequential combination treatments in the future; as was done by Verreault *et al.*[308]. Alternatively, studies have suggested that another 8-hydroxyquinoline analogue, 5-nitro-8-hydroxyquinoline, is much more potent than CQ [18] and future studies could investigate formulations of this analogue. The methodology described is broadly applicable to the synthesis of many different metal-complexes inside liposomes and provides the opportunity to select for formulations that will be better suited for clinical development then the $Cu(CQ)_2$ formulation described here.

5.4 Conclusion

This work examined whether CQ and $Cu(CQ)_2$ could be formulated in a manner suitable for development as an anti-cancer agent. A liposomal $Cu(CQ)_2$ formulation was described that solves the solubility issues plaguing efforts to assess the activity of the highly water insoluble $Cu(CQ)_2$ complex. Further the resultant formulation ensured that therapeutically effective concentrations of CQ or $Cu(CQ)_2$ could be maintained in the plasma compartment over time. However, the resulting formulation was not efficacious whether used alone or in combination with DSF, a drug that is known to be activated in the

presence of copper. While this formulation did not exhibit interesting therapeutic effects *in vivo* the formulation methods are suitable for other analogues of 8-hydroxyquinoline which exhibit more potent anti-cancer activity.

6. Copper Complexes of Bidentate Ligands Exhibit Potent *In Vitro* Anti-Cancer Activity Regardless of Platinum Sensitivity Status*

6.1 Introduction

Pt drugs are the most successful class of inorganic medicinal compounds used to treat cancer [36, 309]. They are a mainstay in cancer therapy, being utilized in approximately 50% of chemotherapeutic regimens [36, 310]. CDDP was first used to treat leukemia in the 1960s, but through several inorganic medicinal chemistry programs, other Pt-based drugs (CBDCA, oxaliplatin, paraplatin etc.) have been produced and approved by the FDA [32, 36]. Mechanistically, these drugs are known to act by forming Pt-DNA complexes that cause DNA damage that accumulate to a point that is beyond repair, ultimately leading to cell death [310, 311]. Pt drugs are currently used as first-line therapy in blood, lung, ovarian, testicular, and head and neck cancers. While some cancers are Pt sensitive and thus respond to Pt drugs [36], many others are Pt-insensitive due to inherent or acquired resistance [310, 311]. There is a need to define drugs capable of treating Pt insensitive cancers [312].

While Pt drugs have been successful for many patients, they also produce serious side effects including nephrotoxicity, neurotoxicity and ototoxicity [313]. To address these adverse effects, many inorganic medicinal chemistry investigations have focused on developing alternatives to Pt-based drugs by replacing Pt with other divalent metals, such as copper [48]. Copper-based therapeutics are generally less toxic owing to the physiological processes that detoxifying excess of copper [62]. In line with this approach, many groups have demonstrated *in vitro* that copper complexes of natural compounds have anti-cancer properties; some examples include dithiocarbamates and analogues of 8-HQ [301, 314]. To date, however, no copper complex has transitioned into clinical use for any indication in humans. While the platins that are used in the clinic are water soluble,

* Adapted from: M. Wehbe, C. Lo, A. Leung, W.H. Dragowska, G. Ryan, M.B. Bally, Copper complexes of bidentate ligands exhibit potent anti-cancer activity regardless of platinum sensitivity status, (2017) *Submitted to Investigational new drugs*

one major challenge of copper-based therapeutics is their inherently poor aqueous solubility [66]. This problem has made *in vivo* testing very challenging and required solvents containing mixtures of DMSO and Cremophor which are not clinically applicable due their high toxicity[214, 315]. Chapter 3 solved the solubility issue of copper-based therapeutics by synthesising copper complexes within liposomes [66]. This technology, referred herein as Metaplex™, was used to examine the effects of copper DDC ($Cu(DDC)_2$) in animals bearing a xenograft tumours [276].

In the present studies, five bidentate copper ligands (Figure 6.1) were selected and screened both as ligands and the corresponding copper complexes in a panel of eight cancer cells. As indicated above DDC [42, 65], Pyr [214], Plum [316, 317], 8-HQ [65, 216, 219] and CQ [65, 191, 217, 301] have all been shown to have anti-cancer activity but have never been tested directly against Pt-resistant cancers. The activities of these complexes were compared to the activity of CDDP and CBDCA (Figure 6. 2). The screen showed that many copper complexes are more active *in vitro* than the Pt controls. It was demonstrated that a Metaplex™ formulation of $Cu(DDC)_2$ was active in animals bearing Pt-resistant A2780-CP xenograft tumours. The results highlight the potential of the copper-based therapeutics as candidates to treat Pt resistant cancers.

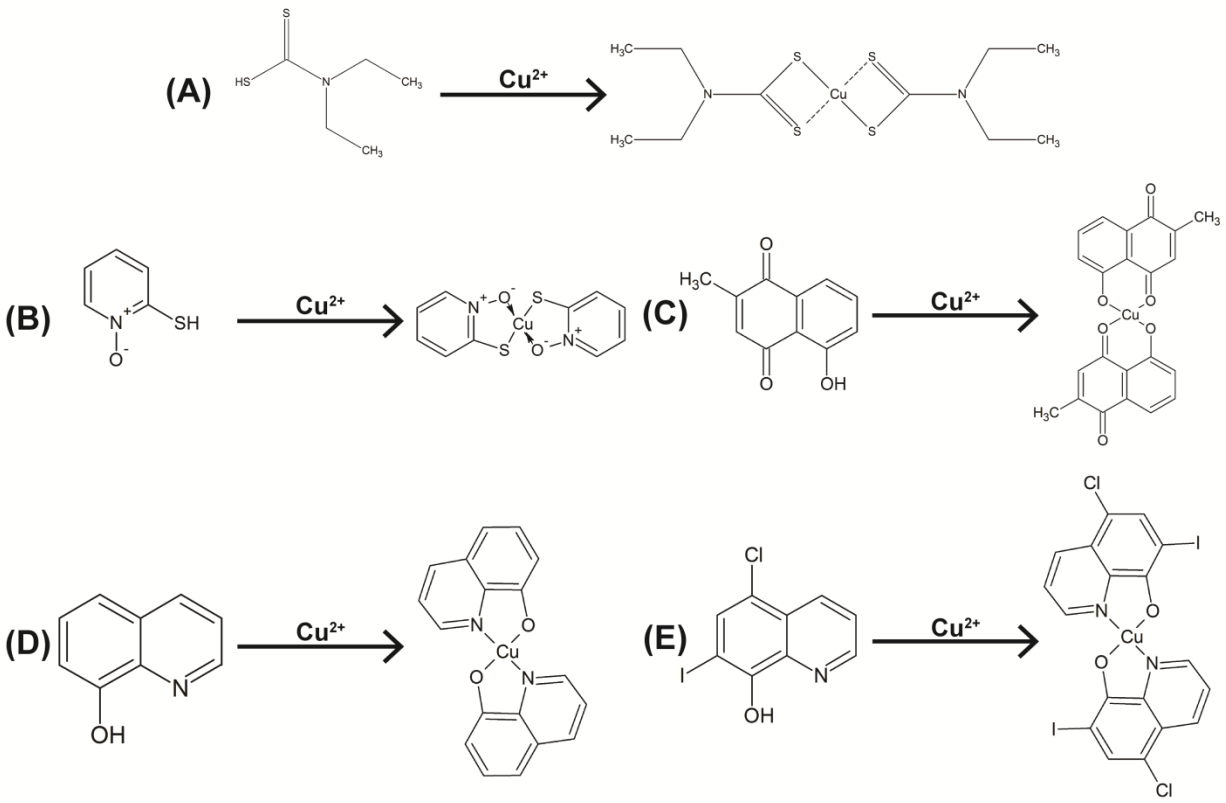


Figure 6.1: Ligands and their respective copper complex structures. (A) DDC and $Cu(DDC)_2$ (B) Pyr and $Cu(Pyr)_2$. (C)Plum and $Cu(Plum)_2$. (D) 8-HQ and $Cu(8\text{-}HQ)_2$ and (E) CQ and $Cu(CQ)_2$.

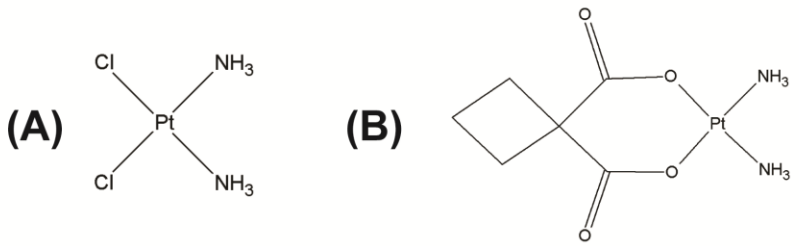


Figure 6. 2: Structures of commonly used platinum drugs. (A) CDDP and (B) CBDCA.

6.2 Results

6.2.1 Pt-Resistance Does not Impact Sensitivity of Cancer Cells to Cu(DDC)$_2$ Complex

CDDP and CBDCA, two commonly used Pt-based therapeutics were tested in a pair of isogenic ovarian cancer cells: A2780-S cells are the parental cells that are sensitive to CDDP while A2780-CP cells are platinum-resistant. As shown in Figure 6.3A and B, the IC_{50} of CDDP and CBDCA was 3.7-and 8.5-fold greater, respectively, in the Pt resistant cells when compared to Pt sensitive cells. To determine if Pt resistance impacts activity of copper complexes, A2780-S and A2780-CP cells were treated with Cu(DDC)$_2$. The data in Figure 6.3C show that the IC_{50} value of the copper complex was not different for Pt sensitive or resistant cells. These data suggest that copper complexes may be effective regardless of Pt sensitivity status.

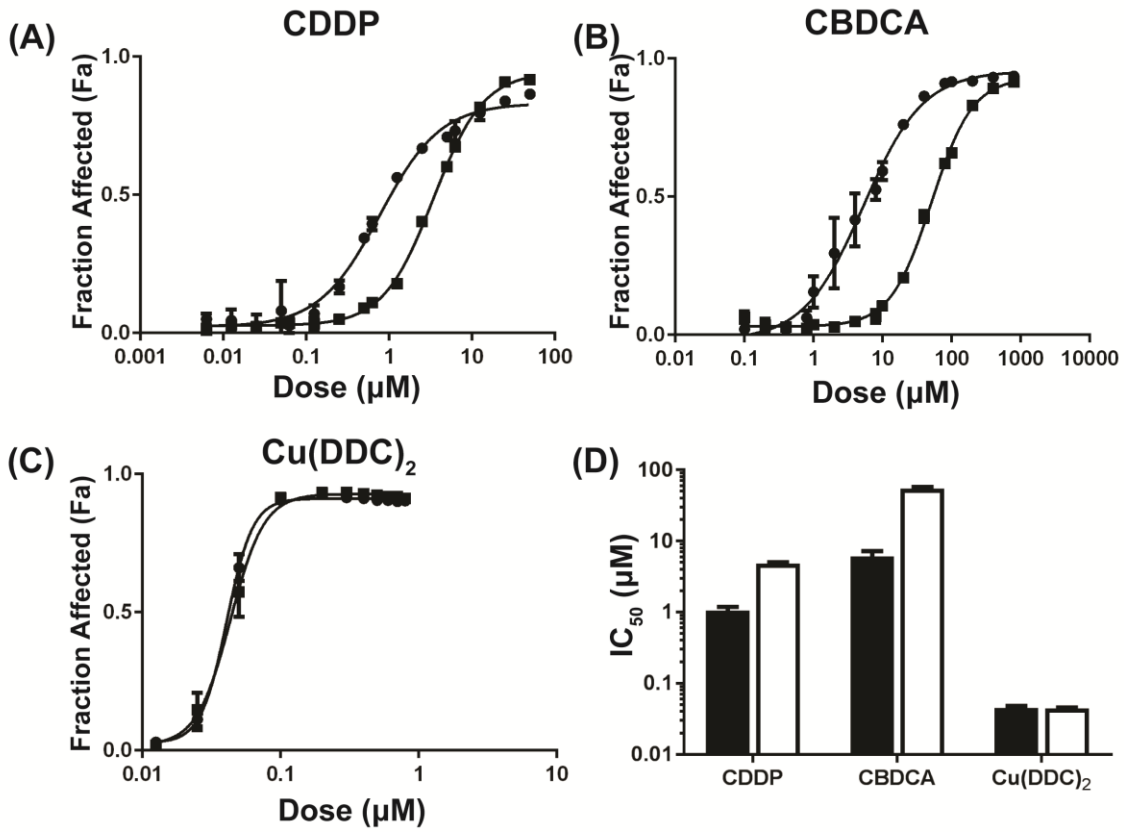


Figure 6. 3: Cytotoxicity profiles of Pt sensitive A2780-S (●) and resistant A2780-CP (■) ovarian cancer cells (or) following 72 hr treatment with (A) CDDP, (B) CBDCA and (C) Cu(DDC)$_2$. Fraction affected cells was assessed based on viability data normalized to vehicle controls. (D) The IC_{50} ± 95% CI for

A2780-S (black) and A2780-CP (white) after treatment with CDDP, CBDCA and $Cu(DDC)_2$. Data are presented as the mean of 3 independent experiments ± SEM.

6.2.2 Activity of Copper Complexes in Cancer Cells with Different Pt Sensitivity

The IC_{50} values ± 95% confidence intervals for the ligands and their corresponding copper complexes are shown in Figure 6.4. Cells were treated with the ligand/drug or complex (2:1 ligand:Cu) for 72 hrs. All cell lines were also exposed to $CuSO_4$ as a control to ensure that copper alone did not impact viability (IC_{50} > 10μM for all cell lines, data not shown). The results in Figure 6.4A indicate that treatment of different cells with the ligand DDC results in an IC_{50} ranging from 5-300 μM DDC. In contrast, the IC_{50} for the $Cu(DDC)_2$ ranged from 0.02-0.15 μM corresponding to differences of 90-11,000 fold (Fig. 6.4A). The IC_{50} of Pyr ranged from 1-30μM, and when used as a copper complex ($Cu(Pyr)_2$), the IC_{50} decreased to 0.1-7.4 μM (Fig. 6.4B). The IC_{50} of Plum ranged from 1.5–11 μM while the IC_{50} of and $Cu(Plum)_2$ Ranged from 0.8-3 μM in the tested cell lines. In some cell lines (A2780-S, A2780-CP, MV-4-11, Cal-27, FaDu) there was only approximately a 2-fold difference in activity between Plum and $Cu(Plum)_2$ (Fig. 6.4C). Considering the 2:1 (ligand:Cu) complexation ratio, these data indicate that the copper Plum complex is as active as the uncomplexed ligand, suggesting that the cytotoxic effects observed for Plum is copper-independent. 8-HQ was more active as a copper complex (IC_{50} for $Cu(8\text{-}HQ)_2$ ranged between 0.2-4.5μM compared to 1.5-30μM for 8-HQ); however, in A2780-S and FaDu cells, the toxicity of 8-HQ appeared to be copper independent (Fig. 6.4D). The IC_{50} for CQ and $Cu(CQ)_2$ ranged from 10-350μM and 20-60μM, respectively. Here the dependence on copper complexation for CQ cytotoxicity appears to be cell line-specific: copper-dependent activity was observed in A2780-S, A2780-CP and A549 cells, whereas in MV-4-11, SCC-25 and H1933, the cytotoxicity of CQ was copper-independent. Moreover, CQ appears to be more cytotoxic than $Cu(CQ)_2$ in Cal-27 and FaDu cells (Fig. 6.4E).

The IC_{50}s obtained from cytotoxicity assays for each cell line were arranged as a heat-map in order of Pt sensitivity to CDDP (Fig. 6.5). An IC_{50} cut-off of 10μM was used to distinguish more potent agents from those that were regarded as pharmaceutically inactive. In general, the ligands appear to be less active in the Pt resistant cell lines whereas the copper complexes augmented anti-cancer activity considerably. The Pyr, Plum and 8-

HQ ligands showed activity in most cell lines, however, their activity was enhanced in the tested cell lines when used as complexes with copper; where the greatest increases were observed for $Cu(Pyr)_2$. DDC and CQ were the least active ligands. The $Cu(CQ)_2$ complex did not show improvement in cytotoxicity whereas the copper complex of DDC was the most active in all cell lines. In general, except for $Cu(Pyr)_2$, the activity of the copper complexes used in this study show no correlation with Pt CDDP sensitivity in tested cell lines (Table 6.1). The heatmap indicates that $(Cu(DDC)_2$ is the most effective complex tested against Pt-resistant cancers.

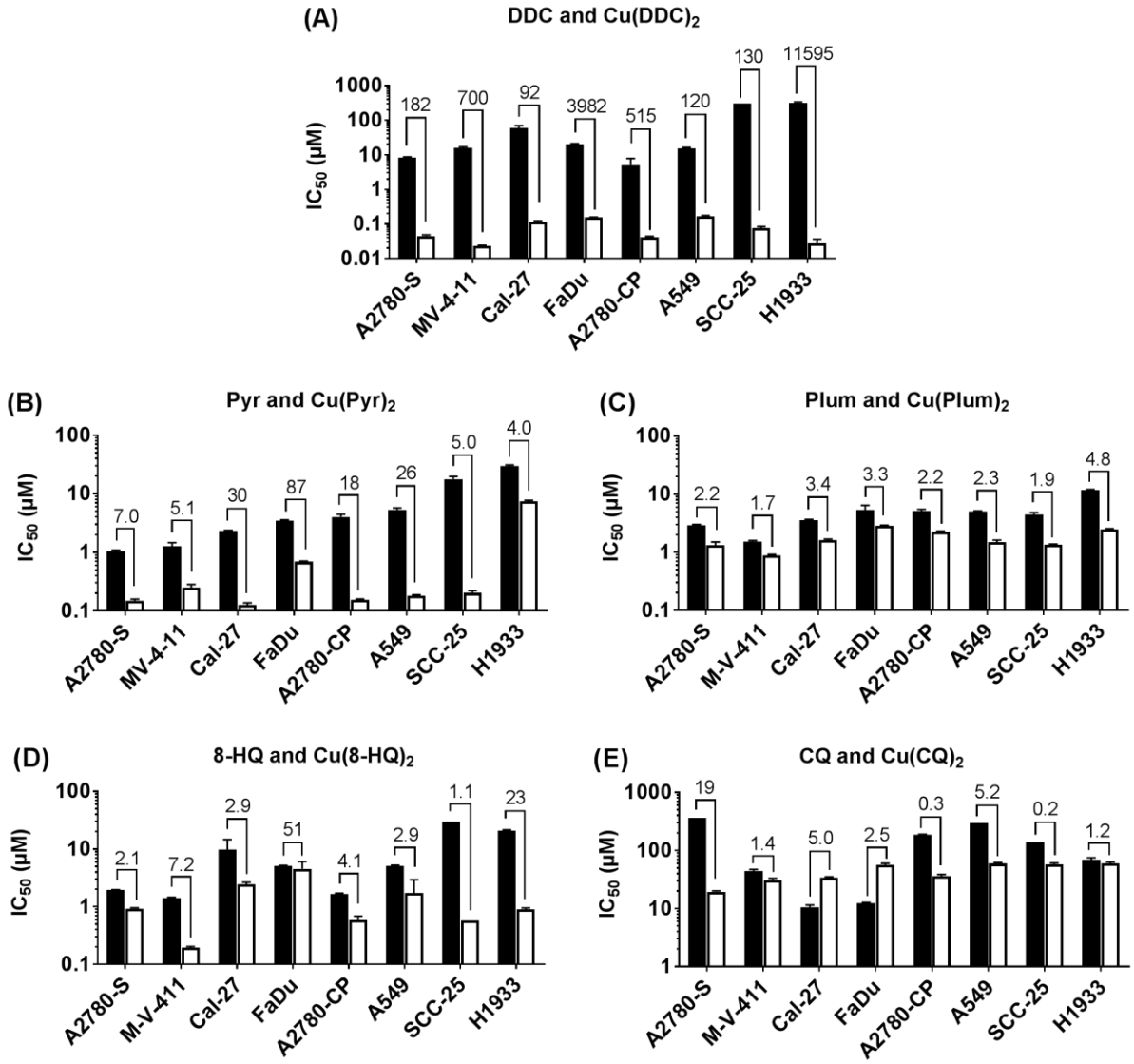


Figure 6. 4: Ligand/drug and copper complex cytotoxicity. A panel of 8 cancer cell lines (A2780-S, MV-4-11, A549, SCC-25, Cal-27, A2780-CP, FaDu and H1933) were treated for 72 hrs with the ligand/drug (■) or copper complex (□): (A) DDC/Cu(DDC)$_2$, (B) Pyr/Cu(Pyr)$_2$,(C) Plum/Cu(Plum)$_2$, (D) 8-HQ/Cu(8-HQ)$_2$ and (E) CQ/Cu(CQ)$_2$. The IC_{50} values were obtained based on the viability data acquired with IN Cell 2200 or PrestoBlue® (M-V-411). The fold difference between the IC_{50} of the ligand and respective copper complex is shown above the histogram bars.

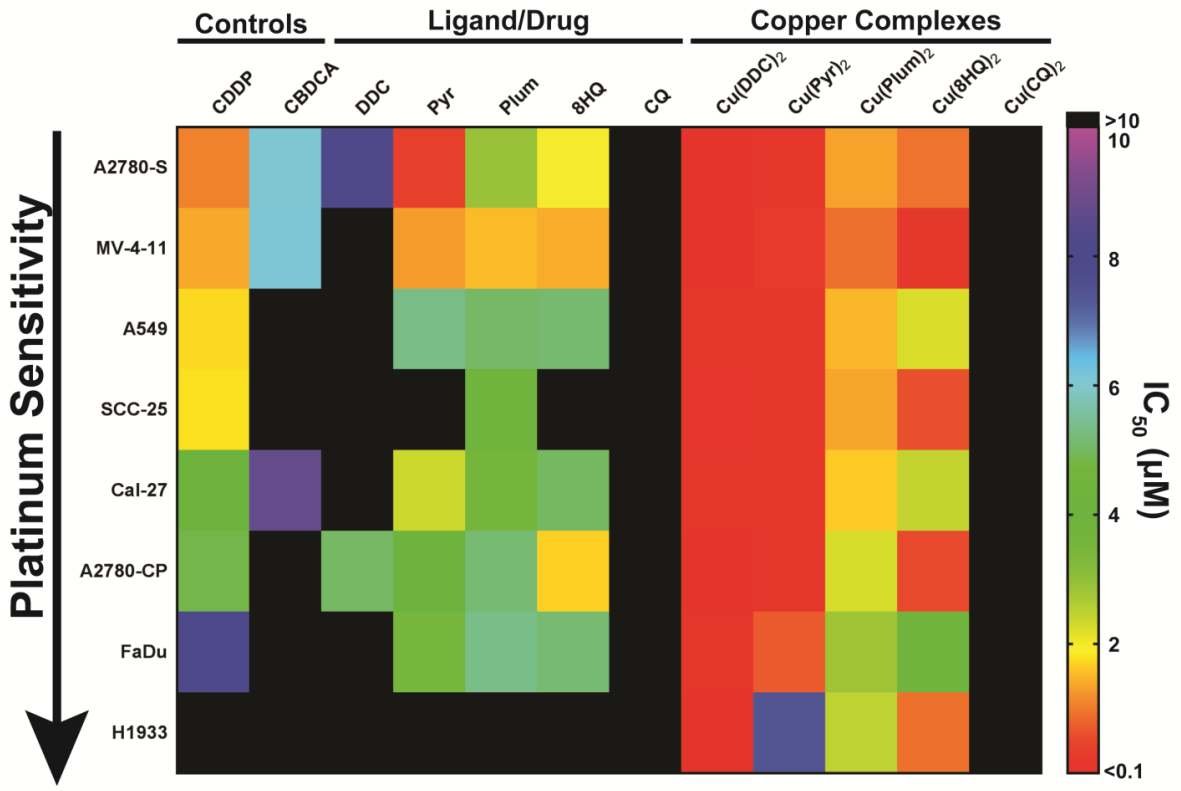


Figure 6. 5: Screen of copper-based therapeutics in cancer cells with different Pt sensitivities. IC_{50} values for Pt drugs (CDDP and CBDCA), ligands (DDC, Pyr, Plum, 8-HQ, CQ) and respective copper complexes are shown for cancer cell lines of differing origin arranged in order of sensitivity to CDDP. IC_{50} values were calculated from viability data (n=3 experiments/each cell line) obtained with IN Cell Analyzer 2200 platform or PrestoBlue™ assay (MV-4-11 cells). The agents with IC_{50} values >10 μM (black) are considered inactive.

Table 6. 1: Correlation between CDDP and copper complex sensitivity based on the IC_{50} values

Drug	$Cu(DDC)_2$	$Cu(Pyr)_2$	$Cu(Plum)_2$	$Cu(8\text{-}HQ)_2$	$Cu(CQ)_2$
Pearson R^2	0.07	0.70	0.10	0.09	0.44
P-value	0.05	0.01	0.44	0.48	0.07
Significant	No	Yes	No	No	No

6.2.3 Efficacy of Liposomal Cu(DDC)$_2$ in a Pt-resistant A2780-CP Tumour Xenograft Model

Cu(DDC)$_2$ has represented a formulation challenge because of its poor aqueous solubility. In Chapter 3, Cu(DDC)$_2$ was synthesized inside a liposomal formulation [66]. This formulation (see Methods) was used to assess the activity of the Cu(DDC)$_2$ in a Pt-resistant A2780-CP subcutaneous tumour xenograft model. The data, shown in Figure 6.6, indicate that Cu(DDC)$_2$ engendered a statistically ($p<0.05$) significant ~50% reduction in tumour burden when compared to the activity of the vehicle control. These data provide proof-of-concept results suggesting that liposomal copper complexes have the potential to be developed as a novel class of therapeutic agents for use in the treatment of Pt-resistant cancers.

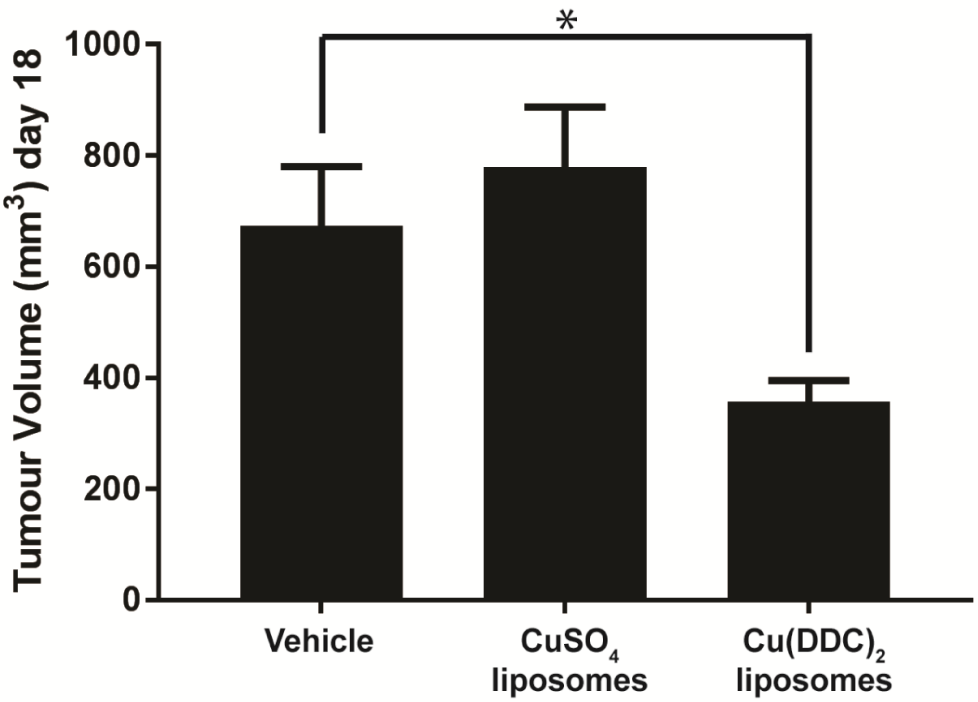


Figure 6. 6: *In vivo* activity of liposomal Cu(DDC)$_2$ in animals bearing Pt resistant A2780-CP tumours. 1 x 10^6 A2780-CP cells were inoculated sc into immune-compromised NRG mice which were subsequently treated intravenously with vehicle, copper containing liposomes and Cu(DDC)$_2$ liposomes using a 3 x per week for 2 weeks dosing schedule. Mean tumour volume was determined on day 18 (the humane endpoint for vehicle treated animals). Data represents the mean ± SEM (n=7). * indicates statistical difference ($p<0.05$)

6.3 Discussion

Pt drugs are standard first-line therapies for the treatment of lung, ovarian, and head and neck cancers [29, 318]. While some cancers are inherently resistant, the widespread use of Pt-based therapies has resulted in the development of resistant cancers, often in the relapse setting where prognosis is poor [312, 318]. *In vitro,* cell lines can be developed to replicate this effect (e.g. A2780-CP), wherein cells are treated with low dose Pt drugs such as CDDP over extended periods of time until resistance is observed through changes in IC_{50} values [319, 320]. The two major mechanisms responsible for Pt resistance are DNA repair and Pt drug transport into cancer cells [36, 321]. Through extensive characterization, it was determined that at equivalent levels of drug accumulation, the A2780-CP cell line is 2-fold more efficient at repairing CDDP-DNA lesions when compared to the parental, Pt-sensitive cell line (A2780-S) [321]. The copper transporter CTR1 is the major influx transporter for cisplatin both *in vitro* and *in vivo* and has been reported to be reduced in cisplatin resistant cancers [318, 322]. This mechanism was attributed to cisplatin influx resulting in the rapid degradation of CTR1[323]. Identifying new therapeutic entities that are active in Pt resistant cancers is a priority to improve treatment outcome for patients.

The ovarian cancer cell line A2780-CP had a clear resistance to CDDP and CBDCA when compared to the activity of these drugs in A2780-S cells. In order to test the utility of CBTs in platinum-resistant cancer cells, preliminary studies investigated $Cu(DDC)_2$ in Pt sensitive and insensitive cell lines. $Cu(DDC)_2$ was active irrespective of Pt sensitivity at equivalent doses. This Pt sensitivity-independent activity could be explained by the $Cu(DDC)_2$ mechanism of toxicity which overcomes resistance mechanisms commonly seen in Pt-resistant cancers. $Cu(DDC)_2$ is able to accumulate in cancer cells irrespective of CTR1 expression [324] and cytotoxicity is mediated through proteosome inhibition [42]. These results suggest that CBTs use mechanisms of cytotoxicity outside of those used by platinums and warrant further investigations in resistant cancers.

Five copper binding ligands (DDC, Pyr, Plum, 8-HQ and CQ) were tested in a panel of eight cancer cell lines of different origins (blood, lung, ovarian, head and neck) and differing sensitivity to Pt-based drugs exemplified by CDDP/CBDCA activity. The *in vitro*

data suggests that the cytotoxicity of ligands DDC, Pyr and 8-HQ appeared to be copper-dependent whereas the cytotoxicity of Plum and CQ were copper independent. This finding is important as DSF (metabolized to DDC *in vivo*) + Cu-Gluconate and CQ were involved in clinical trials with no listed benefit [289, 325, 326]. By knowing the relationship between Cu and the activity of the complex, it can be reasoned that ligands such as DDC, Pyr and 8-HQ should be administered as copper complexes to ensure that the active (copper complexed) species reaches the cancer cells. This is supported by work performed by Katano *et al* which showed that Pt resistant cancers have lower basal levels of copper, suggesting that the uncomplexed ligands would be less active [322]. In the current study, H1933 was the least sensitive to Pt as reflected by a >10 μM IC_{50} cytotoxicity to all the ligands tested. In contrast, Plum and CQ could be used as single agents and pharmaceutical challenges associated with their solubility may be overcome using the Metaplex™ approach used in Chapter 4 to prepare a nanomedicine formulation of $Cu(DDC)_2$ with no loss in activity[66, 276].

This data supports the utility of copper-based therapeutics in the treatment of Pt-insensitive cancer. To date, not a single copper-based therapeutic has received FDA approval. This is in part due to the solubility challenges associated with these complexes. All copper complexes studied as well as the ligands Plum, 8-HQ and CQ are water insoluble (<0.1 mg/mL); in contrast, DDC and Pyr are water soluble as ligands but their therapeutic activity is significantly enhanced in the presence of Cu. All copper complexes had to be dissolved in DMSO (final concentration 0.5%) to allow for *in vitro* testing. The ICH guidelines for residual solvents in a product indicate that DMSO cannot be included at concentrations above 0.5% which would be needed to administer the complexes at relevant doses [64]. In Chapter 3 the Metaplex™ technology as a platform approach to formulate copper complexes inside liposomes in order to overcome solubility issues and to allow for parenteral administration of copper complexed ligands was described [66]. Thus, as a proof-of-concept a liposomal formulation of $Cu(DDC)_2$ was tested in a xenograft tumour model of A2780-CP Pt-resistant ovarian cancer. In this study, it was shown that liposomal $Cu(DDC)_2$ (8 mg/kg) but not Cu-liposomes produced a statistically significant ~50% reduction in tumour burden when compared to the vehicle control. This difference is not considered clinically relevant (based on RECIST criteria [287]) but the results does

suggest that copper complexes are a class of therapeutic that should be further investigated. $Cu(DDC)_2$ pharmacokinetics for the liposomal product show rapid release from the liposome and degradation in the plasma, and as such would require further development to improve circulation lifetime and stability [66]. Also the use of this formulated $Cu(DDC)_2$ preparation in combination with other agents typically combined with platinums (eg. the taxanes [327]) is warranted. The other complexes discussed are proposed as future developments to identify those which have activity as single agents in Pt resistant cancers and as those which can augment therapy in combination with traditional chemotherapeutics.

6.4 Conclusion

Pt drugs have been widely successful in the treatment of cancer. The treatment of Pt-resistant cancers has been a pharmaceutical focus that requires new therapeutics with mechanisms of action differing from those invoked by Pt-based therapies. Copper-based therapeutics represents a new class of drugs that can address many of the challenges associated with Pt-resistant cancers. This data shows that four out of five screened ligands that form copper complexes reached IC_{50} values below 10 μM in the eight cancer cell lines regardless of their Pt sensitivity. Additionally, one of these complexes ($Cu(DDC)_2$) was tested *in vivo* in a Pt-resistant ovarian cancer xenograft model and attained a 50% reduction in tumor volume when compared to vehicle-treated control mice. These data not only provide *in vivo* validation that copper-based therapeutics could be used to treat Pt-resistant cancer but also provides proof-of-concept that the Metaplex™ technology could be used to develop formulations of inherently insoluble copper complexes for pre-clinical and clinical evaluations.

7. Summarizing Discussion

This thesis describes a method by which poorly soluble copper complexes can be synthesized inside the core of liposomes; an approach that has been referred to as Metaplex™. The method is simple and scalable. The technology has been validated for ligands representative of differing functional group classes such as DDC (S-donor), CQ (N,O-donor), Qu (O-donor) and CX-5461 (mixed) (see Figure 1.3 and Table 3.1). Each of these classes can represent a large variety of molecules and I believe that such compounds all have the potential to be formulated using this approach. The synthesis reaction and general approach to preparing such formulations was described in detail in Chapter 3; results which highlight that complexation is solely dependent on the amount of copper entrapped inside the liposome. The copper complex to lipid ratio can thus be estimated by measuring the amount of copper associated with the liposome and the nature of the complexation reactions. The copper complex to lipid ratio can be tuned by changing the concentration of the copper salt solution used to rehydrate the lipids and by the salt form used (i.e. copper sulfate vs copper gluconate) or through changing the lipid composition. With respect to the latter point, it is known that liposomes composed of charged lipids (anionic/cationic) have a higher trapped volumes when compared to liposomes prepared with neutral lipids [240]. This is reflected in the studies summarized in Figure 3.3: as the liposomal lipid composition changed from DSPC/Chol to DSPC/Chol/DSPE-PEG(0.5-5%), the measured copper to lipid ratio increased from 0.2 to 0.45 even though both formulations were prepared using 300mM copper sulfate solutions.

My interest in developing formulations of copper complexes arose fortuitously from an interest to repurpose the anti-alcohol drug DSF for use in cancer. DSF was identified in a screen to be active against brain tumour initiating cells and this activity was enhanced in the presence of copper [116]. An examination of the DSF pharmacokinetic literature showed that DDC is the primary metabolite of DSF and it was well known that DDC is a Cu chelating agent [268, 328]. In chapter 3, it was shown that the activity of DSF + Cu or $Cu(DDC)_2$ are identical (see Figure 3.1). The ability to investigate the anti-cancer potential of $Cu(DDC)_2$,the active agent generated when DSF is mixed with copper, is difficult since it

is sparingly soluble in aqueous solution and this created the pharmaceutical challenge that I focused on. The $Cu(DDC)_2$ formulation first described in Chapter 3 was suitable for further *in vivo* studies and these were summarized in Chapter 4. The formulation of $Cu(DDC)_2$ prepared in DSPC/Chol (55:45) liposomes, when administered i.v. using a dosing schedule of 8 mg/kg (M,W,F x2), did effect a ~50% reduction in tumour burden when using a subcutaneous tumour model. However, the pharmacokinetic studies with this formulation suggested (see Figure 4.3) that the encapsulated $Cu(DDC)_2$ was rapidly released from the injected liposomes. One advantage of this formulation approach is based on the well established understanding that liposomal lipid composition can influence the rate of associated drug release from the liposomes following i.v. administration. I believed that the anti-tumor activity of formulated $Cu(DDC)_2$ could be improved if its retention inside liposomes was improved. By changing liposomal lipid composition it was demonstrated that a 4.2-fold increase in plasma $AUC_{(0-\infty)}$ could be achieved relative to the DSPC/Chol formulation (see Figure 4.7), this increase in plasma AUC was due to the fact that the optimized liposomal formulation exhibited significantly better retention of $Cu(DDC)_2$ following administration . Despite the improvement in drug retention, it was found that the optimized formulation showed equivalent anti-cancer activity when administered at their respective maximum tolerated dose (MTD). In this context, it should be noted that the MTD of the DSPC/Chol formulation was 1.5 times lower than that for the optimized formulation, suggesting that the improved drug retention did reduce the toxicity of the formulated $Cu(DDC)_2$.

A study by Daniel *et al.* examined the use of pyrrolidone diethyldithiocarbamate (PyDDC) and CQ as copper complexes with proteasome inhibitory activity comparable to what has been shown for $Cu(DDC)_2$ (PyDDC is an analogue of DDC) [301]. CQ is an approved topical anti-fungal agent with anti-cancer activity [191]. In my hands, the anti-cancer activity of CQ was enhanced in the presence of copper, but this was not in all cell lines (see Figure 5.1). I choose to develop a CQ formulation because, unlike DDC which is a water soluble ligand that forms a water insoluble copper complex, CQ is a water insoluble ligand which becomes even less soluble when prepared as a copper complex. The development of the $Cu(CQ)_2$ formulation not only enabled the *in vivo* evaluation of $Cu(CQ)_2$, but also provided a unique opportunity to test whether the formulation approach could be

applied to poorly soluble ligands. To prepare the formulation, CQ was added in powder form to Cu-containing liposomes. It this scenario, some portion of the CQ must first dissolve into solution prior to permeating across the lipid membrane where it can then interact to form $Cu(CQ)_2$. For complexation to occur the solution of CQ and copper containing liposomes required heating to at least 40^{o}C (see Figure 5.2). Pharmacokinetic studies with the resulting $Cu(CQ)_2$ formulation suggested that loss of the associate complex from the liposomes following administrtaion was slower then shown for the formulated $Cu(DDC)_2$, but similar to the $Cu(DDC)_2$ formulation the results suggest that the complex, rather than the ligand alone, was released from the liposomes (see Figure 5.3). The formulation developed was suitable for use *in vivo.* Studies were completed to determine whether $Cu(CQ)_2$ exhibited significant anti-cancer effects. Despite using a dose intensive schedule, $Cu(CQ)_2$ failed to cause a significant reduction in tumour burden. The $Cu(CQ)_2$ plasma concentration achieved appeared to reach levels that should have been sufficient to cause anti-cancer effects (seeFigure 5.3), but it should be noted that the levels of $Cu(CQ)_2$ achieved in the plasma are a combination of $Cu(CQ)_2$ inside the liposome and $Cu(CQ)_2$ that has dissociated from the liposomes. In this context, levels of $Cu(CQ)_2$ accessible to tumour cell populations may have been much lower than needed and ultimately tumour levels of $Cu(CQ)_2$ would not be sufficient to cause therapeutic effects. This lack of activity may also be due to the instability $Cu(CQ)_2$ after it was released from the liposomes. I would suggest that other, more potent, analogues of 8-HQ (e.g. 5-amino-8-hydroxyquinoline [314]) could be developed using the approach described in this thesis.

Based on the results in Chapter 5, further investigation of $Cu(CQ)_2$ as a single agent for treatment of cancer is not warranted. Formulations of $Cu(DDC)_2$ and $Cu(CQ)_2$ could prove more therapeutically interesting if used in combination with other drugs. It is known, for example, that the activity of the anti-cancer drug flavopiridol in combination with the proteasome inhibitor bortezomib is enhanced [329]. Thus combinations of flavopiridol and $Cu(DDC)_2$ or $Cu(CQ)_2$ could prove interesting.

As noted in chapter 5, studies have suggested that $Cu(CQ)_2$ can act as a copper ionophore [50]. I believed that it was reasonable to assess the use of $Cu(CQ)_2$ in combination with DSF, with the goal of generating $Cu(DDC)_2$ within the target cancer cell population. As shown in this thesis, the IC_{50} of $Cu(CQ)_2$ is >1μM while the IC_{50} of $Cu(DDC)_2$

is typically <200nM. For this reason, I evaluated the combination and demonstrated that the $Cu(CQ)_2$ and DSF combination resulted in significant cytotoxicity. This was not observed *in vivo* as the $Cu(CQ)_2$ and DSF combination did not result in a statistically significant reductions in tumour growth when compared to vehicle control in a tumour model generated using a sensitive cell line (A2780-CP tumour model; see Figure 5.6). This strategy may require further optimization. For example, Verreault *et al.* compared the effects of sequential versus concurrent dosing of two anti-cancer agents and the results suggested that the combination may require one to coordinate the pharmacokinetic profiles of both agents [308]. Thus, Cu would need to be delivered first to the tumour cells by treatment with $Cu(CQ)_2$. These Cu-"loaded" cancer cells would then be treated with DSF which would be given orally. This sequential strategy should be investigated in the future. Finally, while the $Cu(DDC)_2$ and $Cu(CQ)_2$ formulations did not provide a strong therapeutic response, the studies summarized in Chapter 6 suggest that further efforts with this class of agents should focus on platinum resistant cancers.

7.3 Novelty of Metal-based Complexation Reactions to Prepare Liposomal Drug Formulations

The use of metals and specifically copper in liposome loading of anti-cancer agents has been reported before. This has largely been developed around the campothecins (topotecan, Irinotecan) but has also included the anthracyclines (doxorubicin). With these agents it is difficult to separate the "remote loading" from the complex synthesis. During the development of remote loading it was found that drugs with a protonizible amine functionality could be encapsulated inside the core of liposomes when an electrochemical or proton gradient was present. Thus, the unencapsulated drug would be neutral in charge on the exterior of the liposome core but would become charged once it enters the liposome resulting in its encapsulation. This process is referred to as drug loading and is applicable to both the anthracyclines and campothecins. Both of these drugs classes also contain copper binding motifs which could contribute to the encapsulation and effect drug release.

It is challenging to distinguish between pH gradient loading and encapsulation due to metal complexation. One reason is that attempts to eliminate the pH gradient by

increasing the pH of the metal containing solutions results in formation of insoluble metal hydroxides [330]. This is to say that all unbuffered metal solutions that are used in liposomal drug development are naturally acidic and it is not completely possible to separate the two processes (drug protonization and metal complex formation). Further, much of the work with liposomes containing metal solutions involves the use of metal ionophores which are used to facilitate drug loading. These reduce the amount of metal retained in the liposome and this, in turn, reduces the likelihood that drug-metal complexation can occur. In formulations where the drug has both a protonizable amine and metal binding motif (in the presence of ionophores); one can assume that the main driving force for loading is the pH gradient.

The Cu salts used to prepare liposomes thus far have been $CuSO_4$, $CuCl_2$ and Cu-Gluconate. These salts can be prepared as unbuffered solutions. For example, liposome can be manufactured in 300mM $CuSO_4$ (pH 3.5) [257, 331, 332]. The pH of copper solutions has been increased using appropriate buffers, but these are at best metastable solutions; capable of maintaining the copper in solution for only short periods of time (<1hr). Liposomes with encapsulated buffered Cu-gluconate have been described where triethanolamine (TEA) was used to increase the pH of the copper-gluconate solution to 7 [257]. Irinotecan can be encapsulated via remote loading in these copper gluconate-containing liposomes. However, in these formulations, the amount of TEA entrapped directly correlates to the amount of irinotecan encapsulation. In other words, encapsulated solutions of 90mM TEA would facilitate irinotecan loading to a drug-to-lipid ratio of approximately 0.09 after a 60 minute incubation and 270mM TEA would be able to produce liposomes with a final irinotecan-to-lipid ratio of 0.18 [257]. The copper gluconate concentration for these studies was 100mM and the external buffer used was a sucrose phosphate buffer (300mM sucrose, 40mM phosphate) at pH 7. For these studies there was no evidence to suggest that copper complexation facilitated irinotecan loading and it was proposed that loss of TEA from the inside of the liposome generated a transmembrane pH gradient (interior acidic) that was sufficient to engender irinotecan loading.

Topotecan and irinotecan have been encapsulated in liposomes through the use of entrapped copper in the presence or absence of A23187 [246, 286, 332-335]. It is

important to note that campothecins contain an α-hydroxy-δ-lactone ring moiety, a functionality that is unstable at pH greater than 7. The active form of the drug is believed to be the lactone ring closed structure which is stabilized in acidic pH and is essential for therapeutic activity [336, 337]. The hydrolysis of the lactone yields the carboxylate (inactive) form of the drug [338, 339]. Under conditions where the liposome interior is acidic (<5.0), the drug is stabilized in its lactone form [330, 333]. As indicated, irinotecan and topotecan can be encapsulated in liposomes using pH gradient-dependent methods (e.g. pH 4.0 citrate buffer, ammonium sulfate, $MnSO_4$ buffers with added A23187, etc) [16, 242, 332, 340, 341]. pH gradient loading was achievable because these drugs have protonizable amine functions. However, the chemical structure of irinotecan and topotecan suggests several coordination sites capable of complexing copper, with one of the sites potentially located at the lactone ring. Stabilization of topotecan in an active lactone form was previously attributed to the formation of a coordination complex with copper ions at this site [330, 333]. The sites of topotecan-copper complexation structure, however, have not been elucidated but it was confirmed that a complex is present using titration methods [330]. Interestingly, drug loading appears to be driven by two distinct mechanisms in the presence and absence of A23187 as reported by Taggar *et al*. [330]. In the absence of A23187, the loading occurred both at 40˚C and 60˚C and was driven by Cu-topotecan complexation. In contrast, in the presence of A23187, the loading occurred at higher temperature (60˚C) in response to transmembrane pH gradient. Efficient encapsulation in the presence of Cu^{2+} did not require utilization of A23187. However, when copper complexation was combined with transmembrane pH gradient, substantial improvement in the drug retention was reported [333].

The results summarized above suggest that there is something unique about copper and its potential to bind selected drug candidates, however the examples above have been limited to drugs that contain a protonizable amine functionality as well as copper binding motifs [13]. As another example, mitoxantrone was encapsulated into Cu^{2+}-containing liposomes unbuffered or buffered to pH 7.5 with TEA [342]. Both $CuSO_4$ and $CuCl_2$ resulted in complete drug encapsulation. *Li et al.* attempted to account for drug loading mechanism by using $CuSO_4$ solutions pH adjusted with TEA. They concluded, similar to the research described above, that TEA is membrane permeable and the formulation likely behaved

similarly to $(NH_4)_2SO_4$ remote loading methods where loss of TEA from the liposomes created a pH gradient that facilitates loading. They attempted to use nigericin, an ionophore which exchanges H^+ from the interior of the liposome in exchange for potassium, and showed that mitoxantrone could be encapsulated into $CuSO_4$ liposomes in the presence of nigericin which would suggest encapsulation in a pH gradient independent manner. The $CuCl_2$ containing liposomes could not be used to stably encapsulate mitoxantrone under these conditions.

To clearly highlight the utility of copper-complexation as a method of candidate drug remote loading into liposomes the focus has shifted to compounds that do not have protonizable amine function but do have donor moieties (O, N and S) that can bind copper. This thesis reports that CBTs can be synthesized in liposomes;[66] a strategy that was defined as the Metaplex™ technology (Figure 7.1). This approach eliminates the use of solvents, addresses solubility issues and results in formulations of CBTs (CLCs and CDCs) that are suitable for parenteral administration. The method utilizes the liposome as a nanoscale reaction vessel in which the copper complex is synthesized. Briefly, liposomes are prepared using extrusion in the presence of copper containing solutions and the external copper is removed using column chromatography.[66, 276] The prepared liposomes with copper encapsulated in the core are mixed with the ligand of interest and complexation can be observed in the form of colour changes and spectrophotometric shifts in absorbance. The resulting CBTs remains in suspension and the external buffer in which the liposomes are suspended can be exchanged to one that is suitable for systemic administration.

CBTs represent a class of compounds with potential activity in a wide range of diseases is promising despite the pharmaceutical challenges presented by the inherent solubility issues of these compounds. There is a wide selection of CBTs that have been created as potential drug candidates. This thesis described the Metaplex™ methodology which provides a simple, transformative solution, enabling for the first time, the development of CBTs as viable candidate drugs; drugs that would represent a new class of therapeutics for patients.

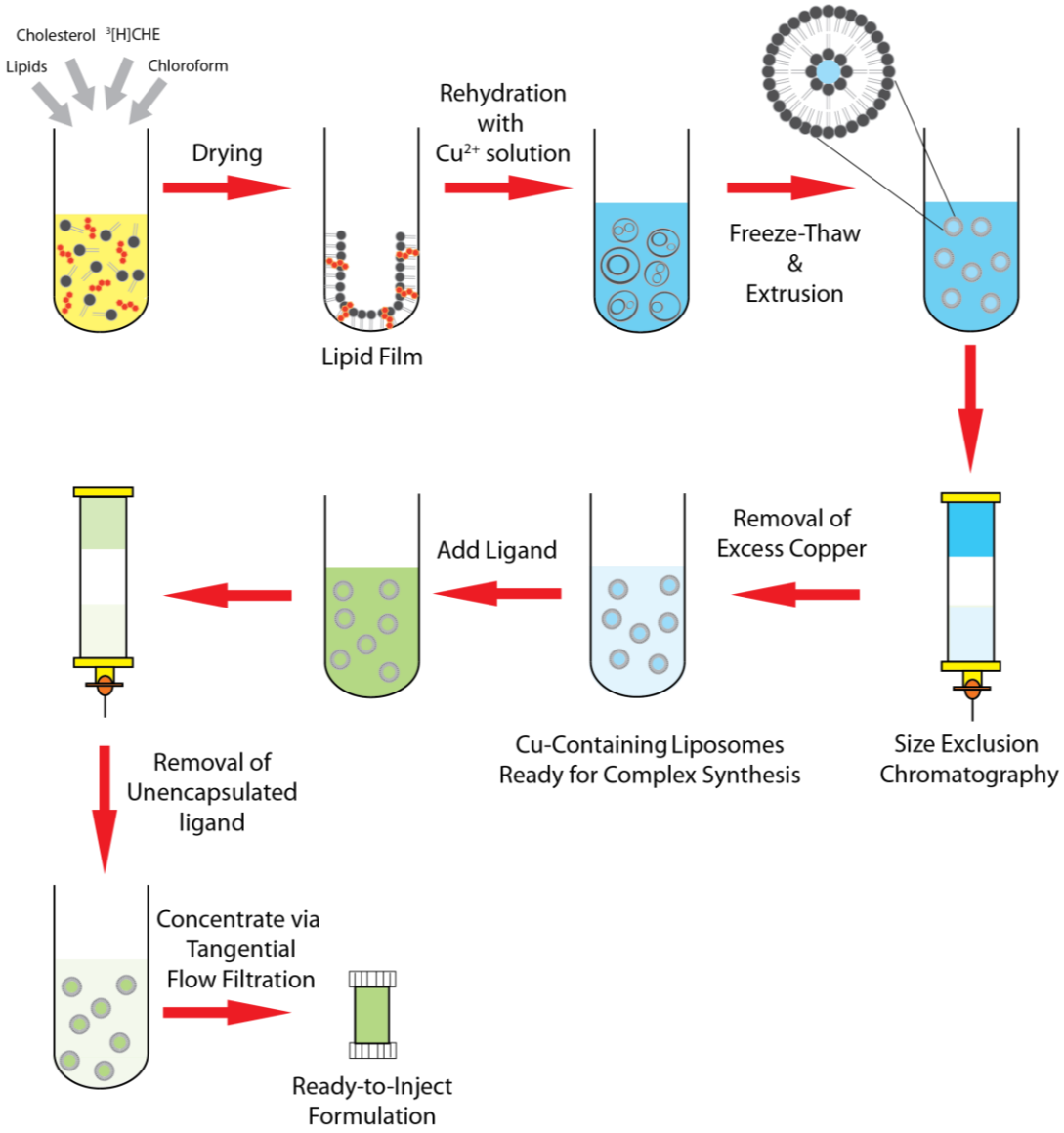


Figure 7. 1: Schematic representation of Metaplex™. The lipid composition of interest is dissolved into chloroform and this is then dried using a rotary evaporator. The lipid film is rehydrated using a Cu containing solution. The resulting multilamellar vesicles (MLVs) undergo five freeze-thaw cycles which is followed by extrusion using 100 nm filters. Size exclusion chromatography was performed using Sucrose/HEPES/EDTA (SHE, 300, 20 and 15 mM) pH 7.4 buffer to remove external unencapsulated copper. The resulting Cu containing liposomes are mixed with the ligand of interest and the unencapsulated ligand was removed using size exclusion chromatography. The liposomes containing the copper complex were concentrated using tangential flow dialysis.

The difference between remote loading with metals (absence of ionophores) and Metaplex™ are three-fold. First, the candidate ligands/drugs do not contain a protonizible

amine functionality and thus cannot be loaded using traditional remote loading method that rely on use of a transmembrane pH gradient. As seen in Figure 3.2C, the removal of the proton gradient did not affect the formation of $Cu(DDC)_2$ inside the DSPC/Chol (55:45) liposomes. Second, there is a subset of CBTs that have been described in Chapter 1, known as CLCs, which have copper dependant activity. Thus, the activity of these ligands requires copper complex formation for activity and this is in contrast to other drugs where the metal was used solely to facilitate encapsulation of the added drug/candidate drug. Third, remote loading has been used on hydrophilic drug candidates; this was later adapted to hydrophobic drugs through dissolution of the drug candidate using solvents such as DMSO [343].The approach has been used specifically in the formulation and development of hydrophobic copper complexes with both hydrophilic ligands (DDC, CX-5461) and hydrophobic ligands (CQ, Qu). While the dissolution of the ligands in a solvent was unnecessary, it could be done to reduce the incubation time during the complexation process. One would need to consider solvents such as DMSO as competitive binders of copper and this could affect complex synthesis.

7.4 Future Work

Owing to the versatility of the platform technology many other possible research projects can be developed around this formulation approach. Here, I will briefly discuss possible the applications which I find most compelling.

7.4.1 Other Metals

While Cu(II) is known to coordinate with N,O and S containing ligands, other metals could be used as alternatives. Specifically, the potential to use Zn(II) as an alternative metal is considerable. Zn complexes may exhibit different formulation attributes (stability, release rates, etc), but more interestingly the activity of the Zn-ligand complex may be different from the Cu-ligand complex. This is illustrated by the data shown in Figure 7.2 and 7.3. The use of Zn(II) did not increase the activity of DDC when compared to $Cu(DDC)_2$ in platinum sensitive and platinum resistant cancer cells (Fig. 7.2), however the activity of $Zn(CQ)_2$ complexes was 1 to 2 orders of magnitude greater then when compared to the

$Cu(CQ)_2$ complexes (Figure 7.3). This is likely related to the specific mechanism of action of each complex formed. The use of Zn or other divalent metals such as Fe, Co, Ni, etc. can be incorporated into liposomes through the addition of unbuffered solutions of these metals and it is reasonable to anticipate that the formation of other divalent metal ion complexes would be comparable to that achieved when using copper loaded liposomes.

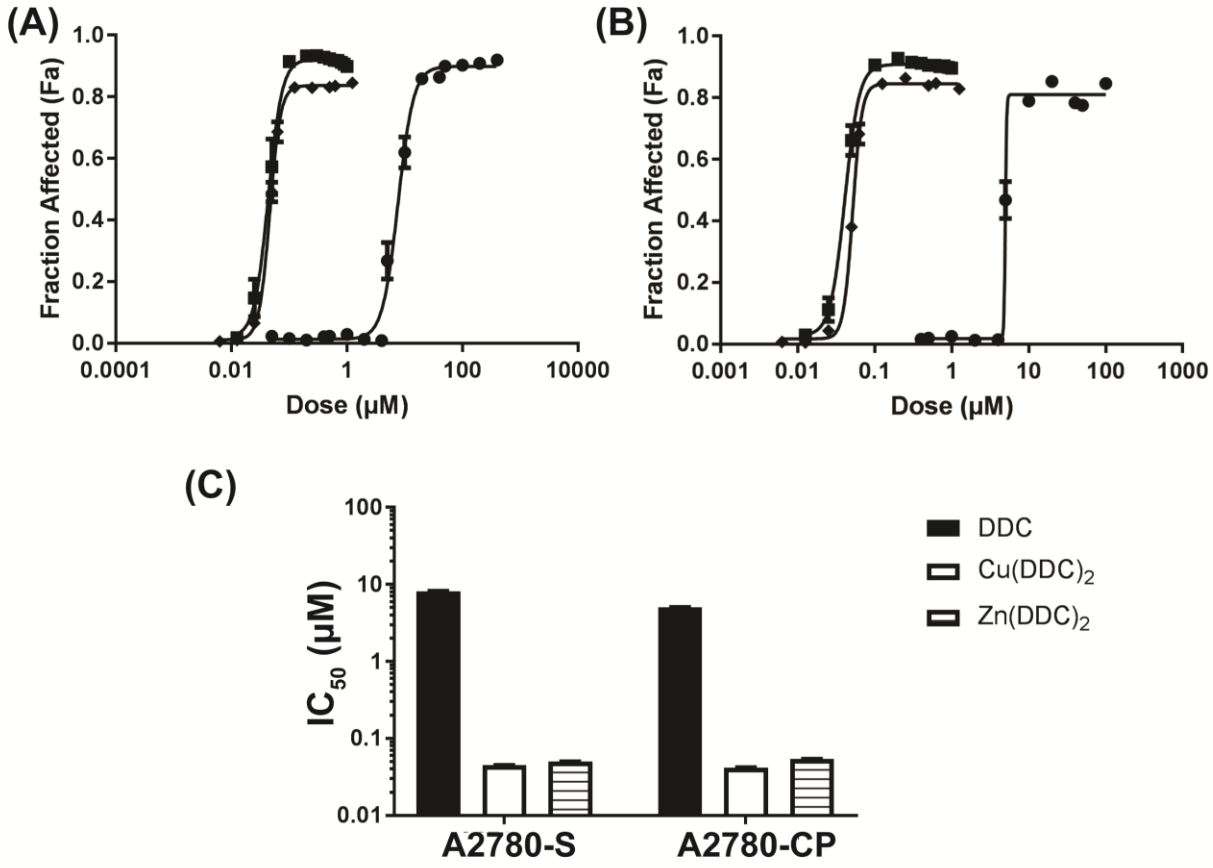


Figure 7. 2: The cytotoxicity of DDC (●), $Cu(DDC)_2$ (■) and $Zn(DDC)_2$(♦) in A2780-S (A) and A2780-CP (B) cells. Cytotoxicity curves were generated in A2780-S and A2780-CP cells after 72 hr treatment with DDC, $Cu(DDC)_2$ or $Zn(DDC)_2$. Cell viability was obtained using the INCell analyzer 2200 where viability was assessed based on loss of plasma membrane integrity 72 hours following treatment; i.e. total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively. (C) IC_{50} ± 95% CI were calculated from cytotoxicity curves generated for DDC, $Cu(DDC)_2$ and $Zn(DDC)_2$ in A2780-S and A2780-CP after 72 hr treatment using Graphpad Prism software. Data is presented as the mean of 3 independent experiments ± SEM

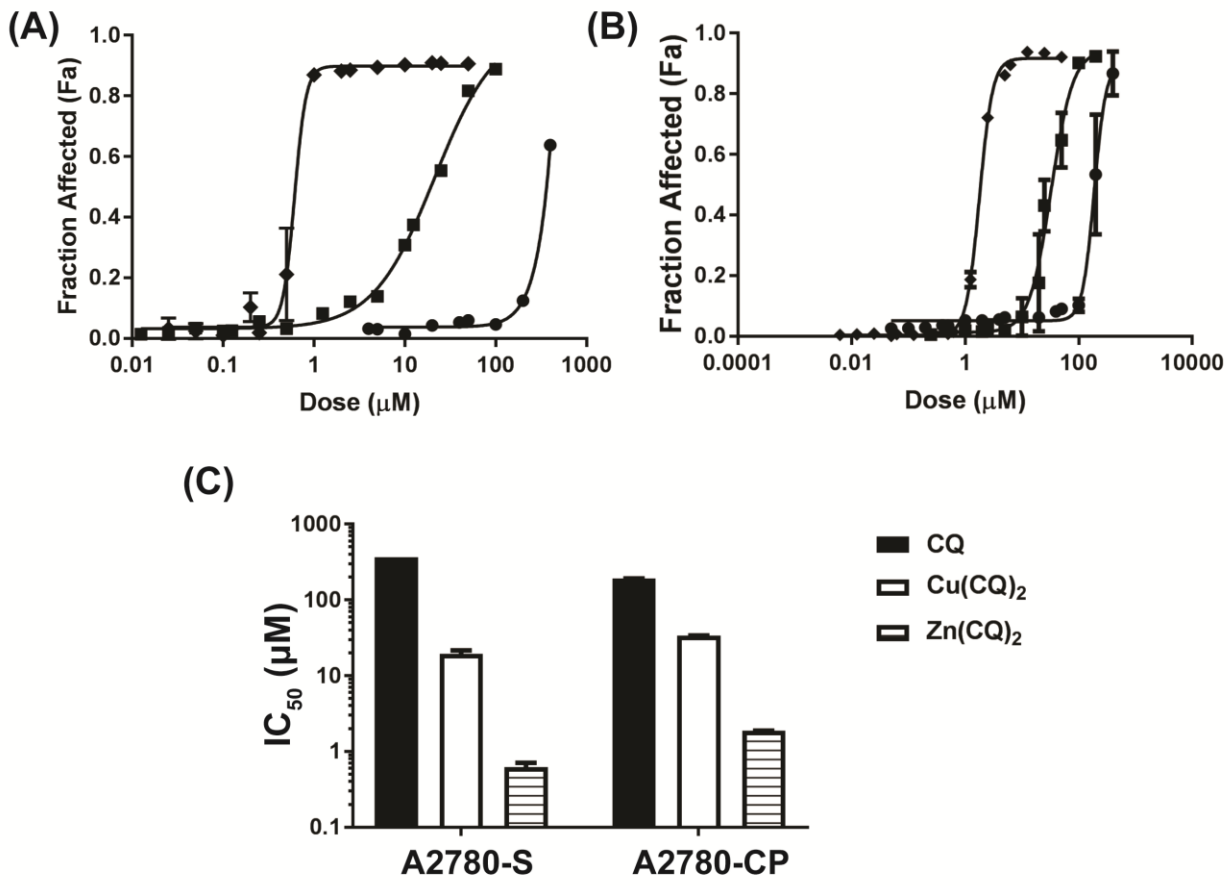


Figure 7. 3: The cytotoxicity of CQ (●), $Cu(CQ)_2$ (■) and $Zn(CQ)_2$(♦) in A2780-S (A) and A2780-CP (B) cells. Cytotoxicity curves were generated in A2780-S and A2780-CP cells after 72 hr treatment with CQ, $Cu(CQ)_2$ or $Zn(CQ)_2$. Cell viability was obtained using the INCell analyzer 2200 where viability was assessed based on loss of plasma membrane integrity 72 hours following treatment; i.e. total cell count and dead cell count were determined using Hoechst 33342 and ethidium homodimer staining, respectively. (C) IC_{50} ± 95% CI were calculated from cytotoxicity curves generated for CQ, $Cu(CQ)_2$ and $Zn(CQ)_2$ in A2780-S and A2780-CP after 72 hr treatment using Graphpad Prism software. Data is presented as the mean of 3 independent experiments ± SEM

7.4.2 Other Diseases and Complexes

Although the focus of this thesis was on copper-based anti-cancer agents, copper complexes with significant anti-microbial, anti-viral and anti-inflammatory activity have also been identified. Although the pharmaceutical development of metal complexes targeting these indications may not require development of injectable solutions (i.e. topical anti-bacterial agents, oral anti-inflammatory drugs) many will be sparingly soluble and formulations could be adapted for these agents.

Additionally, this thesis has focused on bidentate ligands which form dimeric structures which are the simplest case. The method could be altered to develop other types of copper complexes such as NCI-109268 which is a ligand that forms a dichloro-copper complex [39]. The copper complex of NCI-109268 appears to act as a proteasome inhibitor, similar to $Cu(DDC)_2$ and $Cu(CQ)_2$. For a compound like NCI-109268 (Figure 7.3), the liposomes would need to be prepared in $CuCl_2$ solutions instead of $CuSO_4$ solutions and the ligand would be introduced to pre-formed $CuCl_2$ containing liposomes. This is illustrated in Figure 7.4.

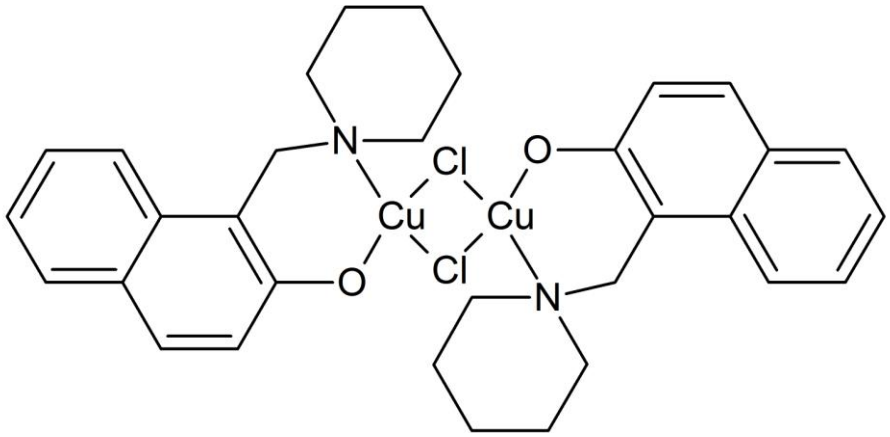


Figure 7. 4: Structures of the copper based proteosome inhibitor NCI-109268

7.5 Conclusion

This thesis research was initiated based on an idea related to repurposing DSF for use in cancer. However, after recognizing that the active agent following administration of DSF was $Cu(DDC)_2$ the focus switched to the development of a formulation of $Cu(DDC)_2$ suitable for intravenous administration. I hypothesized that $Cu(DDC)_2$ could be synthesized inside liposomes and that the resulting formulations would be suitable for further pharmaceutical development as a novel class of anti-cancer agent. I have demonstrated that when DDC is added to copper containing liposomes that $Cu(DDC)_2$ formed inside the liposomes. Perhaps more importantly, I have shown that this approach, where CBTs can be synthesized inside liposomes can be applied to other copper binding ligands, thus defining a novel platform approach from which other CBTs or MBTs could be developed. Of course, addressing the formulation concerns for CBTs represents only one of

many hurdles that must be crossed if this novel drug class will be therapeutically successful. More information about mechanism of action and potential for off target effects will need to be obtained for each candidate CBT being developed. Further the safety profile of candidate CBTs will need to be fully understood. For example, the literature surrounding copper in medicine has to date focused on the use of chelators to correct copper dysregulation but as candidate CBTs are identified it will be necessary to examine their effects on copper homeostasis and associated issues. Regardless there is a wide selection of CBTs that have been created as potential drug candidates and the Metaplex™ methodology provides a simple, transformative solution, enabling for the first time, the development of CBTs as viable candidate drugs; drugs that would represent a new class of therapeutics for patients.

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