Modulatory effects of vitamin B_{12} on doxorubicin accumulation and sensitivity in human HepG2 cells

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Abstract

The most complex naturally occurring molecule, vitamin B_{12} or cobalamin (Cbl), is promising in the treatment of various cancers. Previous research has shown that cobalamin is involved in the downregulation of multi-drug resistance protein 1 (MDR-1), a gene encoding the efflux transport protein P-glycoprotein (P-gp), a transporter heavily implicated in the phenomenon of multi-drug resistance.

As such we initially investigated the time dependence of Cbl on the functionality on another efflux transport protein, multidrug resistance protein 2 (MRP-2) by measuring its effects on cellular accumulation of 5(6)-carboxy-2´,7´dichlorofluorescein (CDCF), a well-defined fluorescent substrate of MRP-2. We then tested the effects of different Cbl concentrations on the efficacy of oxaliplatin, a prominent platinum based anti-cancer drug, as well on accumulation and efficacy of doxorubicin, an anthracycline, in a HepG2 cancer cell line. We hypothesised that the addition of Cbl would lead to the increased accumulation of CDCF, and that increasing concentrations of Cbl would lead to consequent increases in the cytotoxic effects of both oxaliplatin and doxorubicin as well as accumulation of doxorubicin.

To assess CDCF accumulation, cells were pre-treated with 100nM cobalamin for 24, 48 and 72 hours, then tested using cellular accumulation protocols using a MoFlo XDP flow cytometer. The mean cellular accumulation of CDCF is significantly increased by 136% and 72% in HepG2 cells pre-treated with Cbl 100 nM for 24 and 48 hr, respectively; however, pretreatment with Cbl for 72 hr does not result in a statistically significant increase (by 27%) of CDCF accumulation in HepG2 cells.

For testing the effects of Cbl on oxaliplatin cytotoxicity, cells pre-treated with Cbl concentrations of 200, 100 and 50 nM were assessed for oxaliplatin-induced cytotoxicity using MTT cell viability assays. Our results suggest IC_{50} values of cells treated with 100 nM

and 200 nM to be approximately half of those of the control values, but there are no statistically significant differences between control and treated cells.

HepG2 cells pre-treated with 1000, 500 and 100 nM Cbl were incubated with doxorubicin for 2 hours and analysed using a MoFlo XDP flow cytometer. Interesting results were obtained, where 500 nM but not 1,000 nM Cbl induced the greatest doxorubicin accumulation, suggesting a bell-shaped dose response curve. Doxorubicin cytotoxicity assays were carried out using the same concentrations as in the doxorubicin accumulation studies, but instead treated directly in 96-well plates rather than pre-treated. The effects of cobalamin on cytotoxicity of doxorubicin was then tested using MTT cell viability assays. This yielded results consistent with the doxorubicin accumulation studies, where 500 nM Cbl caused the greatest increase in cytotoxicity from doxorubicin, exhibiting a significantly lower IC_{50} value than for other Cbl treatment concentrations.

The effects on CDCF accumulation and oxaliplatin cytotoxicity may be attributed to indirectly to Cbl, as Cbl involvement in human methionine synthase activity is associated with the production of folate, a molecule which effluxes via MRP-2, thus showing the potential of cobalamin to produce a molecule which acts as a competitive inhibitor of MRP-2 in the efflux of oxaliplatin.

The increases in the accumulation and cytotoxicity of doxorubicin in Cbl treated cells may be largely attributed to the down-regulation of MDR-1 and/or ABCC2, and thus P-gp/MRP-2 expression. However, the unusual bell-shaped dose response curve of Cbl may arise from the methionine dependency in cancer cells, where the anti-cancer effects of Cbl could be outweighed by the pro-cancer effects of increased methionine induced by Cbl at an extremely high concentration.

In conclusion, the results presented in this thesis may implicate the concentration of cobalamin as a major determining factor in its potential applicability in a clinical setting. As

such further research may be warranted to more thoroughly investigate the effectiveness of cobalamin as an anti-cancer agent as well as the potential mechanisms behind both its anti-cancer and pro-cancer effects.

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed

Name Jae Heon Kim

Date 14/03/19

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

1.1 HEPATOCELLULAR CARCINOMA (HCC)

Currently liver cancer, including hepatocellular carcinoma (HCC, accounting for 70-85% of liver cancer) and intrahepatic cholangiocarcinoma, is the sixth most common form of cancer, while being the second highest cause of cancer mortality [1]. These cancers are classified into two main groups, primary originating within the liver and secondary liver being a result of metastases from cancers from other areas of the body.

Of primary liver cancers, hepatocellular carcinoma (HCC) is the most prevalent kind, accounting for approximately 80% of all primary liver cancers [2]. HCC itself currently causes the third highest numbers cancer mortality worldwide and results in over 500,000 deaths per year [2].

The most prominent risk factors for HCC are chronic infection with hepatitis B (HBV), hepatitis c (HCV), alcohol abuse and obesity [3]. HBV is the greatest risk factor for HCC, accounting for 52% of HCC cases. Infection with HBV is found in ~350 million people worldwide [4]. In European carriers HCC is mostly in patients with cirrhosis, however in Asian carriers, those without cirrhotic livers are also at high risk of liver cancer [4].

In HCV infected patients, the occurrence of HCC is of highest risk in those with cirrhotic livers [5], with below 5% of HCC cases due to HCV being from non-cirrhotic patients [5,6].

Alcohol abuse is also a very common cause of HCC, as alcohol abuse may cause a cirrhotic liver which leads to HCC [3].

In the treatment of HCC, surgical intervention is the gold standard of treatment, providing a 5 year survivability of ~75% [7], with resection of the liver or a complete transplantation [7]. However, patients who have been found with HCC are often only detected in the advanced

stages and thus less than 20% are eligible for surgical interventions and as such must rely on chemotherapy for treatment [8].

Treatment of advanced HCC using chemotherapy has not yielded very promising results with interventions such as doxorubicin having overwhelming deleterious side effects and oxaliplatin only showing promise in treatment. As such currently the first-line treatment for advanced HCC is sorafenib, a small molecule inhibitor, and even so sorafenib is not beneficial to all patient. For those who it is, sorafenib acts more as a palliative care drug rather than a curative treatment [9]. As such, currently the prognosis for advanced HCC remains dismal.

1.2 CURRENT CHEMOTHERAPY DRUGS USED FOR LIVER CANCER

Although various pharmacological interventions have been investigated, the lack of standard chemotherapy for hepatocellular carcinoma is a significant hurdle to cure hepatocellular carcinoma. Currently doxorubicin, oxaliplatin and sorafenib are used in clinic; however, the highly chemo-resistant nature of the tumour makes hepatocellular carcinoma still a refractory disease.

1.2.1 Doxorubicin

Doxorubicin, previously known as Adriamycin, an anthracycline antibiotic [10], is a popular agent of treatment in various cancers including liver cancers [10]. An initial trial achieved a 79% response rate of hepatocellular carcinoma to doxorubicin. Though doxorubicin has been a mainstay of cancer treatment, there are issues as detrimental side effects accompany dosing and cells have both inherent and acquired resistances to the drug [10]. A major is the cardiotoxicity exhibited by the drug treatment [11], where with extended treatments the cardiac function of patients will decline, eventually to an unmanageable point.

By understanding mechanisms of action, it may be possible to avoid, or mitigate

cardiotoxicity and resistance. Previous research suggests the anti-cancer mechanism of doxorubicin is different to the mechanism of cardiotoxicity [11]. Hence, it may be possible to avoid doxorubicin associated cardiotoxicity, while maintaining anti-cancer effects, by altering the chemical make-up of doxorubicin. Thus, leading to a more effective drug-treatment, which can be administered at higher dosages.

Conversely, the use of other interventions alongside doxorubicin, to either avoid cardiotoxicity or increase the anti-cancer efficacy, may be a means of increasing the survivability of those being treated with doxorubicin.

1.2.1.1 Mechanism of anti-cancer action

The anti-cancer effects of doxorubicin are due to damage to DNA and are attributed to a variety of mechanisms. Of these mechanisms, two are largely accepted; one is due to the formation of DNA adducts increasing torsional stress on DNA and the reduction of activity in the enzyme topoisomerase IIα enzyme (TOP2A)[12], the other is production of free radicals causing damage to DNA, lipid membranes and various proteins [13].

TOP2A is an enzyme which unravels strands of entangled DNA creating and repairing protein-bound double-strand DNA break (DSB)[14]. Doxorubicin acts to stabilise the breaks in the DNA, effectively trapping TOP2A, and thus cause there to be increases in the formation of protein-bound DSBs [13]. These DSBs have a myriad of adverse effects for cells, causing increases in the activation of caspase-dependant apoptotic pathways by up-regulating pro-apoptoic and down-regulating anti-apoptotic Bcl-2 family proteins. As such the anti-cancer effects of doxorubicin are largely attributed to the blocking of this single downstream response of the TOP2A repairing cleaved DNA strands [10].

The other well-defined form of cytotoxicity caused by doxorubicin is due the production free radicals which cause damage to membranes, proteins and DNA [15]. This occurs as

doxorubicin is oxidized into an unstable form, a semiquinone, which is subsequently reduced back into doxorubicin, and a reactive oxygen species (ROS) is generated [15]. The release of ROS is important as ROS are free radical molecules which cause the peroxidation of the lipid membrane, increases in oxidative stress and DNA damage in cell lines, and thus initiate apoptotic pathways [15]. However, due to the reliance of cancer cells on aerobic glycolysis rather than oxidative substrate metabolism in the mitochondria, the levels of doxorubicin required to cause significant cytotoxicity is very high, thus causing this pathway to be less important due to cardiotoxicity from the administration high concentrations of doxorubicin.

1.2.1.2 Cardiotoxicity

Cardiotoxicity resulting from chemotherapy is characterized into 2 types. Type 1 is cardiotoxicity caused by the death of cardiomyocytes and type 2 is caused by the dysfunction of the cardiomyocytes leading reduced cardiac function [11]. The cardiotoxicity resulting from doxorubicin usage can be attributed to type 1 cardiotoxicity as the continued exposure to doxorubicin leads the cardiomyocyte death rather than dysfunction [11]

The extent cardiotoxicity is directly linked to the total cumulative dosing of doxorubicin[11]. As such, cardiotoxicity becomes a major limiting factor in cancer treatment using doxorubicin, especially if cells acquire resistance and require higher doses of the drug.

The understanding of the mechanism of doxorubicin-induced cardio-toxicity is currently incomplete. The prevailing theory suggests that oxidative stress caused by free radicals is the cause of the cardiotoxicity [16].

Specifically, the reduction of doxorubicin in cardiomyocytes, catalysed by NADH dehydrogenase found in the mitochondrial respiratory complex I, causes the release of a semiquinone radical which reacts with oxygen molecules to form a superoxide radical and the superoxide radical is then involved in redox cycling, producing the potent ROS hydroxyl

radical and hydrogen peroxide[17].

There may also be another pathway by which hydroxyl radicals are formed when cells are exposed to doxorubicin, where the reaction of doxorubicin with iron forms oxdorubicin-iron complexes that may catalyse a Fenton reaction where the reduction of hydrogen peroxide is produces a hydroxyl radical [18], increasing levels of ROS in cardiomyocytes. Cardiomyocytes are suggested to be much more sensitive to the oxidative stress caused by increases in ROS levels than in cancer cells, due to the reliance of cardiomyocyte on oxidative substrate metabolism, while cancer cells rely heavily on aerobic glycolysis [16]

1.2.1.3 Resistance in Cancer cells

The resistance of cells to doxorubicin is an interesting area of research. The presence of doxorubicin resistance causes significant decreases in treatment outcomes. Given that doxorubicin treatment is limited by cardiotoxicity, the phenomenon of resistance is especially pertinent in improving treatment outcomes, as increasing the dosage of doxorubicin is not a viable option.

The most important mechanism of resistance towards doxorubicin is the upregulation of efflux transport proteins, with the up-regulation of the MDR-1 gene and the ABCC-1 gene encoding for the drug transporters P-glycoprotein (P-gp) and Multidrug resistance protein-1 (MRP-1) respectively [19]. Both proteins are heavily involved in the removal of doxorubicin from cancer cells, and as such their presence causes reduced accumulation and therefore a reduction in the desired cytotoxicity. Evidence implicating MDR-1 in HCC in doxorubicin resistance is shown by the action of P-gp inhibitors such as verapamil, which have been to restore sensitivity of HCC to doxorubicin in resistant cell lines [19] In addition, MRP-2 (encoded by the ABCC2 gene) is overexpressed in HepG2 cells and doxorubicin has been shown to be a MRP-2 substrate.

In HCC cells another mechanism may be the increased expression of TOP2A, where higher levels of expression cause mutations in the TOP2A enzyme, which directly prevents the doxorubicin induced TOP2A poisoning [10]. Alternatively, cancer cells having elevated levels of TOP2A causes downstream apoptotic pathways to be suppressed, due to decreased DSB. As such TOP2A can act to repair doxorubicin-induced DSB damage [10].

Furthermore, in sensitive cells the addition of doxorubicin causes the induction of apoptotic pathways, where mitochondria release cytochrome c leading to caspase activation and cell death. However, in resistant cells, compared with sensitive cells, increases in anti-apoptotic proteins and decreases in the effectors of apoptosis leading to reduced apoptosis and therefore a reduction in cell death [10].

Of these mechanisms the focus of this thesis is the resistance conferred by transmembrane efflux transporter proteins that reduce the intracellular accumulation of doxorubicin.

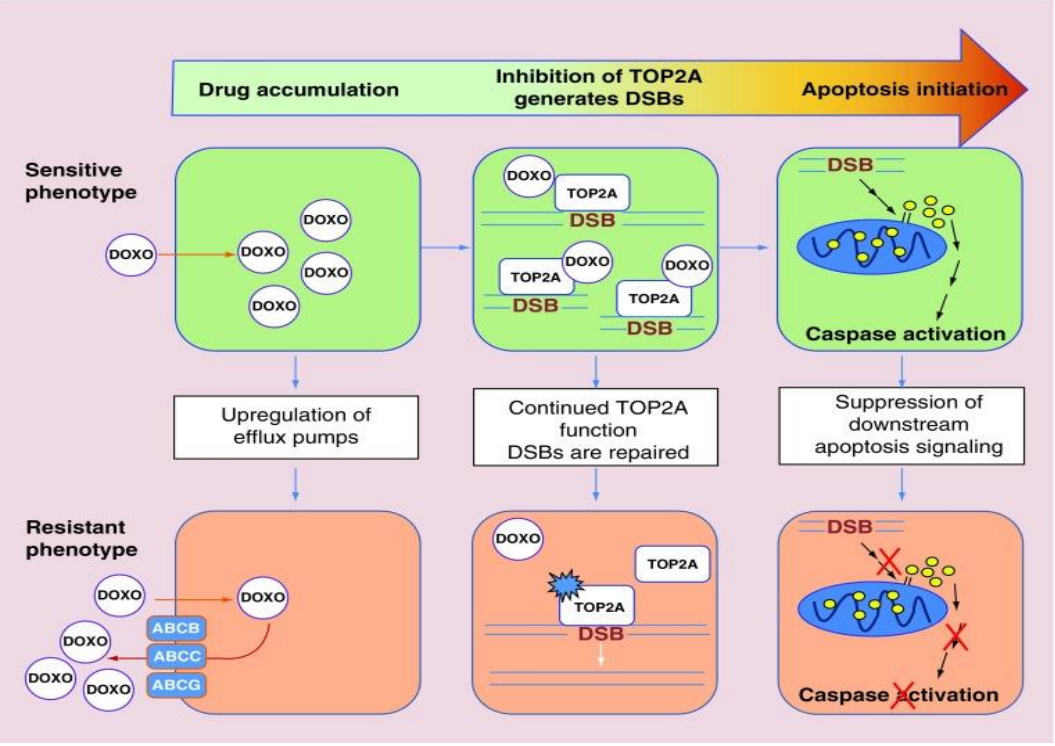

Figure 1.1 Diagram representing the 3 mechanisms of Doxorubicin resistance in cancer cells [10]

1.2.2 Platinum based anti-cancer drugs

Platinum based anti-cancer drugs are a mainstay of cancer chemotherapy, encompassing drugs such as cisplatin, carboplatin and oxaliplatin [20].

The general mechanism amongst these drugs is the formation of platinum-DNA adducts in the nucleus of cancer cells, where they cause the activation of apoptotic pathways.

Amongst the platinum based anti-cancer drugs, the most prominent in a clinical setting is the first-generation drug, cisplatin, a drug which has been used in a range of cancer treatments [20]. Though cisplatin is a widely administered drug, the deleterious side effects, which include neurotoxicity, nephrotoxicity and emeogenesis, cause the effectiveness to be stunted [21] Amongst the side-effects the most significant is neurotoxicity, is the main dose-limiting factor as the others can be managed via hydration and administration [21].

The issues with neurotoxicity ultimately lead to patients having hearing loss, tinnitus and peripheral neuropathy, and as such becomes unmanageable as doses increase [21].

Furthermore, in various cancer cells there is an intrinsic resistance to cisplatin and further acquired resistance when the drug is administered, leading to cisplatin losing efficacy over time and causing tumours to re-establish themselves in patients. As such, to overcome the issues facing cisplatin use, a multitude of different other platinum-based drugs have been synthesized and tested for anti-cancer effects. Amongst these drugs the only other platinum-based drug approved by the FDA is oxaliplatin, a third-generation platinum-based anti-cancer drug.

1.2.2.1 Oxaliplatin

Oxaliplatin shows similar yet differing anti-cancer activity to that of its predecessors cisplatin and carboplatin, avoiding the resistance that cancer cells acquire towards these specific drugs [22]. This is due to oxaliplatin having a different leaving group (oxalate) and carrier ligand

(1,2 diaminocyclohexane (DACH)) [23].

For patients suffering from hepatocellular carcinoma, oxaliplatin is used in combination with a variety of other anti-cancer drugs. For example, the combination of gemcitabine with oxaliplatin (GEMOX)[24], 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX4)[25] and Capecitabine with Oxaliplatin (XELOX) [26] are all prominent drug combinations involving oxaliplatin used in HCC treatments.

1.2.2.1.1 Mechanism of action

The mechanisms of action of oxaliplatin are similar to those found in its predecessor cisplatin and carboplatin [22]. As such most of the cytotoxic effects are exhibited through damage to DNA within cancer cells, where DNA damage causes cell death via apoptosis.

Apoptosis occurs since oxaliplatin-induced cell death is most likely caused by DNA lesions, inhibition of RNA and DNA synthesis and immunological reactions [23].

The DNA lesions through Oxaliplatin exposure occur because of DACH platinum compounds entering cancer cell nuclei, where the compounds bind to the N7 of guanine, causing the formation of DNA monoadducts and bi-adducts [27].

Though monoadducts have negligible apoptosis inducing effects, the bi-adducts cause the inhibition of DNA replication and transcription, thus the cessation of the cell cycle leading to the eventual stimulation of apoptotic pathways [27].

Though the cytotoxic effects of oxaliplatin have been proven to be greater than cisplatin, the formation of these adducts in cisplatin have been shown to be greater. The quantity of these adducts is therefore not the determining factor in the efficacy of oxaliplatin in treating colorectal cancers [27]. As such, it is evident that oxaliplatin holds unique properties in DNA lesions.

The difference in mechanism of action, of oxaliplatin compared with its predecessors, largely occurs to due to the adducts reducing binding with mismatch repair protein complex A. As such the reversibility of the lesions decreases leading to greater apoptotic effects in cancer cells [27]. Furthermore, the DACH ligand is suggested to be more effective in stopping of DNA synthesis compared with the other ligands of platinum complexes [27].

1.2.2.1.2 Resistance in cancer cells

Resistance in cancer cells to oxaliplatin is not yet completely understood, due to the complexity of resistance. [23]. There are numerous factors that play a role in the resistance of cancer cells to oxaliplatin. These include decreases in the uptake of the drug, increases in efflux of the drug, changes in membrane permeability and a reduction of cytotoxicity of oxaliplatin due to its reaction with intracellular glutathione. [23].

The accumulation of oxaliplatin in cancer cells is strongly dependent on the uptake and efflux transporters, and these transporters are heavily implicated in the phenomenon of decreasing sensitivity of cancer cells towards oxaliplatin. The down regulation of uptake transporters of oxaliplatin, copper influx transporter (CTR1) and organic cation transporters (OCTs) [28] may lead to the decreased accumulation initially, while the various efflux transporters, such as the ATP-binding cassette proteins, Copper efflux transporters and multidrug and toxin extrusion transporters, result in the inability of the cell to maintain high levels of oxaliplatin, thus causing the removal/reduced formation of platinum-DNA adducts, ultimately leading to reduced levels of apoptosis.

1.2.3 Sorafenib

During the past 30 years there have been no new especially promising treatment options for advanced HCC, apart from Sorafenib. Sorafenib shows increases in life span by

approximately 5 months [29], a result unmatched in over 100 randomised clinical trials of other new treatments. As such, Sorafenib, though typically used as a palliative care drug, has become the standard for treatment in advanced HCC and remains an important area of research in the scientific community for furthering the possible increases in survivability.

Sorafenib is a multikinase inhibitor, which can be administered orally, and acts to inhibit various tyrosine kinase receptors on cell surfaces and intracellular Raf serine/threonine kinases [29]. By inhibiting various tyrosine kinases such as vascular endothelial growth factors 1,2 and 3 and intracellular Raf kinases, this results in reductions in the proliferation and angiogenesis of tumour cells [29]. As Sorafenib reduces the rate of proliferation in cancer cells, combination therapy is especially useful in advanced hepatocellular carcinoma patients [29]. Various trials have been held with sorafenib in conjunction with prominent anti-cancer drugs such as oxaliplatin and doxorubicin, in which there has been no conjunctive anti-cancer effects [29]. Sorafenib has been reported to be a substrate of MRP-2, which is overexpressed in HCC cells and tissues.

1.3 MULTI-DRUG RESISTANCE IN CANCER

There are two main mechanisms behind resistance to chemotherapeutic agents: disrupting the delivery and accumulation of the anticancer drugs in cancer cells, and mechanisms that are associated with genomic and somatic mutations that cause changes in the cells themselves [30].

With respect to reduced availability by cancer cells resistance arises from factors such as increased levels of drug metabolism and excretion, as well as the poor absorption of orally administered anti-cancer drugs [23]. These factors result in lower concentrations of the drugs in the vasculature. Recent studies have focused on the vasculature of tumour masses, to

ensure the correct concentration/pressure gradients for drugs to diffuse passively into cancer cells [30].

The cellular mechanisms of resistance have been studied much more extensively due to the relative of these experiments. There are two forms of cancer cell drug resistance, intrinsic and acquired resistance. Intrinsic resistance refers to the resistance of cancer cells which is pre-existing, while acquired resistance is resistance acquired after having been exposed to chemotherapeutic agents [30]. The development of acquired resistance is attributed to the plasticity of the cancer cell genome and somatic mutations. This explains the heterogeneity which is exhibited by cancer cells, with large variations even amongst cells of the same cancer type [31].

A further development of acquired resistance is multi-drug resistance (MDR), a phenomenon by which cancer cells become resistant to a chemotherapeutic drug, and followed by resistance to several other chemotherapeutic drugs regardless of their mechanisms of action and structure [23, 32] .

In a clinical setting MDR becomes especially problematic when the myriad of accompanying side effects associated with various chemotherapeutic agents begin to overwhelm patients. At higher doses many drugs have significant anti-cancer effects, though such high doses become too toxic to administer to human patients.

1.4 ABC TRANSPORTERS

One of the main mechanisms that is clinically pertinent in the development of MDR in cancer patients is increased expression of various efflux transport proteins in the membranes of cancer cells, leading to the decreased accumulation of anti-cancer drugs and their analogues [32]. Furthermore, these transport proteins may not only cause efflux of cancer drugs but could also interfere in the targeting of specialised compartments within the cells, such as the

nucleus [32].

These transporter proteins are largely made up of the ATP-binding cassette (ABC) transporter family, a superfamily of transmembrane proteins that use active transport, through ATP hydrolysis, to move substrates across membranes [34]. Currently, ABC transporters have been characterized into seven sub-families, of which various proteins have been proven confer drug resistance in cancer cell lines. As such, these transporters are of considerable interest in the development of more successful cancer treatment outcomes [33]

1.4.1 P-glycoprotein

The MDR-1 gene transcribes the protein P-glycoprotein (P-gp), an ABC transporter gene that is important in cellular homeostasis, whether that be in cancer cells or healthy cells. P-gp is a large protein at 178kD containing 1280 amino acids [35], in two homologous halves which each contain a nucleotide-binding domain (NBD) and six transmembrane domains [34]. These two nucleotide binding domains, which are lined with charged residues, are found in the cytoplasm of cells with a pore on the extracellular half lined with hydrophobic amino acids. At the lipid bilayer there are two extraction sites in the protein, where substrate efflux from the cell.

1.4.1.1 Physiological role

P-gp a polarized transmembrane protein found in cells of various tissues and organs, is involved in various functions throughout the body ranging from protecting the cell and excreting toxins and drugs preventing toxicity and controlling the bioavailability of drugs [34]. In orally dosed drugs, P-gp has an important role in first-class elimination. As they the efflux transporter P-gp is responsible for the removal of drugs in various organs, In the liver P-gp effluxes drugs from the canaliculi at the bile-facing side, leaving the systemic

circulation via biliary excretion [34].

Besides removing drugs, P-gp also plays a role in controlling the permeability of drugs across such barriers as the blood brain barrier and the blood placenta barrier, The absence of P-gp has been shown to increase the uptake through bilipid barriers potentially resulting in toxicity in targeted organs [34].

1.4.1.2 Role in cancer pharmacology

P-gp was discovered in 1970 during experiments investigation MDR, where a cell surface protein (P-gp) was shown to cause MDR. P-gp showed 2500 times increase in the resistance of cells to actinomycin D, and as a result increased resistance to multiple other drugs such as vinblastine, vincristine, doxorubicin and others [35].

P-gp acts to transport a variety of hydrophobic drugs which carry a neutral or positive charge, as well as various other anti-cancer drugs, including but not limited to anthracyclines such as doxorubicin, vinca alkaloids such as vincristine and taxanes such as paclitaxel [35]. However, it has also been shown to not be involved in the transport of platinum based drugs such as oxaliplatin and cisplatin [23]

The mechanism by which P-gp causes this MDR is by the removal of anti-cancer agents from the cellas P-gp acts as an efflux pump. And furthermore, is highly over expressed in various cancer cell lines such as HepG2. [34]

1.4.2 Multidrug resistance proteins (MRPs)

Group C of the ABC transporter superfamily are known as the multidrug resistance protein (MRP) family. The MRP family consists of thirteen ABC transporters, which can transport a multitude of organic anions and some non-anionic compounds [36].

Non-anionic compounds may be transported as sulfate conjugates, or conjugates of glutathione or glucuronide, however, they may also be transported without conjugation cobalamin together with free glutathione. Whereas anionic compounds are directly transported out of the cell [37].

The MRP family includes such transporters such as MRP-1 and MRP-2 which are heavily implicated in the transport of anti-cancer drugs used in the treatment of HCC [23].

1.4.2.1 MRP-1

MRP-1 is the first of the ABCC encoded MRP transporter subfamily, a 190kDa polytopic membrane transport protein [38]. MRP-1 consists of two hydrophobic transmembrane domains and a cytoplasm facing NBD at each domain, with each transmembrane domain consisting of six α-helices [38]

1.4.2.1.1 Physiological role

MRP-1 in non-cancerous physiology plays a role in the efficacy of various drugs such as antibiotics, statins, antivirals and opiates [39]. The MRP-1 protein acts as an efflux transporter for various drugs and their analogues. Furthermore, MRP-1 is shown to be highly expressed in the cells that traverse tissues and the systemic circulation, such as the blood brain barrier and the blood placenta barrier [39].

MRP-1 not only affects the efficacy of certain drugs, but has also been implicated in the occurrence of adverse reactions. Studies have shown an association with ABCC1 single nucleotide polymorphisms and the reduction of cardiotoxicity from use of anthracyclines such as doxorubicin [39]. MRP-1 has also been seen to influence the etiology of various human pathologies, where it may be involved in chronic inflammation, cardiovascular disease and neurological disorders [40].

1.4.2.1.2 Role in cancer pharmacology

When human MRP-1 was first discovered in 1992, it was shown to amplify the resistance of lung cancer cells by at least one hundred times [41]. MRP-1 has since been shown to be strongly affiliated with the phenomenon of MDR, as it causes the efflux of hydrophobic antineoplastic agents such as doxorubicin and vincristine, prominent anti-cancer drugs. The over-expression of MRP-1 and its encoding gene ABCC1 have been shown consistently in observations of various tumour cell lines, and can be a useful indicator of inevitable resistance of tumours to chemotherapy [39].

Amongst the researched tumours associated with MRP-1 the most strongly affected cancer type has been shown as neuroblastomas, where the clinical outcomes of chemotherapy are poor [39]. As a result of MRP-1 being such a common and well-defined efflux transporter, the scientific community has extensively looked into the reversal of drug resistance by targeting MRP-1 using various molecular modalities [40]

1.4.2.2 MRP-2

MRP-2 is a transmembrane transport protein encoded by the gene ABCC2. An important protein in human physiology, MRP-2 is a large protein, with a size of approximately 190kDa and made up of 1545 amino acids [42]. MRP-2 is proposed to be made up of 17 transmembrane segments in three transmembrane domains with two NBDs [42].

1.4.2.2.1 Physiological role

MRP-2 is almost ubiquitous throughout the human body, and is expressed on the apical side of the cellular membrane on various organs and tissues, including in hepatocytes.

In hepatocytes MRP-2 is located on the canalicular membrane and function to excrete glutathione and glutathione conjugated substances, including organic anionic drugs such as anthracycline and platinum-based drugs in the presence of glutathione [42].

The role of MRP-2 can clearly be shown in patients with Dubin-Johnson Sydrome [43], which shows mutations in the ABCC2 and thus no functional MRP-2 in the liver, leading to jaundice and symptoms of accumulation of conjugated bilirubin [43]. As such it is apparent that the MRP-2 protein expressed in the apical membrane functions to remove toxins and drugs from the body to limit toxicity and control bioavailability in these organs.

1.4.2.2.2 Role in cancer pharmacology

As MRP-2 is heavily involved in the removal of drugs in normal healthy cells, controlling bioavailability to limit toxicity, this also applies to cancer cells. Where MRP-2 has been shown to efflux anti-cancer drugs such as oxaliplatin and doxorubicin and several others out of cells. Furthermore, MRP-2 expression is increased in various human cancers [23] and is therefore detrimental to the efficacy of chemotherapeutics. The expression of MRP-2 has also been showed to be significantly increased even in tissues with mild to moderate cancerous legions, implicating MRP-2 in even early stages of cancer [42].

In certain studies, MRP-2 transporters have been shown to heavily influence the accumulation and efficacy of oxaliplatin in HepG2 and Panc1 cell lines. It has been shown that by adding myricetin, a known MRP-2 inhibitor, there is 5 times and 2.8 times increase respectively in accumulation and 3.4 times and 4 times increase in the sensitivity of cells to oxaliplatin [23]. Furthermore, the same studies showed that siRNA-mediated knockdown of MRP-2 in HepG2 cell lines led to significant increases in the accumulation of oxaliplatin derived platinum and increased anti-cancer effects of oxaliplatin in comparison with control SiRNA treated HepG2 cells. The results show an approximately 3-fold increase in the levels of oxaliplatin derived platinum accumulation and as such the cytotoxic effects oxaliplatin towards the HCC are shown to increase aswell [23]. These results are clinically relevant as they were performed using physiologically tolerable concentrations of oxaliplatin ranging from 3.75 to 11.25 µM [23].

1.5 VITAMIN B_{12}

Vitamin B_{12} or cobalamin (Cbl) is the generic name for a group of vitamins that are the most chemically complex vitamin of naturally occurring origin [45]. Structurally, Cbl is defined by a cobalt ion surrounded six coordination sites of which four are provided by a corrin ring and one by a dimethylbenzimidazole group. The final site, the upper axial position of Cbl, can be occupied by various ligands, which are important in the physiological function of Cbl [45]. The four major biologically relevant forms of Cbl are methylcobalamin (Me-Cbl), cyanocobalamin (CN-Cbl), hydroxycobalamin (OH-Cbl) and adenosylcobalamin (Ado-Cbl) [46], of which CN-Cbl is the only non-naturally occurring vitamer [46].

Cbl is an extremely important vitamin with crucial functions within the body; furthermore, its deficiency is implicated in a plethora of different diseases.

1.5.1 Physiological role

Cbl has various roles within the human body. The most well-defined roles pertain to acting as cofactors for the enzymes methionine synthase and methylmalonyl CoA mutase [45]. Cbl has also been seen to have potential antioxidant effects and is also implicated in MDR [45].

1.5.1.1 Synthesis

The synthesis of Cbl due to its complex structure is a very involved process requiring near 30 enzymes and 20 different genes [47]. This process, however, does not occur in humans or any other mammals, and only occurs in certain prokaryotic cells [47].

Because of the essential nature of Cbl in certain physiological functions, humans have mechanisms to acquire Cbl, external sources of Cbl such as from milk, fish and meat; and supplements, both oral and intramuscular, provide the bulk of human Cbl [48].

1.5.1.2 Absorption

In the transport of Cbl from oral dosage to cells there are three carrier proteins, in order of encounter, haptocorrin (HC), intrinsic factor (IF) and transcobalamin (TC) [49].
The first of the carrier proteins, HC from saliva binds to Cbl and remains bound until the proteolytic enzymes of the pancreas degrade HC, resulting in the Cbl binding to IF [49].
In the ileum, the most distal region of the small intestine, the IF-Cbl complex is absorbed by endocytosis into ileal enterocytes and exits the cell into the blood stream at the basolateral surface, at which point Cbl is bound to TC [47]. In the liver, the internalisation of TC-Cbl complex from blood into the cells is achieved through binding to an TC receptor where endocytosis occurs; a receptor which has been shown to be upregulated in cancer cells cultured in methionine-deficient medium [47]. Once the TC-Cbl complex containing vesicles have reached inside the cell, the acidic environment of lysosomes causes the breakdown of the TC to its constituent amino acids and Cbl ultimately ends up in the cytoplasm for use in metabolic enzymatic processes such as in methionine production [50]

Cbl is mostly stored in the liver and deficiency in such stores can have detrimental side effects and is associated with anaemia and neurological dysfunction [50]. The administration route of Cbl is an important consideration. As described above, oral dosages must go through various stages to reach cells, whereas intramuscular dosing bypasses these events. As such, currently the gold standard treatment for Cbl deficiency is administering 1000µg Cbl intramuscularly to patients once every 3 months [51]. However due to the process being extremely painful for patients, using other methods is very appealing. Furthermore, the limit to tolerability of Cbl has yet to be determined and as such Cbl can be administered at mega-doses, suggesting that greater doses of oral Cbl can be taken to account for the differences in absorption in individuals [51].

There are two forms of Cbl that are generally used to dose in humans, CN-Cbl and OH-Cbl, both of which must first be converted to Me-Cbl and Ado-Cbl in cells before use as cofactors in enzymatic reactions [46].

In a clinical setting CN-Cbl is currently used more often as it is more easily synthesised than OH-Cbl and is thus a much cheaper alternative, even though OH-Cbl is more easily converted in the body and accounts for approximately 60% of physiological Cbl [46,51].

In the conversion of other forms of Cbl to Me-Cbl and Ado-Cbl, the cobalt ion Co^{3+} must be reduced to Co^{+} in order for Me-Cbl to be formed in the cytoplasm and Ado-Cbl in the mitochondria. In mitochondria the CN molecule from CN-Cbl is remove, and the cobalt ion reduced Co^{2+} then adenosylated to give Ado-Cbl via a transient Co^{+} intermediate [46].

In Me-Cbl formation in the cytoplasm CN-Cbl is again decyanated and the cobalt ion reduced to an intermediate oxidation state of Co^{2+} that binds to the enzyme methionine synthase. Once bound to methionine synthase the methyl group is added to Cbl from S-adenosyl methionine (SAM) via a transient Co^{+} intermediate forming Me-Cbl[50].

1.1.1 Role in cancer pharmacology

Given that Cbl is involved in the production of methionine, this presentsan interesting opportunity to exploit this in the in the prevention, treatment and progression of various cancers.

1.1.1.1 Antioxidant effects

Previous research have shown that Cbl may have physiological effects in addition to its cofactor role in the two Cbl dependant enzymes. Cbl has been proposed to have antioxidant effects, through modulation of cytokine and growth factor expression [52]. Evidence includes patients with Cbl deficiency having raised levels of TNF-α and decreased epidermal growth factor levels in cerebrospinal fluid, which is reversable by Cbl supplementation [52].

Cytokines such as TNF-α are inflammatory biomarkers, and as such Cbl seems to act as an antioxidant. These properties are suggested to arise from both direct and indirect means. Cbl plays an integral role in methionine synthase activity, possibly leading to downstream effects. Cbl also reacts directly with ROS and reactive nitrogen species and modifies certain signalling molecules such as NF- κB, a molecule involved in the induction of stress responses [52]. These antioxidant are often observed with thiolatocobalamins rather than the other vitamers of Cbl. As such Cbls, especially thiolatocobalmins, may be useful in the prevention of cancer as the onset of various cancers is linked to the presence of high levels ROS, causing genetic mutations leading to the rapid proliferation of cancer cells [52].

1.1.1.2 MDR reversal

Previous research has shown that treatment with Cbl activates methionine synthase (MS) and increases in the activity of phospholipase D (PLD) and hence increased levels of phosphatidic acid (PA) which down regulates the MDR-1 gene and thus decreases expression of P-gp in cancer cells [56]. In hepatocytes, phophatidylethanolamine methyl transferase (PEMT) catalyses the reaction that transfers methyl groups from SAM onto phosphatidyl-ethanolamine (PE) creating phosphatidylcholine (PC) [57]. The terminal diester bond of PC is hydrolysed by a reaction catalysed by PLD, this hydrolysis causes the release of PA and choline [57]. PA functions as an intracellular signalling molecule and is involved in the activation of protein kinases and phospholipases. Previous research has also implicated PA in the downregulation of MDR-1 and thus a decrease in P-gp expression in HepG2 cell lines [57].

In HepG2 cells the second pathway of methionine production, the betaine Hcy methyltransferase pathway, is not functional and as such the production of methionine and consequently SAM is completely reliant on Cbl-mediated MS [57]. As SAM is a limiting

factor of PEMT activity and thus production of PA, it is likely that Cbl is responsible for the down-regulation of MDR-1 and decreased expression of P-gp [57].

1.1.1.3 Cancer progression

1.1.1.3.1 Methionine dependency

Methionine dependency in cancer cells is the impaired ability of cells to proliferate in the absence of methionine [58]. Cancer cells have the phenotype of being methionine dependent rather than having a built-up dependency throughout proliferation, as both new tumour cells and malignant cells cannot proliferate in growth medium lacking methionine but in the presence of homocysteine (Hcy), the direct precursor to methionine [59]. However, in normal healthy cells, proliferation occurs normally in methionine deficient, Hcy rich culture medium [59].

The biochemistry of this phenomenon of cancer cells being dependent on methionine is not yet completely understood; however here are several theories, including the increased demand for methionine by cancer cells or dysfunctional MS in cancer cells.

Cancer cells are extremely methionine dependent, since cancer cells rapidly proliferate. Rapid proliferation causes the availability of methionine to be rate limiting, as methionine produces SAM, which is the methyl donor in various reactions in the cell, including those involving DNA and protein synthesis [59]. Whereas, the dysfunctional MS theory proposes that MS is unable to convert Hcy into methionine, and as such the levels of methionine are depleted and exogenous sources of methionine become important [59]. However, the increased demand for methionine seems to be more likely as previous studies have shown that the increase of methionine production in cancer cells via the addition of Cbl, consistent with MS catalysing the methylation of Hcy to provide methionine.

1.6 HYPOTHESES AND AIMS

The following are thesis hypothesis:

1. Modulation of the drug efflux pump MRP-2 by Cbl may increase cellular accumulation of its model substrates such as 5(6)-carboxy-2´,7´dichlorofluorescein (CDCF) or doxorubicin in HepG2 cells;.
2. Modulation of MRP-2 by Cbl may enhance the sensitivity to doxorubicin or oxaliplatin in HepG2 cells.

The aims of this research include 1) characterise the possible modulatory effects of Cbl on cellular accumulation of a model substrate CDCF, in HepG2 cell line; 2) to evaluate the effects of Cbl on oxaliplatin cytotoxicity in HepG2 cells; 3) to evaluate the effects of Cbl on doxorubicin accumulation and cytotoxicity in HepG2 cells.

The hypothesized results for this experimentation was the increase in both CDCF accumulation and oxaliplatin cytotoxicity due to the blocking of MRP-2 transporters and the same for doxorubicin accumulation and cytotoxicity due to the down-regulation of MDR-1 by addition of Cbl shown in previous research.

2 MATERIALS AND METHODS

2.1 MATERIALS

HepG2 Cell line was purchased from ATCC. CDCFDA (5(6)-Carboxy-2´,7´Dichlorofluorescein diacetate), vitamin B12, dimethyl sulfoxide (DMSO) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide) were obtained from Sigma-Aldrich. Oxaliplatin powder (Actavis) was purchased from Onelink (Auckland, NZ) and doxorubicin hydrochloride from Abcam (Sydney, AU), RPMI1640 cell culture medium, L-glutamine, penicillin-Streptomycin (10,000 U/mL), phosphate Buffered Saline (PBS), trypan blue stain (0.4 %)and TrypLE^{TM} (Trypsin) were purchased from Life Technologies (Auckland, New Zealand) and pure ethanol from Thermo Fisher Scientific (Auckland, NZ).

2.2 CELL CULTURE

Cell culture medium (CM) was prepared by supplementing RPMI with 100 units/mL of penicillin-streptomycin, 4 mM L-glutamine, and 10 % fetal bovine serum.

2.2.1 Cell Thawing

Step 1: HepG2 cell lines were kept in 2 mL aliquots in -80^{o} C freezer until required, and thawed by briefly immersing the aliquots in a 37^{o} C water bath and gently rocking back and forth until thawed.

Step 2: Thawed aliquots were wiped with 70 % ethanol and contents transferred to a T25 flask with 5 mL of CM and gently rocked back and forth to distribute cells evenly on the surface. Cells were then placed in a 37° C, 5 % CO_2, 95 % humidified incubator for 24 hours for cells to attach to growth surface.

Step 3: After 24 hours cells were observed under an inverted microscope to ensure cells had adhered well to the surface, and that they were displaying healthy morphology. Subsequently the medium was removed then replaced with fresh pre-warmed CM.

2.2.2 Cell Splitting

Step 1: Cells were split at approximately 80 % confluency. The medium was removed from the flasks, and 2 mL and 4 mL of pre-warmed PBS was added to T25 and T75 flasks, respectively, and gently rocked back and forth for around 30 seconds. PBS was removed.

Step 2: Cells were trypsinzed by adding 2 mL and 4 mL of TrypLE express solution for T25 and T75 flasks respectively. Flasks were then incubated for 8 minutes, or until cells detached, with a maximum time of 12 minutes in a 37° C, 5 % CO_2, in a 95 % humidified incubator.

Step 3: Trypsinisation was stopped by adding an equal amount of CM to the previously added TrypLE.

Step 4: Cells were transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form cell pellets.

Step 5: Then the medium was removed, and the cell pellet re-suspended in 2 mL of CM per tube, pipetting gently up and down to redistribute cells.

Step 6: Then cells were counted using a cell haematocytometer and seeded at approximately 500,000 and 1,000,000 for T25 and T75 flasks, respectively. Flasks were kept at 37° C, 5 % CO_2, in a 95 % humidified incubator until needed for experimentation or required to split.

2.3 VITAMIN B_{12} TREATMENT

Cbl stock solution was freshly prepared by dissolving 5 mg Cbl powder into 5 mL sterile milliQ water.

Step 1: Cells grown to around 80% confluency in T25 and T75 flasks were washed with PBS, gently rocking back and forth for ~30 seconds.

Step 2: Cells were then trypsinized by adding 2 mL and 4 mL of TrypLE to T25 and T75 flasks respectively, and incubating at 37^{o} C, 5 % CO_2 in 95% humidified air for 8 minutes, or until detached, with a maximum of 12 minutes.

Step 3: Trypsinization was stopped by adding an equal amount of CM to TrypLE.

Step 4: Then ells were transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form cell pellets.

Step 5: Then the medium was removed, and the cell pellet re-suspended in 2 mL of CM per tube, pipetting gently up and down to redistribute cells.

Step 6: Then cells were counted using a cell haematocytometer and seeded into several 6 well plates at 300,000 cells per well with 2 mL of CM. The cells were incubated at 37^{o} C, 5 % CO_2 in 95 % humidified air for 2 days, until cells reached approximately 70 % confluency.

CDCF accumulation

Step 7: For Cbl treated plates, Cbl was added to each well at a final concentration of 100 nM. For control plates, no Cbl was added.

Step 8: Plates were incubated at 37^{o} C, 5 % CO_2 in 95 % humidified air for 24, 48 and 72 hours before use in CDCF accumulation studies.

Oxaliplatin cytotoxicity

Step 7: Cbl was added to achieve final concentrations of 200 nM, 100 nM, 50 nM. A control with no Cbl was added as well.

Step 8: Plates were incubated at 37^{o} C, 5 % CO_2 in 95 % humidified air for 24 hours before use in oxaliplatin cytotoxicity assay.

Doxorubicin accumulation

Step 7: For doxorubicin accumulation Cbl was added to a have final concentrations of 1000 nM, 500 nM,100 nM. A control with no Cbl was added as well.

Step 8: Plates were incubated at 37^{o} C, 5 % CO_2 in 95 % humidified air for 24 hours before use in CDCF accumulation studies.

2.4 DETERMINATION OF TIME TO STEADY STATE ACCUMULATION OF CDCF IN HEPG2 CELLS

Step 1: Cells grown to around 80 % confluency in T25 and T75 flasks were washed with PBS, gently rocking back and forth for ~30 seconds.

Step 2: Cells were then trypsinized by adding 2 mL and 4 mL of TrypLE to T25 and T75 flasks, respectively, and incubating at 37^{o} C, 5 % CO_2 in 95 % humidified air for 8 minutes, or until detached, with a maximum of 12 minutes.

Step 3: Trypsinization was stopped by adding an equal amount of CM to TrypLE.

Step 4: Cells were transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form cell pellets.

Step 5: Then the medium was removed, and the cell pellet were re-suspended in 2 mL of foetal bovine serum-free and phenol red-free RPMI per tube, pipetting gently up and down to redistribute cells.

Step 6: Then cells were counted using a cell haematocytometer and seeded. An aliquot of 1 mL cell suspension was added into a 15 mL centrifuge tube at a density of approximately 5 × 10^5 cells per ml. The cells were incubated at 37° C in a water bath for 10 minutes.

Step 7: The cellular accumulation of CDCF was initiated with spiking CDCFDA into the cell suspension (final concentration of 5 µM, 0.1% DMSO). The samples were gently vortexed and then incubated at 37° C for six different time points as listed in the table below.

Samples	Time points (min)
	HepG2
1	0
2	5
3	10
4	20
5	30

Table 2.4: Time course of CDCF accumulation in HepG2 cells

Step 8: After incubation for 5, 10, 20, and 30 min at 37° C, the accumulation was stopped by immediately placing the samples on ice and quickly adding 3 mL of ice-cold PBS.

Step 9: The cells were centrifuged at 1200 r.p.m. for 5 min at 4°C and again re-suspended with ice-cold PBS. The cells were washed twice with PBS to remove the excess fluorescent probe.

Step 10: The cells were reconstituted in 500 µl of ice-cold PBS and placed into ice immediately.

Step 11: The intracellular level of CDCF was analysed using the Moflo XDP flow cytometer (Beckman Coulter, Auckland), equipped with a standard laser for excitation at 488 nm. A bandpass filter at 525 nm was used to detect CDCF fluorescence. The cells incubated with DMSO only represent a control sample.

Multiple gating of cells was performed to gate out cell debris and doublets. Cells of a defined range of sizes and only single cells were detected in each flow cytometric analysis. Single cells were selected by gating on the events along the diagonal in the signal pulse height versus area plots for forward scatter (FSC) and side scatter (SSC) (Figure 2.4). An example of data acquisition and definitions of gated populations are shown in Figure 2.4. Fluorescence

intensity was expressed as the geometric mean value of 10,000 events from gated population C.

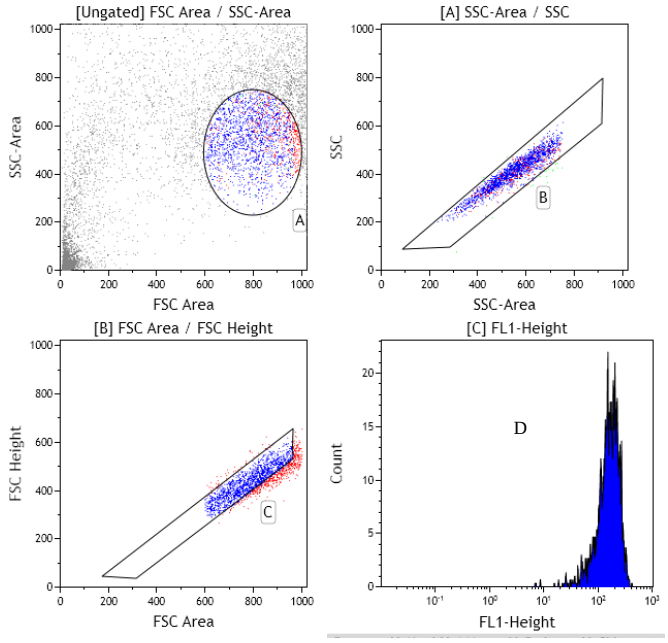

Figure 2.4 An example of data acquisition using the software Kaluza

Figure 2.4 A) A dot plot of analysed cells based on signal pulse area of the forward scatter (FSC) and side scatter (SSC). A population of cells were selected so that only cells that were in the gating were analysed. These cells were designated as population A. B) Population B was gated as the signal pulse height versus area of the SSC. Single cells, which were located along the diagonal of the plot, were designated as population B.C) the same procedure as in in B) was repeated for FSC, and the selected cells were marked as population C. D) Population C was used in the analysis of cellular fluorescence intensity.

2.5 Effect of Vitamin B_{12} on CDCF Accumulation

Step 1: On the day of experimentation, medium from each well was removed and then washed twice with PBS, adding 1 mL PBS per well, rocking back and forth gently for ~30

seconds, and then removing PBS.

Step 2: Cells were then trypsinized, adding 1 mL of TrypLE Express solution to each well and incubating at 37º C, 5 % CO_2 in 95 % humidified air for 8 minutes or until detached, with a maximum of 12 minutes. Trypsinization was stopped by addition of 1 mL of FBS free and phenol-red free RPMI.

Step 3: Then cells were transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form a cell pellet. Then the medium was removed, and cell pellets re-suspended in FBS free and phenol-red free RPMI at a cell density between 0.5-1 x 10^6 cells per mL.

Step 4: Then 1 mL of cells from each treatment concentration was incubated with 0.5 µM of CDCF (0.1% DMSO) and no CDCF for 30 minutes protected from light. The accumulation was stopped by adding 3mL of ice-cold PBS and centrifuging at 1200 r.p.m. for 5 minutes.

Step 5: Then cells were washed again with in 3mL of ice-cold PBS and centrifuged at 1200 r.p.m. for 5 minutes. The cells were then re-suspended in 0.5 mL of ice-cold PBS and protected from light

Step 6: Then the fluorescence exhibited by cells was analysed using the MoFlo XDP flow cytometer equipped with a standard laser for excitation at 488 nm and a bandpass filter at 525 nm, to test the accumulation of CDCF and to confirm no auto fluorescence of Cbl.

2.6 Effect of Vitamin B_{12} on Oxaliplatin cytotoxicity

Oxaliplatin (Actavis, New Zealand) stock solution at 5 mg/ml was prepared by dissolving 100 mg powder into 20 ml MiliQ grade water. After filtration through a 0.22 µm Millipore filter, the stock solutions were immediately aliquoted and stored at -20° C. The stock solutions were used within one month after preparation. MTT stock solution (12 mM) was

prepared by mixing 1 mL of sterile PBS per 5mg of MTT powder. The solution was protected from light to avoid degradation and discarded if older than 1 week. The effect of oxaliplatin on HepG2 proliferation was undertaken as follows.

Step 1: On the day of experimentation, medium from each well was removed and then washed with PBS, adding 1 mL PBS per well and rocking back and forth gently for ~30 seconds. Then PBS was removed.

Step 2: Then cells were then trypsinized, adding 1 mL of TrypLE Express solution to each well and incubating at 37º C, 5 % CO_2 in 95 % humidified air for 8 minutes or until detached, with a maximum of 12 minutes. Trypsinization was stopped by addition of 1 mL of FBS free and phenol-red free RPMI.

Step 3: Then cells were then transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form a cell pellet. Then the medium was removed, and the cell pellet was re-suspended in 1 mL of fresh, pre-warmed CM.

Step 4: Then cells were counted and diluted with pre-warmed CM to 80,000 cells per mL and seeded into 96 well plates at 8000 cells per well by adding 100 µL of cells to each well.
Then plates were incubated for 24 hours in 37º C, 5 % CO_2 at 95 % humidity.

Step 5: Then cells were treated with Oxaliplatin by adding 100 µl of drug solution to each well, with a maximum final concentration of 200 uM in the final row of the plate, and having a serial dilution across the plate, halving the concentration at each row.
Then the plates were incubated for 2 hours at 37º C with 5 % CO_2, 95 % humidified air.

Step 6: Drug exposure was then terminated by removing all medium in each well, and replacing with pre-warmed, drug-free growth medium.

Step 7: Then cells were allowed to grow at 37º C with 5 % CO_2 ,95 % humidified air for 72 hours.

Step 8: The medium was removed from each well, and replaced with 100 µL of fresh phenol-red free culture medium

Step 9: 10 µL of 12 mM MTT stock solution was added to each well, a negative control was made by adding 10 µL to 100 µL medium only.

Step 10: Then cells were incubated at 37ºC with 5% CO_2 ,95% humidified air for 3 hours.

Step 11: Then all but 25 µL of medium was removed from wells, 150 µL of DMSO added, and gently mixed using an orbit plate shaker while protected from light.

Step 12: Plates were incubated at 37º C with 5 % CO_2 ,95 % humidified air for 10 minutes

Step 13: Then samples were briefly shaken in the plate reader and absorbances measured at 540 nm and 680 nm (reference wavelength)

2.7 EFFECT OF VITAMIN B_{12} ON DOXORUBICIN ACCUMULATION

Doxorubicin stock solution was prepared by dissolving 10 mg powder into 1 ml MiliQ grade water. After filtration through a 0.22 µm Millipore filter, the stock solutions were immediately separated into aliquots and stored at -20° C. The stock solutions were used within three months after preparation.

Step 1: On the day of experimentation, medium from each well was removed and then washed with PBS, adding 1 mL PBS per well, rocking back and forth gently for ~30 seconds, and then removing PBS.

Step 2: Then cells were trypsinized, adding 1 mL of TrypLE Express solution to each well

and incubating at 37° C, 5 % CO_2 in 95 % humidified air for 8 minutes or until detached, with a maximum of 12 minutes. Trypsinization was stopped by addition of 1 mL of FBS free and phenol-red free RPMI.

Step 3: Then cells were transferred to 15 mL tubes and centrifuged at 1200 r.p.m. for 5 minutes to form a cell pellet. Then the medium was removed, and the cell pellet re-suspended in FBS free and phenol-red free RPMI at a cell density between 0.5-1 x 10^6 cells per mL.

Step 4: Then 1 mL of cells from each treatment concentration was incubated with 4 µM of the drug doxorubicin and no drug, respectively, for 30 minutes protected from light. The accumulation was stopped by adding 3 mL of ice-cold PBS and centrifuging at 1200 r.p.m. for 5 minutes.

Step 5: Then cells were washed again with 3mL of ice-cold PBS, and centrifuged at 1200 r.p.m. for 5 minutes. The cells were resuspended in 0.5 mL of ice-cold PBS and protected from light.

Step 6: The fluorescence exhibited by cells was analysed using aMoFlo XDP flow cytometer equipped with a standard laser for excitation at 488 nm and a bandpass filter at 525 nm, to test the accumulation of doxorubicin and to confirm no auto fluorescence of Cbl.

2.8 EFFECT OF VITAMIN B_{12} ON DOXORUBICIN CYTOTOXICITY

A preliminary study was conducted to optimize Cbl pre-treatment conditions. Interestingly, we found that HepG2 cells with Cbl pre-treatment directly in 96-well plates showed better and more reproducible results compared with those pre-treated with Cbl in a separate plate. Accordingly, protocols were modified to better suit the conditions present and cell lines used. MTT stock solution (12 mM) was prepared by mixing 1 mL of sterile PBS per 5mg of MTT powder. Solution was protected from light to avoid degradation.

Step1: Cells grown to around 80 % confluency in T75 flasks were washed with PBS, gently rocking back and forth for ~30 seconds.

Step 2: Cells were trypsinized by adding 4 mL of TrypLE express solution and incubated at 37º C, 5 % CO_2, 95 % humidified air for 8 minutes, or until detached, with a maximum of 12 minutes.

Step 3: CM was then added to stop trypisinization, transferred to 15 mL test tubes and then centrifuged at 1200 rpm for 5 minutes to form a cell pellet. The medium was then removed, and cells re-suspended in 2 mL CM by carefully pipetting gently up and down several times

Step 4: Cells were then seeded in 96 well plates with 12,000 cells per well in 100 µL of CM

Step 5: After attachment to cell culture plate, cells were exposed to Cbl at 1000 nM, 500 nM and 100 nM concentrations and incubated for 24 hours at 37º C with 5 % CO_2 95 % humidified air.

Step 6: The cells were then exposed to doxorubicin by adding 100 µL of drug solution to each well with a final concentration of 40 µM in the last row as the highest concentration. A serial 1 in 2 dilutions were made to prepare drug solutions in sterile tubes. An aliquot of 100 µL of drug solution was added into each well, and six replicates were used for each doxorubicin concentration, plates were then incubated for 48 hours at 37º C with 5 % CO_2 95 % humidified air.

Step 7: Drug exposure was then terminated by removing all medium in each well and replacing it with 100 µL pre-warmed drug-free growth medium.

Step 8: The medium was removed from each well and replaced with 100 µL of fresh phenol-red free medium

Step 9: 10 µL of 12 mM MTT stock solution was added to each well. A negative control sample was prepared by adding 10 µL to 100 µL medium only.

Step 10: Cells were then incubated at 37º C with 5% CO_2 ,95 % humidified air for 3 hours.

Step 11: All but 25 µL of medium was then removed from wells, 150 µL of DMSO added, and gently mixed using an orbit plate shaker while protected from light.

Step 12: Plates were incubated at 37º C with 5 % CO_2 ,95 % humidified air for 10 minutes

Step 13: Samples were then briefly shaken in the plate reader and absorbance measured at 540 nm and 680 nm.

2.9 STATISTICAL ANALYSIS

All the data were analysed using Prism 7 software (GraphPad, San Diego, CA, USA), and were presented as a mean value with a standard deviation of the mean (mean ± SD). All results were from two to four independent experiments unless otherwise stated in each experiment. To determine whether or not the observed values were statistically significant, mostly unpaired Student's t-test and one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests were applied. A p-value <0.05 was considered to be statistically significant.

For IC_{50} calculation, non-linear regression analyses were applied to determine IC_{50} values of oxaliplatin or doxorubicin using Prism software

3 RESULTS

3.1 CDCF ACCUMULATION

CDCF is a substrate for MRP-2. Figure 3.1.1 gives the time course for CDCF accumulation in HepG2 cells. It appears that cellular CDCF accumulation achieved steady-state readily 5 minutes after the initiation of uptake. Within 30 minutes, the cellular CDCF accumulation appeared to be stable. 30 min was chosen as a time-point to represent the steady-state accumulation of CDCF.

3.1.1 Time-course of CDCF accumulation in HepG2 cells

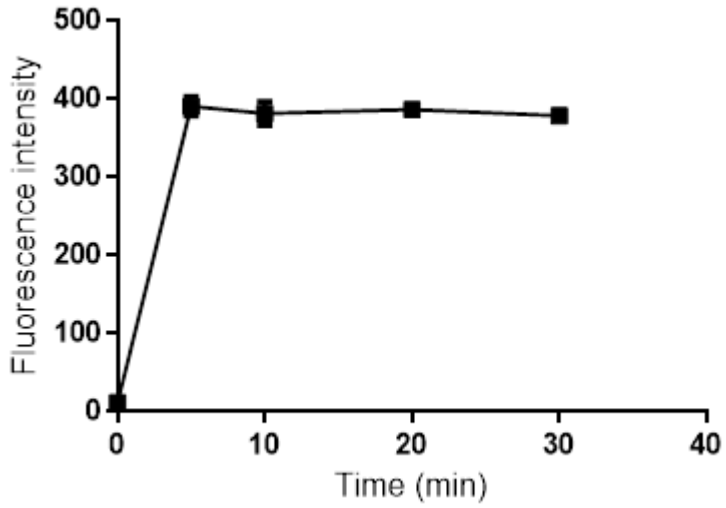

Figure 3.1.1 Time course of CDCF accumulation in HepG2 cells.

3.1.2 Effects of vitamin B_{12} on CDCF accumulation in HepG2 cells

In order to investigate the effects of Cbl on the MRP-2 transporters, a major transporter involved in oxaliplatin efflux, accumulation studies were carried out using CDCF as a substrate of MRP-2 .

Proceeding with a Cbl concentration of 100 nM, a concentration suggested in the literature, (1) cells were exposed and incubated for 24, 48 and 72 hours. After reading fluorescence via the flow cytometer, results showed a significant increase of accumulation at 24 hours in comparison with the untreated cells. The subsequent two-time treatments also showed significant increases, however much less than that of the 24-hour treatments, with decreases from 24 to 48 and from 48 to 72 hours.

As shown in Figure 3.1.2, compared with control, the mean cellular accumulation of CDCF is significantly increased by 136% and 72% in HepG2 cells pre-treated with 100 nM Cbl for 24 and 48 hr, respectively. While 24 and 48 hr results are statistically significant, the pre-treatment with Cbl for 72 hr does not show a statistically significant increment (by 27%) of CDCF accumulation in HepG2 cells. Such time-dependant effects have laid good foundations

for the subsequent experiments. An exposure time of 24 hr was chosen for evaluation of the effect of Cbl in HepG2 cells due to exhibiting a *P*-value of <0.001.

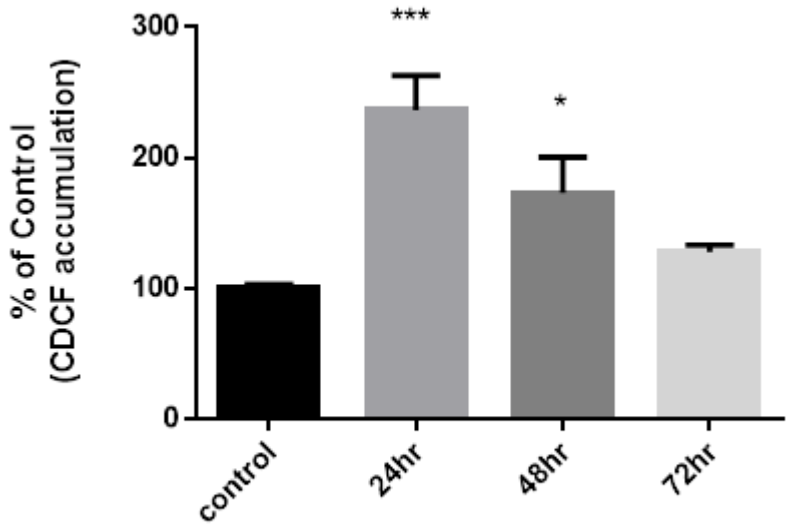

Figure 3.1.2: Time-dependant effect of Cbl pre-treatment on CDCF accumulation in HepG2 cells. All data are normalized to the percentage of control and presented as means ± SD (n=4). Asterisks are *P* values (***, *P*<0.001; * *P*< 0.05) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

	24hr	**48hr**	**72hr**
Mean (%)	236.4	173.1	127.8
SD	26.5	27.2	5.4
CV	11.2	15.7	4.2
***P* value**	< 0.001	< 0.05	> 0.05

Table 3.1. 2: CDCF accumulation in HepG2 cells exposed to Cbl for differing incubation times compared with untreated cells (tabulated data). All data is normalized to the percentage of control,

and presented as means ± SD (n=4). P values are from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

3.2 Effect of Vitamin B_{12} on Oxaliplatin cytotoxicity

Having confirmed 24 hours to be the optimal time of exposure to Cbl as stated by the literature (1), focus was shifted to testing the optimal concentrations of Cbl to be used in combination with oxaliplatin.

As such, cells were treated with Cbl at 50 nM, 100 nM and 200 nM in combination with exposure to oxaliplatin at concentrations of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM in triplicate repeats on 96 well plates.

Though the results appeared to show a decrease, with progressively lower IC_{50} values from treating with progressively higher doses of Cbl, statistical analysis showed that the changes were not statistically significant as the variances between tests were too high.

Only the IC_{50} for 100 nM falls within the acceptable range ($P < 0.05$) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control. The one-way ANOVA analysis gave a P value of 0.5538, which is significantly above the threshold of ($P < 0.05$), confirming that the differences between treatments IC_{50} values are not statistically significant.

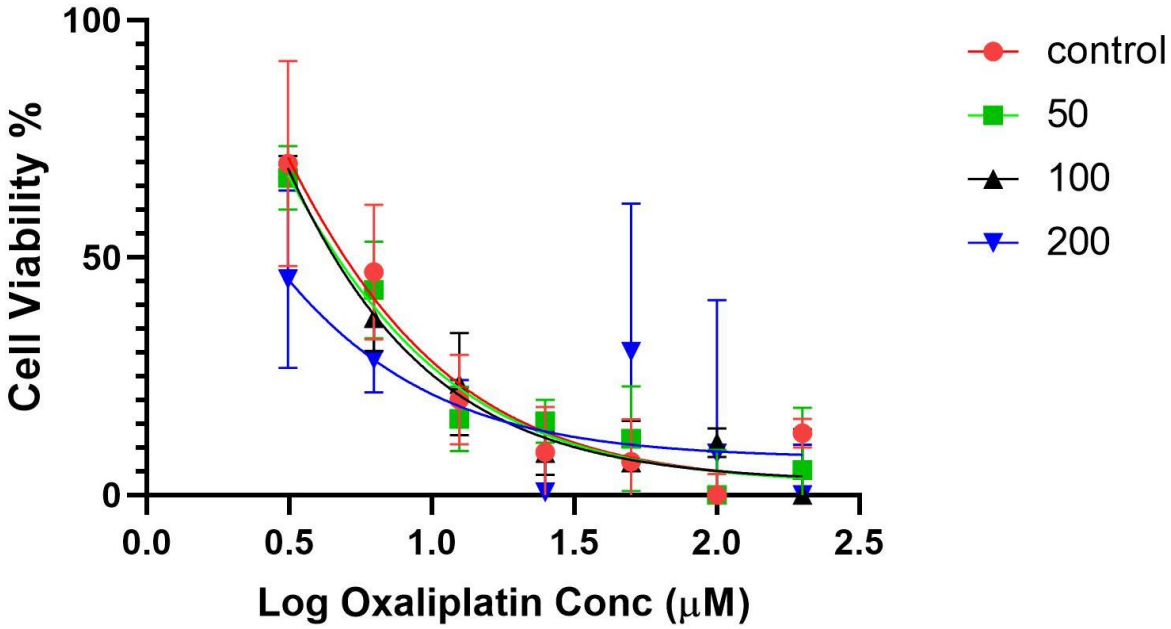

Figure 3.2.1:Growth inhibition by oxaliplatin in control and Cbl treated HepG2 cells (trial 1). All data are presented as means ± SD (n=3).

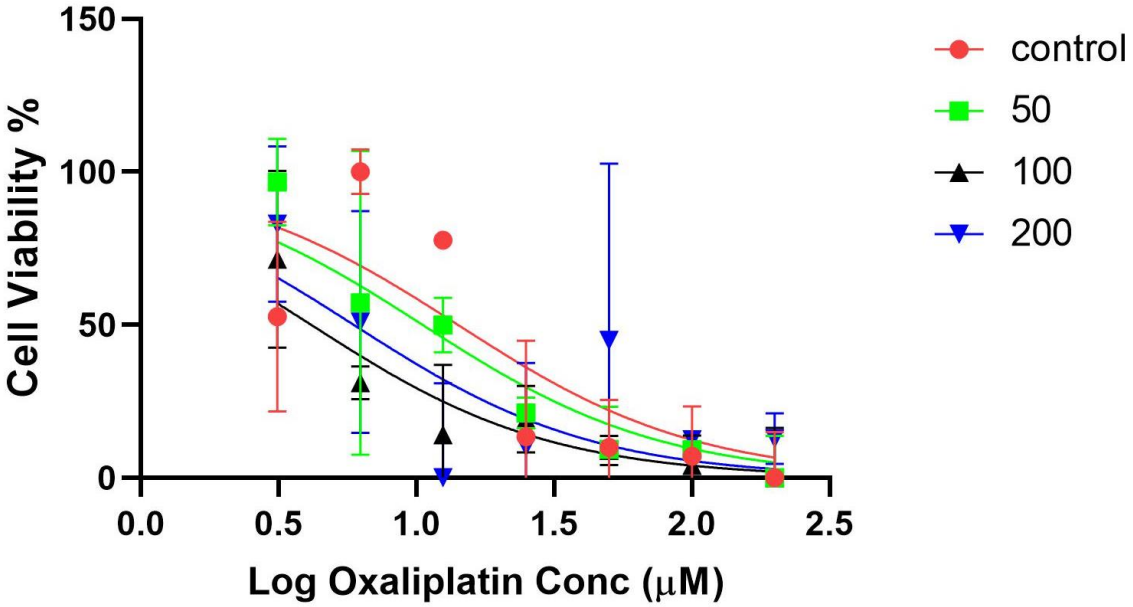

Figure 3.2.2 Growth inhibition by oxaliplatin in control and Cbl treated cells (trial 2). All data are presented as means ± SD (n=3).

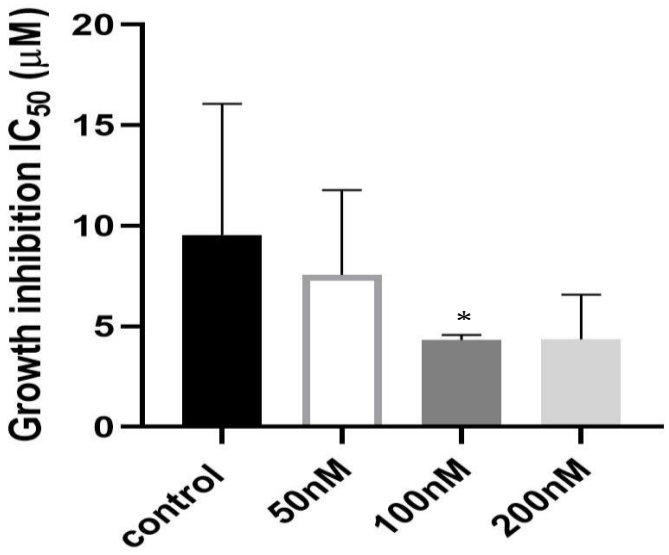

Figure 3.2.3: Mean oxaliplatin IC_{50} values determined in control and Cbl-treated HepG2 cells. All data are presented as means ± SD (n=4). Asterisks are P values (* P< 0.05) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

	Control	**50nM**	**100nM**	**200nM**
Mean	9.53	7.57	4.32	4.36
SD	6.53	4.21	0.24	2.22
CV	68.46	55.62	5.64	50.88
P value		> 0.05	< 0.05	> 0.05

Table 3.2: Mean oxaliplatin IC_{50} values determined in control and Cbl-treated HepG2 cells .

3.3 EFFECT OF VITAMIN B_{12} ON DOXORUBICIN ACCUMULATION

Doxorubicin, another prominent drug in hepatocellular carcinoma treatment, was chosen to investigate the effects of Cbl pre-treatment on doxorubicin accumulation and sensitivity in HepG2 cells.

To test for the accumulation of doxorubicin in HepG2 cells, cells pre-treated for 24 hours with Cbl at various concentrations (1000 nM, 500 nM, 100 nM) and untreated cells were incubated with doxorubicin for 2 hours, which has been previously reported to be time when steady-state accumulation is reached [60]

Figure 3.3 shows that at 100 nM Cbl there was almost no change, and there was only a slight increase of 6 % in average accumulation at the highest concentration of 1000 nM. Surprisingly, 500 nM Cbl pre-treatment yielded the greatest increase. Here accumulation of doxorubicin increased by 19.6 %.

As shown in Figure 3.3 and Table 3.3, the cellular accumulation of doxorubicin was statistically significantly increased by 500 nM Cbl and 1000 nM Cbl pretreatment for 24 hours.

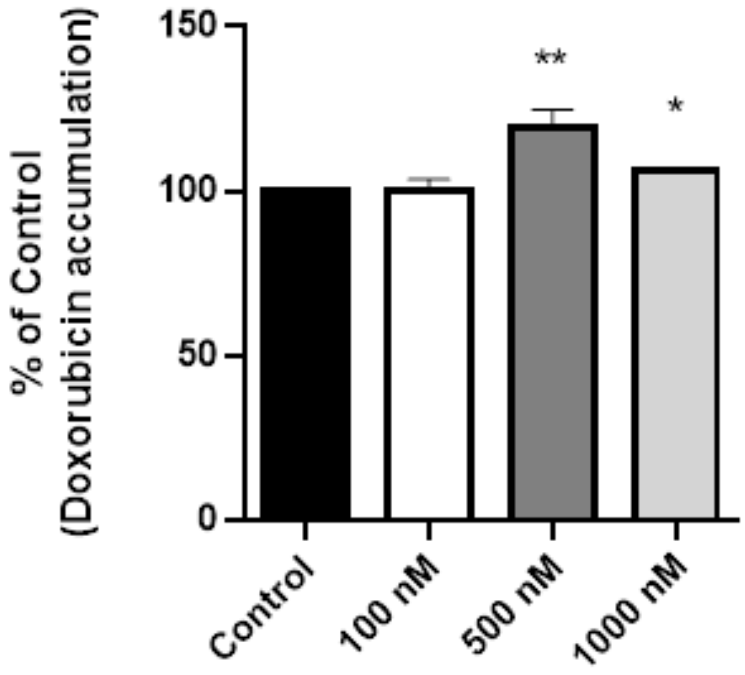

Figure 3.3: Concentration-dependant effect of Cbl on doxorubicin accumulation in Cbl pre-treated HepG2 cells compared with control. All data are normalized to the percentage of control and presented as means ± SD (n=4). Asterisks are P values (**, P<0.01; * P< 0.05) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

	100nM	**500nM**	**1000nM**
Mean	99.97	119.61	106.21
SD	3.65	5.10	0.60
CV	3.65	4.26	0.56
P value	> 0.05	<0.01	<0.05

Table 3.3: Doxorubicin accumulation in Cbl pre-treated cells. All data is normalized to the percentage of control (without Cbl-pretreatment) and presented as means ± SD (n=4).

3.4 EFFECT OF VITAMIN B_{12} ON DOXORUBICIN CYTOTOXICITY

HepG2 cells treated with 1000nM, 500nM and 100nM Cbl were compared with controls to determine the effects of Cbl on the cytotoxic efficacy of doxorubicin in HepG2 cells. Results showed that, as with the accumulation studies, 500nM Cbl had the greatest effect.

All concentration cell viability curves fit well with a sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by a relatively steep drop of cell viability. Figure 3.4.3 shows that pre-treatment with 500 nM Cbl significantly increases sensitivity of doxorubicin in HepG2 cells compared with control. It is apparent that at the lowest doxorubicin IC50 value is with exposure to 500nM Cbl. However, in the 1,000nM Cbl treatment has no significant effects on doxorubicin cytotoxicity in HepG2 cells

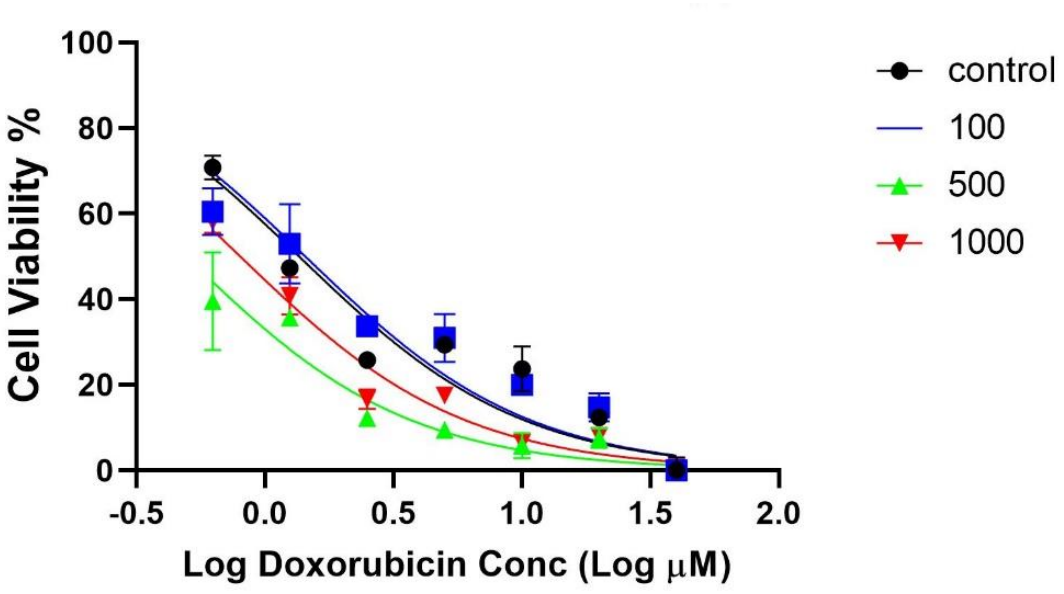

Figure 3.4.1 : Concentration-dependant effects of Cbl pre-treatment on doxorubicin cytotoxicity in HepG2 cells. (Expt 1). *All data are presented as means ± SD (n=3).*

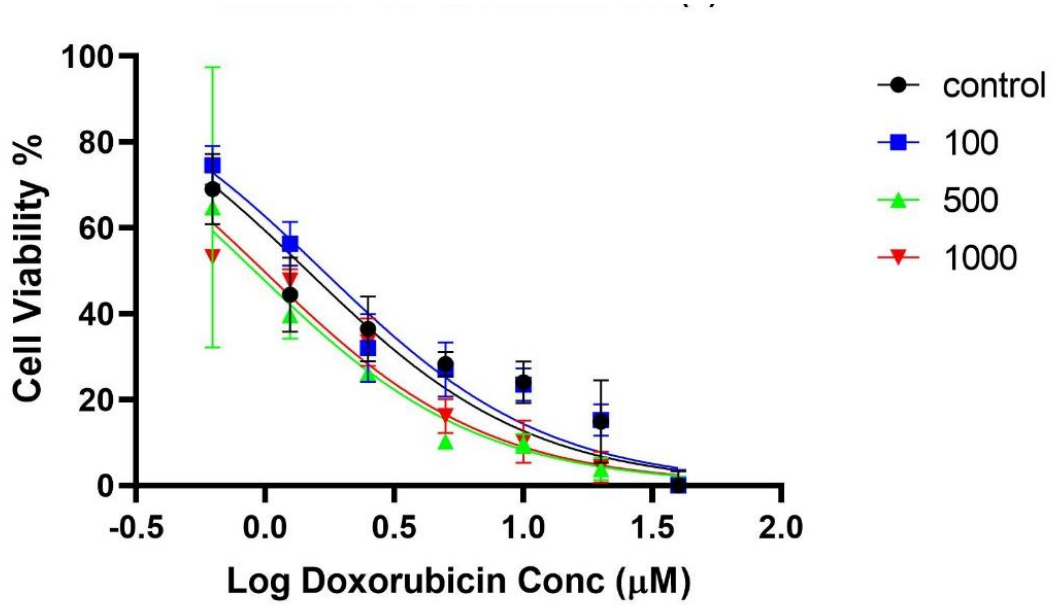

Figure 3.4.2: Concentration-dependant effects of Cbl pre-treatment on doxorubicin cytotoxicity in HepG2 cells (Expt 2). *All data are presented as means ± SD (n=3).*

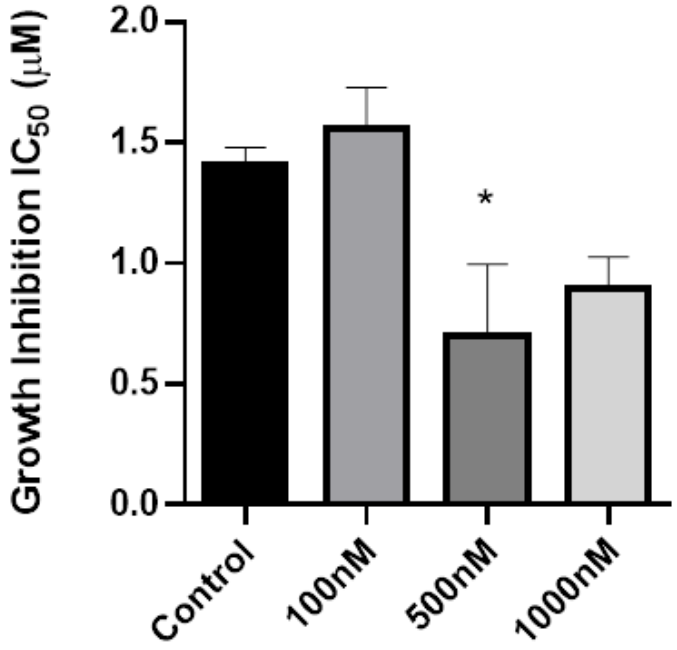

Figure 3.4.3: Mean Doxorubicin IC_{50} values determined in control and Cbl pre-treated HepG2 cells. All data are presented as means ± SD (n=2). Asterisks are P values (* P< 0.05) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

	Control	100nM	500nM	1000nM
Mean	1.41	1.56	0.70	0.89
SD	0.07	0.17	0.29	0.13
P value		P > 0.05	P < 0.05	P > 0.05

Table 3.4: Mean Doxorubicin IC_{50} values determined in control and Cbl-pretreated HepG2 cells. P values from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all treatment samples with control.

4 DISCUSSION

4.1 CDCF ACCUMULATION STUDIES

In CDCF accumulation studies, pre-treatment of 100nM Cbl showed significant increases in the accumulation of CDCF at 24 and 48 hours but not at 72 hours of exposure to Cbl, with the greatest increase being shown at 24 hours of exposure. The increase in accumulation of CDCF, a substrate of MRP-2, may be due to the competitive inhibition of the MRP-2 transporter. The competitive inhibition may not be by Cbl but could instead be a result of the binding of folates. Folates are a product of the release of the methyl group from 5-methyltetrahydrofolate by the action of Cbl mediated MS, to NBDs of MRP-2, preventing efflux of CDCF [61]. Evidence suggesting that folates are substrates of MRP-2 is shown in MRP-2 deficient rats. In these rats, the biliary excretion of endogenous tetrahydrofolate and tetrahydrofolate cofactors is markedly decreased, suggesting that absence of MRP-2 causes increases in accumulation of intracellular folates, this shows that MRP-2 is important in the efflux of intracellular folates [62].

The largest increase in accumulation of CDCF is in cells treated for 24 hours with Cbl, with longer exposures gradually decreasing the effects on CDCF accumulation. This may be due to a variety of Cbl-related reasons. Firstly, the usage of Cbl in catalysing MS reactions may have led to a gradual reduction in concentrations of intracellular Cbl. Consequently, reducing the activity of MS catalysed reactions, and therefore decreasing the intracellular folate level, decreases the level of competitive inhibition. In addition, the efflux of Cbl may also have played a part in the reduced effect on CDCF accumulation at longer times. A major efflux transporter of Cbl, MRP-1 [63] is found in the membrane of HepG2 cells, although at comparatively low levels with other efflux transporters such as MRP-2 and P-gp. The efflux

of Cbl over time would lead to the same effect on CDCF accumulation as with the usage of Cbl by MS, as the intracellular folate levels would decrease with the gradual drop of MS activity, consequently lowering the inhibition of MRP-2 efflux transporters and increasing efflux of CDCF from cells.

4.2 OXALIPLATIN GROWTH INHIBITION ASSAY

In oxaliplatin growth inhibition assays, MTT assays were used to test the effect of three Cbl concentrations, 200, 100 and 50 nM, on the cytotoxic efficacy of oxaliplatin on HepG2 cell lines. The addition of Cbl caused IC_{50} values to decrease, with 100 and 200 nM showing the lowest IC_{50} values. However, these results were not statistically significant changes in IC_{50} values. Furthermore, due to significant variance between plates of the control studies (no Cbl added), no accurate conclusions could be drawn. The seemingly lower IC_{50} values may have been due to the control values being skewed by inaccurately high IC_{50} values.

If these results had been substantiated by statistical significance, it could be concluded that a decrease in IC_{50} with Cbl treatment may be due to oxaliplatin being a substrate of MRP-2, as proposed by recent research. Research that showed knocking down MRP-2 SiRNA in HepG2 cells caused 2-3 fold increases in the accumulation and sensitivity of cells to oxaliplatin when compared with normal HepG2 cells [64]. The slight lowering of the IC_{50} values in cells treated with 200 and 100 nM Cbl may be due to the same mechanisms as in CDCF accumulation.

CDCF is an anionic MRP-2 substrate and is affected only by MRP-2. Oxaliplatin involves not only MRP-2 transporters, but many other influx and efflux transporters such as copper influx transporters (CTR1) and organic cation transporters [23]. Therefore, the lack of statistically significant reductions in IC_{50} values of oxaliplatin sensitivity may have been a

result of off-target effects of Cbl on another transporter such as CTR1. Such transporters being implicated would not impact the accumulation of CDCF, but may reduce the accumulation of oxaliplatin. There is a discrepancy between CDCF and oxaliplatin accumulation in HepG2 cells treated with one of MRP-2-siRNA sequence [64], which could also be explained by this off-target effect. To test this hypothesis, we tested the effects of Cbl on accumulation and sensitivity of another MRP-2 substrate, doxorubicin, in HepG2 cells.

The potentially increased anti-cancer effects of oxaliplatin on HepG2 cells when treated with Cbl may also be caused by MRP-1, as studies have shown that the efflux of oxaliplatin is also reliant on MRP-1, a major pathway of Cbl efflux [63]. Both being substrates of MRP-1 may lead to further competitive inhibition of transporters, leading to further increases in the accumulation, and thus increases in cytotoxicity of oxaliplatin in HepG2 cells. However, since MRP-1 is comparatively low in expression in HepG2 cell lines, this may not be a significant effect.

Interestingly, a prominent transporter that is overexpressed in HepG2 cell lines, P-gp, would not be involved in the proposed increases in accumulation and cytotoxicity of oxaliplatin. Previous studies have shown that although oxaliplatin resistant cell lines exhibit overexpression of P-gp, there is no apparent increase of its activity in comparison to its parental cell lines [23]. This suggests that P-gp does not transport oxaliplatin or oxaliplatin derived platinum, but instead may be evidence of the phenomenon of drug exposure causing resistance to other drugs with other unrelated mechanisms of action.

4.3 DOXORUBICIN ACCUMULATION AND CYTOTOXICITY

In the accumulation and cancer cell cytotoxicity experiments for doxorubicin in HepG2 cell lines treated with Cbl, both accumulation and cytotoxicity showed a non-linear, dose-dependent relationship. In this relationship a bell-shaped curve was observed, where up to 500 nM Cbl both accumulation and cytotoxicity increased, but above this concentration both accumulation and cytotoxicity were reduced.

The increases in both accumulation and cytotoxicity in HepG2 cells treated with Cbl may largely be due to MDR-1 down regulating the gene coding for P-gp [56]. P-gp is the most prominent efflux pathway for doxorubicin found in HepG2 cell lines.

The down regulation of MDR-1 in HepG2 cells treated with Cbl, counterintuitively, is suggested to be a result of increased activity of MS, the enzyme responsible for methionine and SAM production, which cancer cells are dependent on for rapid proliferation.
Though the increased production of methionine and SAM may encourage proliferation, SAM also plays a role in down-regulating MDR-1. This phenomenon occurs when PEMT catalyses the reaction of SAM transferring methyl groups to PE, creating PC [57]. PC is then hydrolysed by the enzyme PLD, creating PA and choline. PA then causes down-regulation of the MDR-1 gene. The mechanism of down-regulation is suggested to use intracellular signalling that leads to the activation of protein kinases and other phospholipases [56]. This explains how pre-treating cells with Cbl causes an increase in both accumulation and cytotoxicity of doxorubicin. Cbl is a cofactor for MS activity, and thus increasing levels of SAM results in the down-regulation of MDR-1, causing reduced expression of P-gp.

Interestingly, both methionine and PLD are implicated in the increase of cancer progression and carcinogenesis. Methionine is an amino acid that cancers are dependent upon, as methionine is converted to SAM, which acts as the primary source of methyl groups for DNA

synthesis and protein synthesis [57]. Both of these are increased in cancer cells in comparison to healthy cells, due to the rapid rate of proliferation characteristic of cancer cells. PLD is involved in several cellular functions besides that of hydrolysing PC. PLD works in cell migration, exocytosis and endocytosis, cytoskeletal reorganization and membrane trafficking [57]. Since both of these factors are involved in the progression of cancer, this suggest that there is another factor which limits the pro-cancer effects of cancer cells. As observed in the case of down regulation of MDR-1, this is seemingly reliant on PEMT[57].

PEMT acting as the limiting factor of the upregulation of cancer cell proliferation is due to a few mechanisms in which PEMT is involved. Firstly, PEMT depletes levels of SAM, by catalysing the reaction of transferring a methyl group from SAM to PE to produce PC. As SAM also has effects that promote cancer cell proliferation, PEMT catalysing the transmethylation reaction works to deplete SAM levels. Furthermore, as PEMT produces PC, PEMT may also be important in limiting the pro-cancer effects of PLD, as PLD is used to catalyse hydrolysis of PC into PA. These may add to the explanation of the decreased cytotoxicity from 500 nM to 1000 nM of Cbl, where PEMT is saturated, thus allowing both SAM and PLD to upregulate the proliferation of HepG2 cells, and have reduced effects on down-regulating MDR-1.

Doxorubicin is also a substrate of MRP-2, a transporter overexpressed in HepG2 cells [15], and as such modulatory effects of Cbl on MRP-2 may also play a significant role, similar to competitive inhibition by folates as explained in section 4.1 and 4.2. Another major efflux transporter of doxorubicin, MRP-1, is not highly expressed in HepG2 cell lines [23], and will probably play a minor role in the increased accumulation of cytotoxicity of doxorubicin. [15]

Limitations and Improvements

In the undertaking of this research, there were a few limitations which may have impeded the results of experimentation, as well as certain improvements that should have been made. Areas where the experimentation was compromised was the consistency of experimentation, to unequivocally establish the effects of Cbl on CDCF, oxaliplatin and doxorubicin. The mechanisms of differentiated effective Cbl concentrations on different MRP-2 substrates should be further investigated throughout the experimental procedures.

In addition, the effects of Cbl on surface expression (e.g. by flow cytometric analysis of immunostained cells) or total protein expression (e.g. by using western blotting) was not determined. Such identification of Cbl effects on the expression of the various transporters MRP-1, MRP-2 and P-gp in the membrane were not determined, leaving a gap in the potential research.

Furthermore, transcriptional regulation of MRP-2 and/or P-gp expression by Cbl was not characterized in HepG2 cells. Our functional results could be due to the decreased expression of the MRP-2 and/or MDR-1 gene. Many drugs (e.g. dexamethasone, 2-acetylaminofluorene, cisplatin, cycloheximide, phenobarbital, clotrimazole) increase MRP-2 mRNA and protein within 24 h in primary cultures of rat hepatocytes. The promoter regions of the human MRP-2 gene contain several putative consensus binding sites for various transcription factors [65]. CCAAT-enhancer binding protein β control gene expression through binding their promoters in HepG2 cells. To detect the effect of Cbl on mRNA expression of ABC transporters, simultaneous quantitation of various gene expression should be undertaken by using real-time PCR or gene microarrays.

4.4 FURTHER RESEARCH OPPORTUNITIES

The results of this experimentation offer opportunities for continued further research. Although the results above for the MTT cell viability assays using oxaliplatin showed too great a variation to have statistical significance, the increase in the accumulation of CDCF in addition to the oxaliplatin IC_{50} values decreasing by approximately half suggest that there is room for further investigation. In future studies, a wider range of Cbl concentrations could be used to see if there is a dose dependent relationship between the levels of accumulation and cytotoxicity. The same could also be carried out for doxorubicin. The use of either surface staining or western blotting to check if the addition of Cbl down-regulates the ABCC2 gene to reduce MRP-2 expression may be potentially interesting similar to research which has been undertaken for the role of Cbl in down regulating MDR-1 [56].

Regarding further doxorubicin studies, trials could be undertaken using different cell lines, with higher expression of MRP-1 and knocking down P-gp to test the effects of Cbl on MRP-1. Greater concentrations of Cbl could also be used to treat cells, to further establish whether there is a parabolic relationship of dosage and accumulation or an inverse parabolic relationship with IC_{50} values.

The role of PEMT in the effectiveness of Cbl as a modulator of cancer drug efficacy should also be further investigated as there may be potential for its use with Cbl to reverse MDR which results from the reduced expression of P-gp in cancer cells.

5 CONCLUSIONS

As a result of the data presented in this thesis, it was concluded that there is promising potential for Cbl to be used in the reversal of MDR in cancers. The fact that Cbl has an effect on cancer is apparent in both the literature and the experimental results above.

As we set out to understand the effects of Cbl mainly on MRP-2 in HepG2, we were unable to define the relationship, requiring further research to understand the direct and indirect effects of Cbl on MRP-2. The results showed that by pre-treating cells with Cbl there were significant increases in the accumulation of CDCF, a substrate of MRP-2, suggesting that there is an effect of Cbl on MRP-2 transporters. However, when testing with MTT cell viability assays to understand the effect of Cbl on oxaliplatin cytotoxicity, no conclusive results were obtained, due to large amounts of variance. Although the results did show some promise of reducing IC_{50} values, the lack of statistical significance meant we could not confirm the increase in cytotoxicity. Due to only testing the functionality and not undertaking experiments such as western blotting or surface staining we could not ascertain if MRP-2 expression was downregulated by Cbl, and as such the reduction in CDCF accumulation was attributed to the gathering of folates as a by-product of increased MS activity and Cbl concentration.

The effects of Cbl on doxorubicin accumulation and cytotoxicity in HepG2 cells were more apparent. The HepG2 cells pre-treated with 1000, 500 and 100 nM Cbl showed interesting results, where the accumulation and cytotoxicity of doxorubicin increased up until 500 nM and then decreased from 500 to 1000 nM. The increases in accumulation and cytotoxicity are largely attributed to the effects of Cbl on P-gp, MRP-2 and MRP-1, the most prominent effect being the down-regulation of MDR-1 genes, causing reduced P-gp expression.

The literature in conjunction with the experimental results suggests there may be a narrow concentration range in which Cbl could be useful in the treatment of cancers, including HCC. As the results above have shown, a lower concentration of Cbl was able to produce greater effects in the reversal of MDR. Due to Cbl having both pro and anti-cancer effects, the dosages require further research to determine at which concentrations Cbls promote cancer. Furthermore, the balance between being useful versus promoting cancer seems to hinge on the availability of PEMT and as such more investigations into the relationship between PEMT and MDR should also be pursued.

Further research must be undertaken, to better understand the effects of Cbl on cancer cells to progress towards a clinically applicable outcome, especially given that there is potential for Cbl to cause increased cancer cell proliferation rather than reversing MDR in cancer cells.

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