The role and mechanism of P-glycoprotein (ABCB1) in collateral sensitivity of multidrug resistant cells

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I dedicate this thesis to my role models, my parents Mary and Peter, who shaped me to be the woman I am today.

"I was taught that the way of progress was neither swift nor easy."

-Marie Curie

ABSTRACT

It is predicted that one out of two Canadians will be diagnosed with cancer in their lifetime. Current treatments rely on surgery, radiation therapy and chemotherapy, with varying degrees of success. One of the most prevalent reasons for poor prognosis of chemotherapy is the emergence of multidrug-resistant (MDR) cancers. ATP-binding cassette (ABC) transporters are a family of proteins that are responsible for much of the drug-efflux mechanisms seen in MDR phenotypes in both the clinical and laboratory setting.

The first chapter of this thesis is a comprehensive literature review unfolding the roles and mechanisms of ABC transporters in relation to drug resistance. More specifically, ABC protein subclass B1 (ABCB1, P-glycoprotein) is described as one of the major contributors to clinical MDR, and its roles, structure and substrate specificity are explained in detail. Finally, the concept of collateral sensitivity (CS), a hypersensitivity of MDR cells to certain compounds, emerges as a potential therapeutic tool in the treatment of MDR malignancies.

The second chapter of this thesis aims to describe the mechanism by which some multidrug-resistant cells are able to survive in the presence of the CS drugs tamoxifen (TAM) and verapamil (VP). Our results show that while most MDR cells are more sensitive to TAM and VP compared to the drug-sensitive parental cell lines, some of the MDR cells survive. In clonal cell lines, we show that this survival is due to a downregulation of ABCB1 protein and mRNA. This downregulation decreases MDR cells' abilities to survive in the presence of chemotherapeutic drugs, and also possibly affects expression of the glycolytic

enzyme α-enolase. Potentially, drug cycling with collateral sensitivity compounds may decrease the emergence of ABCB1-overexpressing MDR cells.

The third chapter of this thesis points to the widespread practicality of our CRISPR-Cas9-mediated ΔABCB1 cell lines as tools for studying MDR and CS. In this chapter, we describe the protocol for knocking out ABCB1 in our MDR $CHO^{R}C5$ cell lines, and the subsequent validation of the roles of P-glycoprotein in the collateral sensitivity of MDR cells. We show that the ROS-mediated mechanism of CS is absent in ABCB1 knockouts, and P-glycoprotein is shown to be absolutely necessary for both drug resistance and collateral sensitivity to occur. We also discuss possible roles for sorcin and other transporters in drug resistance and accumulation. This is the first ever paper addressing the total knockout of ABCB1 in a collateral sensitivity context.

Finally, the fourth chapter of this thesis uses the aforementioned tools to explore a novel collateral sensitivity drug, trifluoperazine (TFP). TFP is a clinically approved compound used in the treatment of psychosis. Using our CRISPR/Cas9 cell lines, we show that MDR cells are collaterally sensitive to clinically relevant concentrations of TFP in an ABCB1-dependent and ROS-mediated mechanism. Additionally, we validate our earlier mechanism of ABCB1-mediated collateral sensitivity, showing that TFP stimulates the ABCB1 ATPase and increases ROS levels in MDR cells. This increase in ROS induces apoptosis in the MDR cells, but not in the parental cell lines. We propose the use of TFP as a collateral sensitivity drug in the treatment of MDR cancers.

Overall, this work clarifies our knowledge of ABCB1-mediated collateral sensitivity, and proposes new tools and compounds that might be used to further study this phenomenon.

ABRÉGÉ

On prévoit qu'un Canadien sur deux recevra un diagnostique de cancer au cours de sa vie. Les traitements actuels contre les tumeurs reposent sur la chirurgie, la radiothérapie et la chimiothérapie, avec plus ou moins de succès. L'une des raisons pour un mauvais pronostic lors d'un traitement chimiothérapeutique est l'émergence de cancers multirésistants (MR). Les transporteurs de cassettes de liaison à l'ATP (ABC) sont une famille de protéines qui sont responsables pour une grande partie des mécanismes d'efflux de médicaments dans les phénotypes MR, tant en milieu clinique qu'en laboratoire.

Le premier chapitre de cette thèse est une revue de la littérature qui aborde les rôles et les mécanismes des transporteurs ABC en relation avec la résistance aux médicaments. Plus précisément, la protéine ABCB1 (P-glycoprotéine) est décrite comme l'une des raisons principales pour la multirésistance. Ses rôles, sa structure et la spécificité de ses substrats sont expliqués en détail. Enfin, le concept de sensibilité collatérale (SC), une hypersensibilité des cellules multirésistantes à certains composés, apparaît comme un outil thérapeutique potentiel dans le traitement des tumeurs malignes MR.

Le deuxième chapitre de cette thèse vise à décrire le mécanisme par lequel certaines cellules multirésistantes sont capables de survivre en présence des médicaments SC, tels que le tamoxifène (TAM) et le verapamil (VP). Nos résultats montrent que la plupart des cellules MR sont plus sensibles au TAM et au VP que les lignées cellulaires parentales sensibles, mais certaines des cellules MR survivent. Dans les lignées cellulaires clonales, nous montrons que cette survie est due à une régulation négative de la protéine ABCB1 et de l'ARNm. Ceci diminue la capacité des cellules MR à survivre en présence de médicaments chimiothérapeutiques conventionels et affecte peut-être également

l'expression de l'enzyme glycolytique énolase α. Potentiellement, le cyclage de médicaments avec des composés SC peut diminuer l'émergence de cellules MR surexprimant ABCB1.

Le troisième chapitre de cette thèse souligne le caractère pratique de nos lignées cellulaires ΔABCB1, médiées par CRISPR-Cas9, comme outils pour étudier la multirésistance et la sensibilité collatérale. Dans ce chapitre, nous décrivons le protocole pour éliminer ABCB1 dans nos lignées cellulaires MR $CHO^{R}C5$. Nous dépeignons aussi la validation ultérieure des rôles de la P-glycoprotéine dans le contexte MR et SC. Nous avons montré que le mécanisme de la sensitivité collatérale médié par ROS est absent dans les cellules sans ABCB1, et la P-glycoprotéine est absolument nécessaire pour que la résistance aux médicaments et la sensibilité collatérale se produisent. Nous discutons également des rôles possibles pour la sorcine et d'autres transporteurs dans la résistance et l'accumulation de médicaments. Il s'agit du tout premier document traitant de l'élimination totale de ABCB1 dans un contexte de sensibilité collatérale.

Enfin, le quatrième chapitre de cette thèse utilise les outils susmentionnés pour explorer un nouveau candidat induisant la SC, la trifluopérazine (TFP). TFP est un composé cliniquement approuvé utilisé dans le traitement de la psychose. En utilisant nos lignées cellulaires CRISPR/Cas9, nous montrons que les cellules MR sont collatéralement sensibles aux concentrations cliniquement pertinentes de TFP dans un mécanisme dépendant de la P-glycoprotéine. De plus, nous validons notre mécanisme antérieur de sensibilité collatérale médiée par ABCB1, montrant que la TFP stimule l'ATPase de ABCB1 et augmente les niveaux de ROS dans les cellules MR. Cette augmentation de ROS induit l'apoptose dans les cellules MR, mais non dans les lignées cellulaires parentales. Nous

proposons l'utilisation de la TFP comme médicament de sensibilité collatérale dans le traitement des cancers multirésistants.

Dans l'ensemble, ce travail clarifie nos connaissances sur la sensibilité collatérale induite par ABCB1 et propose de nouveaux outils et composés qui pourraient être utilisés pour poursuivre l'étude de ce phénomène.

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CONTRIBUTION OF AUTHORS

The experimental work described in the chapters of this thesis were designed and completed by the author, Georgia Limniatis, under the supervision and guidance of her supervisor, Dr. Elias Georges, who contributed to the design, data analysis and editing of the manuscripts.

The first manuscript (Chapter 2) is a follow-up to the identification of tamoxifen as a collateral sensitivity drug by the work of a previous MSc. Student, Rowa Bakadlag. Nevertheless, the author of this thesis, Georgia Limniatis, performed all of the experiments described in this chapter.

In the third manuscript (Chapter 4), the ATPase activity assay of trifluoperazine was performed by Dr. Elias Georges. All other experiments in this chapter were performed by the author of this thesis.

STATEMENT OF ORIGINALITY

The following aspects of this thesis are considered significant and original contributions to the body of knowledge on ABCB1-mediated multidrug resistance and collateral sensitivity:

Manuscript 1: Georgia Limniatis and Elias Georges. Tamoxifen down-regulates ABCB1 expression and up-regulates enolase I expression in multidrug resistant cells. (Manuscript in preparation).

Previous work by R. Bakadlag identified tamoxifen as a CS agent. This current manuscript is the first work describing tamoxifen as a modulator of ABCB1 expression, and is also the first manuscript showing differences in resistance and sensitivity profiles of clones after being treated with collateral sensitivity agents. In this study, we show that the collateral sensitivity drugs target multidrug resistant cells, decrease ABCB1 expression and sensitize cells to conventional chemotherapy. We also suggest a relationship between ABCB1 energy needs and α-enolase expression.

Manuscript 2: Georgia Limniatis and Elias Georges. ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells completely reverses the drug resistance and collateral sensitivity phenotypes. (Manuscript in preparation).

The second manuscript (Chapter 3) is the first work describing the use of the CRISPR/Cas9 gene editing technique in the study of ABCB1-mediated collateral sensitivity. It is also the first work using this system to stably knock ABCB1 out of the commonly used multidrug resistant cell line, CHO. In this chapter, we solidify the working mechanism of verapamil- and progesterone-mediated collateral sensitivity. We show that despite the

other up-regulated proteins that arise during drug selection in chemotherapy, ABCB1 is absolutely necessary for both the drug resistance and collateral sensitivity phenotype. Additionally, we propose the use of the CHO^{R}C5 ABCB1 knockouts as a practical tool for studying both drug resistance and collateral sensitivity.

Manuscript 3: Georgia Limniatis and Elias Georges. Collateral sensitivity of multidrug resistant cells to the phenothiazine trifluoperazine is dependent on ABCB1 expression and ATPase activity. (Manuscript in preparation).

This is the first work describing trifluoperazine as a stand-alone collateral sensitivity drug and elucidating the mechanism involved. We show that trifluoperazine acts as a collateral sensitivity agent at clinically relevant concentrations, and that it synergizes with another well tolerated drug, tamoxifen.

LIST OF ABBREVIATIONS

ABC	ATP binding cassette transporter
ABCB1	ATP binding cassette transporter B1 (P-gp, MDR1)
ΔABCB1	ABCB1 knockout
ABCC1	ATP binding cassette transporter C1
ABCG2	ATP binding cassette transporter G2
ATP	Adenosine triphosphate
CDDP	Cis-platinum
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CHO^{R}C5 (C5)	Chinese hamster ovary cells selected in 5 μg/ml colchicine
CS	Collateral Sensitivity
CRISPR	Clustered regularly interspaced short palindromic sequences
DPPE	N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine
GSH	Glutathione (reduced)
IC50	Inhibitory concentration (50%)
MDA$^{400\ nM\ Doxo}$	MDA-MB-231 cells selected in 400 nM doxorubicin
MDR	Multidrug resistance or multidrug resistant
MDR1	Multidrug resistance protein 1 (ABCB1, P-gp)
NAC	N-acteyl-L-cysteine
NBD	Nucleotide binding domain
OCTN1	Organic cation transporter N1
OxPhos	Oxidative phosphorylation
PARP	poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein (ABCB1/MDR1)
PI	Propidium iodide
PRO	Progesterone
qPCR	Quantitative polymerase chain reaction

Rh123	Rhodamine 123
ROS	Reactive oxygen species
Sorcin	Soluble resistance related calcium binding protein
SRB	Sulforhodamine B
TAM	Tamoxifen
TFP	Trifluoperazine
TMD	Transmembrane domain
VP	Verapamil

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Chapter 1: Literature review.

Chapter 2: Tamoxifen down-regulates ABCB1 expression and up-regulates enolase I expression in multidrug resistant cells.

Chapter 3: ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells completely reverses the drug resistance and collateral sensitivity phenotypes.

Chapter 4: Collateral sensitivity of multidrug resistant cells to the phenothiazine trifluoperazine is dependent on ABCB1 expression and ATPase activity.

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GENERAL INTRODUCTION

Cancer affects a large proportion of Canadians: one in two will be diagnosed in their lifetime, and one in four are projected to succumb to this disease. [1] Though many treatment options exist, including surgery, radiation therapy and chemotherapy, drug resistance remains an obstacle in the clinical management of cancer. It is estimated that approximately 40% of cancers will present some form of multidrug resistance (MDR) to chemotherapy [2], wherein the tumors are resistant to first line treatment and develop cross-resistance to many other compounds. One of the major contributors to clinical multidrug resistance is P-glycoprotein (MDR1), a plasma membrane transporter responsible for effluxing compounds out of cells. P-glycoprotein is an ATP binding cassette transporter (ABCB1) that relies on energy from ATP hydrolysis to function [3, 4].

Efforts to overcome ABCB1-mediated drug resistance have led to the development of three generations of ABCB1 inhibitors. Unfortunately, inhibition of ABCB1 function failed to provide any clinical benefit. The transporter is thought to have a protective function [5, 6], and its inhibition caused high toxicity and altered pharmacokinetic properties of treatment in patients [7]. Therefore, inhibition of ABCB1 function is not a feasible approach to overcoming the prevalent multidrug resistance setbacks in the clinic.

Several reports show that ABCB1-expressing multidrug resistant cells are hyper sensitive, or collaterally sensitive (CS), to a variety of different compounds which drug-sensitive cells are able to circumvent. These compounds, which exploit the function of ABCB1 instead of inhibiting it, include verapamil [8, 9], progesterone [10], DPPE [11], and NSC73306 [12] which target MDR cells in an ABCB1 ATPase-dependent mechanism as

shown by the use of ABCB1 inhibitors or siRNA. However, not all of the MDR cells exposed to these compounds are eradicated.

The main objective of this project was to further understand ABCB1-mediated drug resistance and collateral sensitivity in multidrug resistant cells, using the Chinese hamster ovary (CHO) cell lines. The major hypothesis for this project is that despite other cellular changes arising during drug-selection and development of the multidrug resistance phenotype, P-glycoprotein expression is necessary for both drug resistance and collateral sensitivity. As such, P-glycoprotein-mediated collateral sensitivity has potential as a therapeutic strategy against clinical multidrug-resistance.

The first manuscript addresses the effects of the collateral sensitivity drugs tamoxifen and verapamil on surviving MDR cells. The objective of this study was to determine the effects of drug selection in surviving MDR cells, and determine whether this affects cellular response to conventional chemotherapy.

The second manuscript presented in this thesis describes a valuable tool we developed, the CHO ΔABCB1 knockout cell line. This provides us with a convenient, specific and long-term cell line to study MDR and CS. Previous studies on collateral sensitivity used ABCB1 inhibitors, which may affect cells beyond just ABCB1-inhibition, and siRNA, which is more specific but transient, and does not allow for long-term studies. This manuscript confirms the working mechanisms of collateral sensitivity and characterizes the CHO knockout cell lines.

The third and final manuscript in this chapter supports the final objective of this work, to identify and characterize a collateral sensitivity drug that has already been approved for clinical use. This chapter identifies and confirms the mechanism of the novel

collateral sensitivity drug, trifluoperazine. We show that trifluoperazine has potential as a way to prevent or manage the emergence of drug resistance in the clinical treatment of cancer.

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Literature review

Contents

1. Cancer

1.1. Definition

Cancer is the collective name given to a variety of related diseases, generally defined as the unregulated growth and division of abnormal cells in the body. Normal regulated cell growth and apoptosis is disrupted via the dysregulation of vital genes, wherein old or damaged cells survive and new cell division is initiated without reason. Two major classes of genes are responsible for this genetic basis of cancer: proto-oncogenes and tumor suppressor genes, also known as DNA repair genes.

Proto-oncogenes are genes responsible for producing proteins that are involved in normal cell growth and regulation. Mutations in proto-oncogenes may increase protein expression or involve a gain of function – when this occurs, the gene is referred to as an *oncogene*. Examples of such genes include *HER2* [1, 2], *RAS* [3-7], *BRAF* [8-11], *Cyclin E* [12-16], *beta-Catenin* [17], $^{\text{BCR-ABL}}$ [18] and *Myc* [19-23].

Tumor suppressor genes are responsible for producing proteins that inhibit cell proliferation. Mutations in tumor suppressor genes may decrease protein expression or involve a loss of function. Examples of such genes include *RB1* [24-26], *BRCA1 [27-29]*, *p16 [30, 31]* and *TP53 [32-35]*. Mutations in the latter, producing a less active form of p53, occur anywhere from 38-50% of all cancer types [36].

1.2. Epidemiology and Causes

Cancer is the second leading cause of death in the United States [37] and the leading primary cause of death in Canada – where is it attributed to 30% of deaths, and is projected to affect almost 1 in 2 Canadians in their lifetime [38]. Over the next 30 years,

cancer incidence rates are expected to increase in Canada [38], underlining the importance of understanding the causes, prevention strategies and treatments options of a cancer diagnosis. Globally, the most common cancers diagnosed are prostate and lung cancer in men, and breast and lung cancer in women [39], all of which are susceptible to the development of drug resistance.

According to the World Health Organization, between 30-50% of cancer cases are preventable [40]. This aligns with the findings from the recent ComPARe study by the Canadian Cancer Society, which describes that 4 in 10 cancer cases in Canada can be prevented by making improved lifestyle choices [41]. Other groups claim that up to 95% of cancer cases are attributable to environmental and lifestyle factors, and only 5-10% of cancer cases are due solely to genetic susceptibility [42].

The link between inherited mutations and susceptibility to cancer was first established by Alfred Knudson during his studies on familial retinoblastoma [43], though it was observed anecdotally much earlier in the works of Paul Broca, who described a family prone to cancers of the breast and liver [44]. It is now well established that mutations in proto-oncogenes such as *HER2* [1, 2], *RAS* [3-7] and *Myc* [19-23], and tumor suppressor genes such as *RB1* [24-26], *BRCA1* [27-29] and *TP53* [32-35] can be inherited and can increase the chances of cancer development. For example, in the case of *BRCA1*, a woman with an inherited mutation of the gene carries a breast or ovarian cancer lifetime risk of 70% [45]. Other links exist between reproductive factors and hormones and an increased risk of cancer. In the cases of breast cancer, there are increased risks associated with women who start menarche early or who reach menopause later in life, most likely due to the prolonged exposure to

estrogen and progesterone during this time [46]. Whether the genetic susceptibility of cancer is related to inherited mutations or disparities in natural biological functions, a clear link exists.

Strong links have also been established between certain environmental factors and increased incidence of cancer. Some examples include asbestos exposure [47], outdoor [48] and indoor [48] air pollution, arsenic-contaminated soil [49, 50], parasitic infections [51, 52], bacterial infections such as *H. pylori* [53, 54], viral infections such as the Human Immunodeficiency Virus [55-57], Hepatitis B Virus [58-60] and Human Papilloma Virus [61-64], food contaminants [65-67] and UV radiation [68-71].

Though lifestyle choices such as tobacco use [72-76], tanning bed exposure [77, 78] and alcohol consumption [79-83] are well established to increase the risk of cancer development, recent trends have shifted towards understanding how obesity [84-87] and certain dietary choices such as red meat [88, 89] also contribute to cancer.

1.3. Treatment and Therapy

Cancer treatment can be divided into three major groups: chemotherapy, radiation therapy and surgery. Depending on the stage, grade or overall severity of the cancer, any combination of these three is typically used to treat the patient. Standard chemotherapy involves the use of one or several different compounds that typically target different checkpoints in rapidly dividing cells; nonselective alkylating agents act by adding an alkyl group to guanine bases in dividing cells, preventing successful division; anti-microtubule agents interrupt microtubule function in the mitotic spindle apparatus; topoisomerase inhibitors prevent ligation during DNA replication, leading to

apoptosis; antitumor antibiotics typically act as DNA intercalating agents; antimetabolites are often metabolite analogs that inhibit the availability of metabolites and enzymes during DNA synthesis. It is important to note that many chemotherapeutic agents are nonspecific, in that they target both rapidly-dividing cancer cells but also healthy somatic cells. Additionally, not all cancer cells are rapidly dividing, and so conventional therapies may be rendered redundant. Many side effects are associated with the use of standard chemotherapy, including fatigue, hair loss, nausea, cardiotoxicity, memory loss, neuropathy, secondary infections and the development of new neoplasms.

Immunotherapy is a subclass of chemotherapy that involves the use immune checkpoint inhibitors to enhance cancer killing. Human monoclonal antibodies (mAbs) are also commonly used in targeted chemotherapy, and they typically target proteins that enhance growth, metastasis and angiogenesis. Such examples include EGFR, HER2, CD20 and VEGFR-2 [90].

Radiation therapy, also called ionizing radiation, is a type of treatment that uses ions to deposit energy in the cells it comes into contact with. This contact can lead to apoptotic cell death in the cells directly, by causing sufficient DNA damage to delay or prevent cellular proliferation altogether, or indirectly by increasing the production of free radicals in the tumor [91]. Radiation therapy is often used in combination with chemotherapy or surgery.

Finally, surgery involves the physical excision of a tumor or neoplasm and surrounding tissue.

1.4. Metabolism in cancer cells

Cancer cells have been shown to exhibit increased rates of glycolysis compared to normally functioning cells, even in the presence of high O_2 concentrations [92, 93]. This process of aerobic glycolysis, also known as the Warburg effect, is the mechanism by which cancer cells undergo the fermentation of glucose to lactate as opposed to the complete oxidation of glucose via glycolysis, the TCA cycle and oxidative phosphorylation [94]. The mechanisms by which cancer cells accomplish this phenomenon can be through the up-regulation of glycolytic enzymes including the glucose transporter, hexokinase, phosphofructokinase-1 and enolase, by decreasing the expression of mitochondrial oxidative enzymes, lowering the amount of mitochondria or inhibiting oxidative phosphorylation (Crabtree effect) [95]. While not all cancer cells exhibit the Warburg effect, it is important to note that the ones that do can do so even in the presence of healthy and intact mitochondria [95]. There are several hypotheses as to why tumor cells do this. The first is that rapid ATP production only becomes a need for a cell when resources such as glucose are scarce, which is seldom the case in the human body [96]. The second theory states that the metabolic needs of rapidly proliferating cells –acetyl-CoA, glycolytic intermediates for amino acid synthesis and ribose for nucleotides- can be synthesized from glucose [96]. Furthermore, the regeneration of NAD+ pools during lactate fermentation can be useful for anabolic pathways such as the production of nucleotides and NADPH from 3-phosphoglycerate to serine [97]. This hypothesis has also been supported by the findings that several anabolic pathways that branch off of glycolysis are up-regulated in cancer cells, including the pentose phosphate pathway [98] and the serine synthesis pathway [99].

Another theory to support the evolutionary advantage of aerobic glycolysis for cancer cells is that the acidified tumor microenvironment created due to lactic acid accumulation may enhance invasiveness [100-102] and pro-tumor immunity [103]. Lastly, the earliest and most substantiated theory is that tumor cells typically grow under hypoxic conditions, and thus are forced to undergo fermentation simply due to the lack of oxygen [94, 95].

There is increasing evidence that suggests that cancer cells have increased baseline levels of reactive oxygen species (ROS) compared to normal cells [104-106]. Therefore, while a small increase in ROS has been known to promote cell proliferation and cancer initiation, excessive levels of ROS in cancer cells by the addition of exogenous agents would make the latter even more vulnerable to ROS.

2. Drug Resistance

2.1. Cancer and Chemotherapy

Drug resistance, a well-characterized phenomenon by which cells or organisms have a reduced susceptibility to treatment, is one of the primary contributors to the failure of anti cancer therapy and high recurrence rates. For example, glioblastoma has a nearly 100% recurrence rate [107], which is attributed largely to the difficulty in treating tumors in the brain due to presence of the blood-brain barrier, the highly efficient protective layer formed by a network of endothelial cells that selectively prevent certain molecules from reaching the brain. Correspondingly, the blood-brain barrier is rich in multidrug resistance proteins. Relapse rates due to drug resistance are also high in ovarian cancers and soft tissue sarcoma, at 85%[108] and up to 100%[109],

respectively. All in all, it is estimated that at least 40% of all cancers develop multidrug resistance [110], making MDR a major obstacle in the treatment of cancer.

2.2. Mechanisms of Drug resistance

There are several different documented mechanisms of drug resistance to cancer chemotherapy. Drug inactivation involves the modification or degradation, partially or completely, of a compound intended to kill cancer cells. This has been documented with the use of cytarabine (AraC), a nucleoside drug that is used in the treatment of myelogenous leukemia. AraC is activated following multiple phosphorylation steps, converting it to AraC-triphosphate – any mutation of down-regulation events in this pathway would lead to a loss in the activation of AraC [111, 112]. The alteration of drug targets involves changes of expression levels or mutations in the molecular target of a drug. One example of a mutated drug target is topoisomerase II, which is responsible for forming a transient complex with DNA in order to prevent coiling stress. In cancer treatment, topoisomerase II-targeting drugs stabilize this complex and cause DNA damage. However, some cells develop resistance to treatment through the mutation of topoisomerase II [113, 114]. DNA damage repair is a response by tumor cells wherein they are able to successfully repair the DNA damage caused by alkylating agents such as Cisplatin [115, 116], causing a form of drug resistance to these agents. Inhibition of apoptosis through the up-regulation of intrinsic anti-apoptotic proteins such as Bcl-2 and Bcl-xL has been documented in the clinic: high levels of Bcl-2 expression following chemotherapy are associated with poor prognosis [117]. Epithelial-mesenchymal transition (EMT) metastasis is the mechanism by which tumors which are solid in

origin become metastatic i.e. they spread to different areas of the body, independently of their site of origin. Although the role of EMT in cancer drug resistance is not completely clear, there are several studies linking the two, suggesting that EMT may play a role in drug resistance by the up-regulation of drug-resistance associated genes [118-120]. Finally, altered membrane transport (including compartmentalization) is responsible for the decreased accumulation of chemotherapeutic drugs in target cells due to the presence or absence of specific drug transporters on the surface of the cell membrane. For example, a decrease in expression of the reduced folate carrier (RFC), a drug influx protein, has been associated with methotrexate resistance [121]. In contrast, the overexpression of certain drug efflux proteins, notably the members of the ABC protein subfamily, is one of the most well studied mechanisms of cancer chemotherapy resistance.

2.3. ABC transporters

The ATP binding cassette (ABC) protein family is the largest family of trans-membrane proteins, involved in the transport of structurally and functionally diverse xenobiotics, endogenous molecules, lipids and metabolites across membranes [122, 123]. This active transport of molecules across the membranes is ATP-dependent, modulated by highly conserved nucleotide-binding domains (NBD) containing Walker A and B motifs, as well as an ABC signature C motif found in all of the members [122]. All of the 48 fully functional members of the ABC transporter family contain two NBDs, which bind and hydrolyze ATP, and two trans-membrane domains (TMD), which recognize and transport the substrates [122]. ABC transporters are found in all living

organisms, and are thought to function in a protective mechanism by effluxing toxic compounds out of cells [123, 124]; however they have also been implicated to mediate drug resistance in a large variety of diseases and disorders, including HIV/AIDS [125], malaria [126, 127] and cancer (Table 1).

Cancer	ABC Transporters
Colorectal	ABCB1, ABCC3
Breast	ABCG2, ABCB1
Lung	ABCC3, ABCC5, ABCG2
Prostate	ABCC4
Melanoma	ABCC1, ABCG2
Ovarian	ABCB5, ABCA1, ABCC3
Pancreatic	ABCA1, ABCA7, ABCC3
Leukemia	ABCB5
Brain	ABCC3
Liver	ABCG2, ABCC1, ABCB1, ABCB5

Table 1. ABC transporters in different cancers. Adapted from: H. Amawi, H.-M. Sim, A.K. Tiwari, S.V. Ambudkar, S. Shukla, ABC Transporter-Mediated Multidrug-Resistant Cancer, in: X. Liu, G. Pan (Eds.) Drug Transporters in Drug Disposition, Effects and Toxicity, Springer Singapore, Singapore, 2019, pp. 549-580. [334]

Three ABC transporters are responsible for the vast majority of cancer multidrug resistance due to the efflux of chemotherapeutic compounds: ABCG2, ABCC1 and ABCB1, also known as P-glycoprotein/MDR1. See Figure 1 by Takano *et al.*, for structures [128].

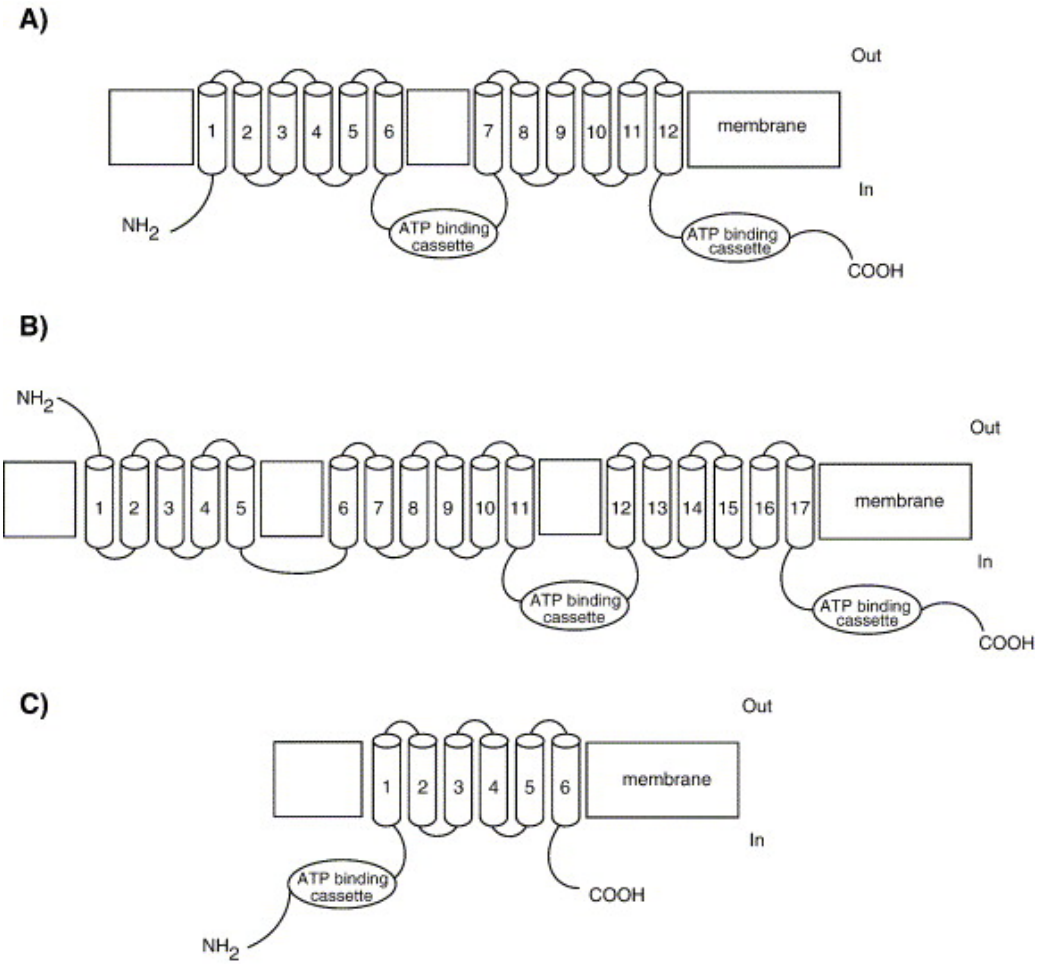

Figure 1. Structures of three major ABC transporters. (A) ABCB1; (B) ABCC1; (C) ABCG2. Takano, M.; Yumoto, R.; Murakami, T., Expression and function of efflux drug transporters in the intestine. *Pharmacology & therapeutics* **2006,** *109* (1-2), 137-161.

2.4. The cancer stem cell

The stem cell hypothesis for cancer initiation and progression states that only a subpopulation of cancer cells – between 12% and 60% are tumorigenic [129, 130]. These cells, deemed to behave like stem cells because of their undifferentiated status and capacity to contribute to tumor evolution, have self-renewal ability and allow the proliferation of progeny end-point tumor-cells, which cannot evolve [129]. However, differentiated tumor cells can revert to the stem cell genotype by the activation of a combination of transcription factors [131]. Based on the cancer stem cell (CSC) hypothesis, therapies are successful if they are able to target these cancer progenitor cells, and failure to do so is what leads to relapse [132]. Problematically, CSCs have genetic and cellular adaptations that allow them to innately exhibit the MDR phenotype [132]. In fact, CSCs also overexpress ABC transporters, including ABCB1 [133]. (Figure 2 and Figure 3).

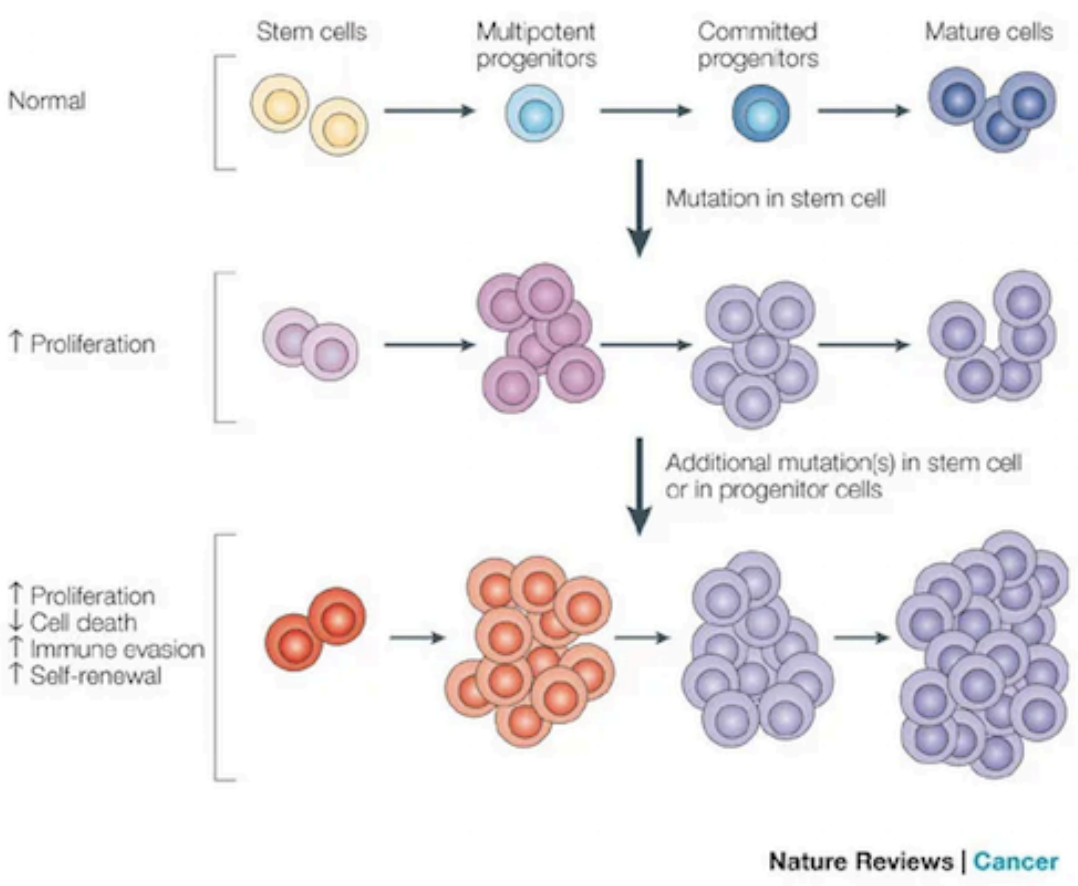

Figure 2. Cancer stem cell hypothesis. Dean, M.; Fojo, T.; Bates, S., Tumour stem cells and drug resistance. *Nature Reviews Cancer* **2005,** *5* (4), 275-284.

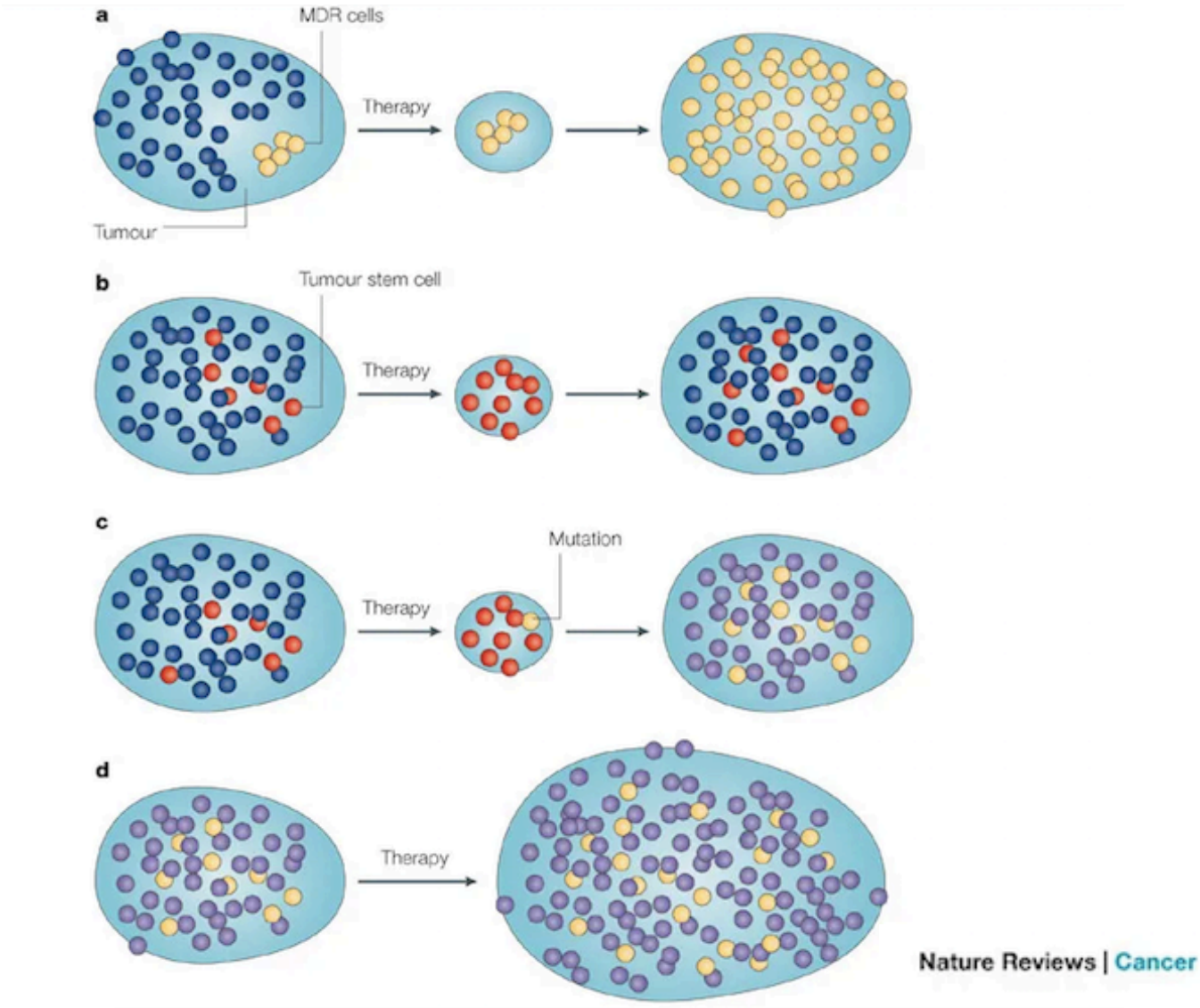

Figure 3. Multidrug resistance in cancer stem cells. Dean, M.; Fojo, T.; Bates, S., Tumour stem cells and drug resistance. *Nature Reviews Cancer* **2005,** *5* (4), 275-284.

3. P-glycoprotein/ABCB1

3.1. Discovery and early observations

Early days of *in vitro* research were heavily focused on finding suitable cell lines that would be stable enough for prolonged cell culture, and have a high cloning capacity and could develop variant phenotypes that were genetically linked. The Chinese hamster ovary cell line, also known as CHO, was chosen for its ease in culture, manipulation and observation in response to different changes in environment [134]. This led Victor Ling's group to use colchicine, an anti-mitotic drug that binds and prevents the polymerization of tubulin, to select CHO cells in order to create a class of cell division mutants with altered tubulin [135]. And so, colchicine-resistant CHO clones, known as CH^R, were selected and studied and found to be cross-resistant to unrelated drugs (daunomycin and puromycin). These drugs do not interact with microtubule formation, and so it was determined that another mechanism of resistance was at play, one that involved reduced cell permeability [135], so the cells were said to be membrane permeability mutants. In addition, this drug resistance was shown to be energy-dependent, reversible with the addition of metabolic inhibitors and rescued with the addition of sugars [136]. It did not take long for Dr. Ling's group to identify a ~165 kDa permeability protein in CH^R mutants, now known as P-glycoprotein, that was absent in wild-type CHO cells and decreased in revertants [137]. Research into the discovery of the resistance protein took off, and ABCB1 was identified as the causative resistance protein in several different cell lines, including CEM/VLB_{100}. Shortly thereafter, the National Cancer Institute tasked a group of researchers with the responsibility of determining the molecular basis of multidrug resistance in cancer, and in 1983, ABCB1

expression in a variety of other MDR cell lines was shown [138, 139]. Homology studies with a bacterial hemolysis transport protein suggested that ABCB1 functioned as a drug efflux pump [140], confirming earlier hypotheses as to how one protein could be responsible for changing cell membrane permeability towards a large variety of unrelated compounds. ABCB1 was subsequently shown to be encoded by the MDR1 gene [141], which was transfected into ABCB1 negative cell lines and shown to confer drug resistance [142, 143]: this was the first such instance where a direct link was established between multidrug resistance and ABCB1. It was in the early 1990s where the widespread expression of ABCB1 in human cancers was solidified [144], prompting what would be decades of attempts at reversing its function.

The role of ABCB1 in clinical practice has been extensively studied, and has consistently shown relationships between MDR1 expression and poor prognosis. While expression of ABCB1 overall may vary in different tumor types, cancers that have long been considered as primarily chemoresistant - meaning they exhibit baseline multidrug resistance to chemotherapy- such as gastrointestinal, liver and renal cell cancers, frequently show higher than average baseline levels of ABCB1 [145, 146]. Furthermore, even cancer types with lower levels of expression of ABCB1, such as breast cancer and leukemia, tend to have up-regulated ABCB1 after treatment [146]. As seen in Figure 4, ABCB1 remains a problem in the clinic, as it is often up-regulated post-chemotherapy and can lead to poor clinical prognosis. It is estimated that close to 90% of bladder cancer [147] and cervical cancer [148, 149] patients are ABCB1 positive, with some older studies on colon and liver cancer going as far as showing 100% of samples to be ABCB1 positive [150], which could have great implications for the types of

chemotherapy that can successfully be administered to these groups. Therefore, understanding the mechanism of action of ABCB1, and elucidating methods to circumvent its contribution to clinical multidrug resistance, is of utmost importance.

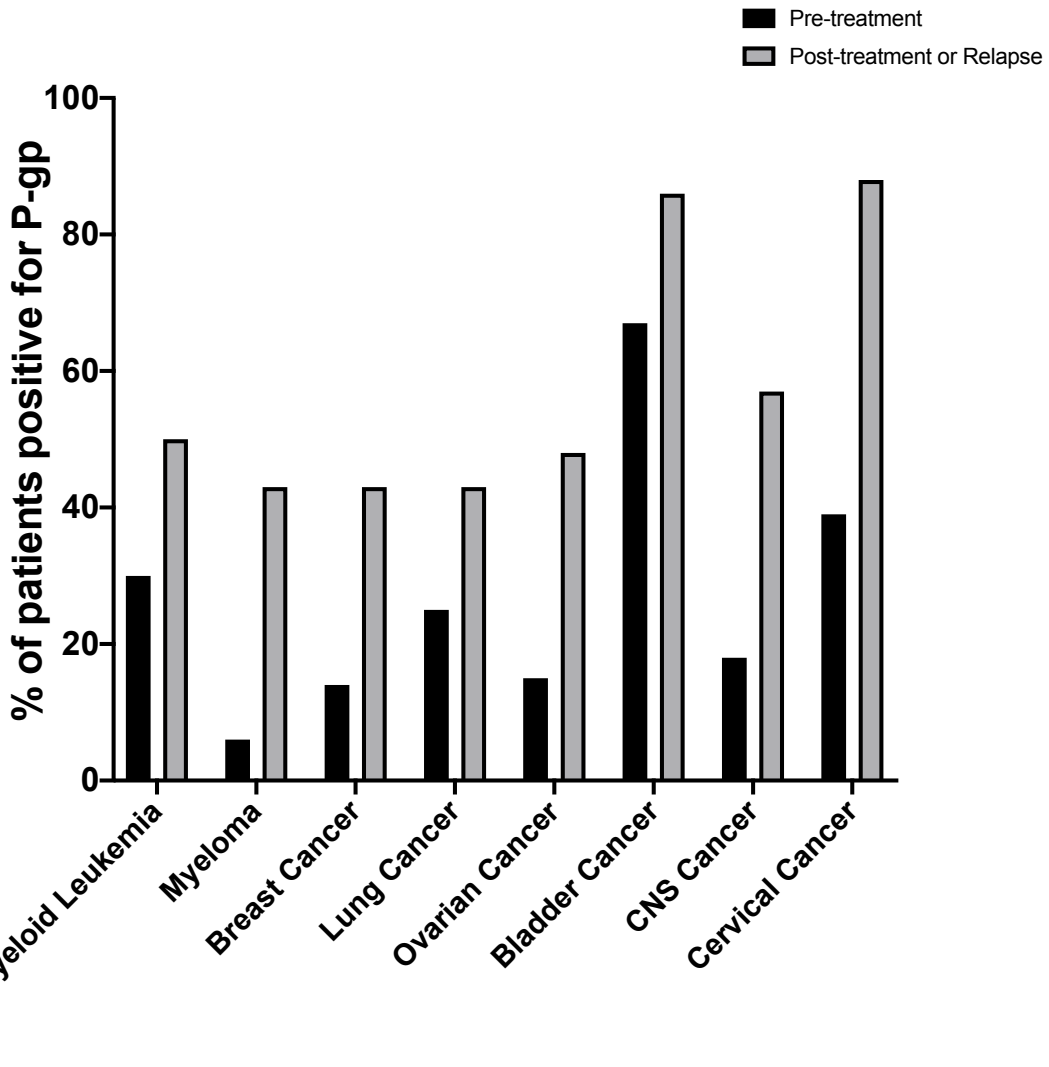

Figure 4. ABCB1 expression and drug resistance in cancers. Adapted from Leonard, G. D.; Fojo, T.; Bates, S. E., The role of ABC transporters in clinical practice. *The oncologist* **2003,** *8* (5), 411-424.

3.2. Mechanism of action

Like all ATP binding cassette transporters, the mechanism of ABCB1-mediated efflux is ATP-dependent, and relies on the two nucleotide-binding domains (NBD) that act as the functional units and two transmembrane domains (TMD) involved in the recognition and transport of substrates. It is also known that ABCB1 exhibits baseline levels of ATPase activity, in the absence of drug substrates, and that the activation energy for ATPase activity in ABCB1 is decreased in the presence of substrate [151]. However, despite decades of research on the molecular mechanism of ABCB1, there is still controversy surrounding the order and details in the steps leading to drug extrusion or sequestration.

Early works on ABCB1 function yielded several different hypotheses as to how P-glycoprotein could transport such a vast number of different compounds in an ATP-dependent manner. The 'hydrophobic vacuum cleaner' model stipulated that ABCB1 provided a hydrophobic path or pore, which allowed substrates to pass through the cell membrane, out of the cell [152]. The 'flippase' model proposed that the substrate interacts with the lipids in the cell membrane bilayer and gained access to the drug binding site in the transporter directly from the lipid phase, in between the leaflets. The drug could then be flipped to the outer leaflet of the bilayer, much like a flippase [152].

Consensus on the energy-dependability of ABCB1 was attained by [3H]-vinblastine affinity studies, showing that ATP binding induced conformational changes that allowed the transmembrane domains to extrude drugs out of cells, and the energy from ATP hydrolysis resets ABCB1 for more transport cycles [153]. It is also well accepted that the transport of substrates by P-glycoprotein occurs when ABCB1 switches from the

inward-facing (IF) (drug recognition and binding) to outward-facing (OF) (drug release) conformation (Figure 5), and that conformational changes at the NBDs during ATP binding are shifted to the TMDs, leading to drug movement. However, the difficulty in elucidating a putative, proven mechanism of ABCB1-mediated transport stems from the lack of any available crystal structure of human ABCB1, debates on proper reconstitution of functional ABCB1 in liposomes vs. nanodiscs vs. micelles and difficulty in studying the transporter in its ATP- and substrate-bound states. To date, several different mechanisms of transport have been suggested for ABCB1.

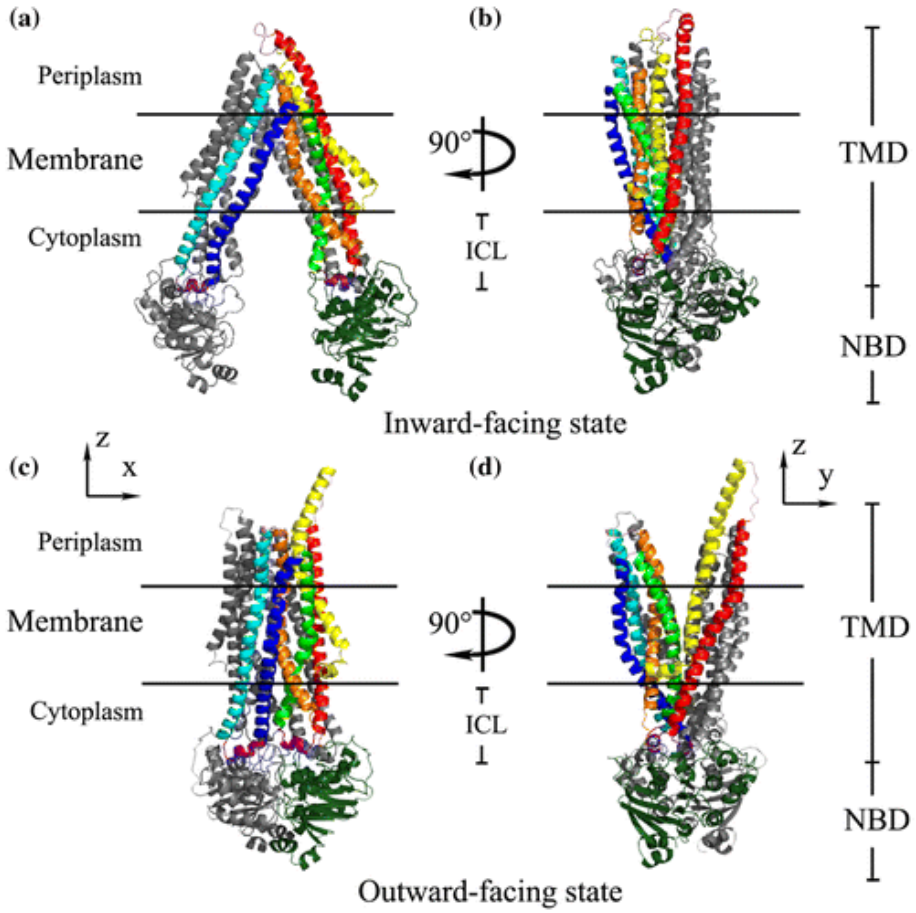

Figure 5. Conformations of ABCB1. From Zhang, Y.; Gong, W.; Wang, Y.; Liu, Y.; Li, C., Exploring movement and energy in human P-glycoprotein conformational rearrangement. *Journal of Biomolecular Structure and Dynamics* **2019,** *37* (5), 1104-1119.

The two proposed 'alternating catalytic site' models suggest that only one of the two NBDs hydrolyses ATP at any given moment and the two NBDs alternate during one catalytic cycle. The first model by Higgins and Linton, also known as the 'ATP switch model', proposes that the dimerization of the two ATP sites is what powers substrate transport [154]. In it, a drug would bind to a high affinity site in the TMD in the IF conformation, and would increase the affinity for ATP at the NDBs, where ATP would bind when the NDBs dimerize. This dimerization would cause a conformational change that moves the drug to a low affinity location on the extracellular side of the membrane, leading to the OF conformation. The hydrolysis of the two ATPs would reset the molecule to its original conformation, IF. The second 'alternating catalytic site' model proposed by Sauna *et al*. suggests that ATP hydrolysis is what powers drug transport [155]. In this model, binding of ATP is unaffected by the presence of another substrate for ABCB1. When an ATP bound to the NBD of ABCB1 hydrolyzes, the subsequent conformational change decreases the affinity for a substrate that is bound to a NBD, thus releasing the substrate. The second ATP bound at the other NBD hydrolyses and returns ABCB1 back to its original conformation.

It is also important to note that ATPase activity in ABCB1 may be different in baseline vs. induced instances. Alternative models of the mechanism in the nucleotide binding domains have been proposed for ABCB1 function in the presence and absence of transport substrate. In the 'monomer/dimer' model, emulating the absence of substrate, ATP hydrolysis completely resets the dimerization of the NBD [156]. On the other hand, in the 'constant contact' model, hypothesized to happen in the presence of

substrate, the NBDs remain in close contact during the catalytic cycle, keeping ATPase activity high [157].

Despite the perplexity surrounding the exact mechanism of action of ABCB1, there is consensus surrounding the four major steps leading to transport: (1) initiation of the transport event is caused by ATP or drug binding; (2) the necessary binding of ATP causes a conformational change in the NBDs [158], and a dimerization or 'sandwiching' between the two halves of the ATP site, which triggers the (3) conformational changes at the TMDs where substrates interact, leading to a low-affinity conformation for the substrate in question and (4) resetting the pump, either by release of a nucleotide or ATP hydrolysis.

3.3. Structure

Fully functional human ABCB1 is found on the cell plasma membrane, and is 1280 amino acids in length, organized into two mirror tandem repeats connected by a linker domain [159]. Each of the two sections of the transporter contains an N-terminal transmembrane domain (TMD) spanning 6 transmembrane (TM) α-helix segments, followed by a nucleotide-binding domain (NBD). The TMDs are responsible for drug binding and transport, while the linker domain is thought to help with cell-surface localization of ABCB1 and ATP hydrolysis [160]. Each NBD of ABCB1 contains several conserved domains, typical of many ABC transporters; Walker A (the P loop), Walker B and a signature motif LSGGQ [161], in addition to A- D- H- and Q-loops [159]. Some studies suggest that both NBD catalytic sites need to be functional in order for ABCB1 to efflux substrates [162, 163], whereas others suggest that a single catalytic site is enough

to stimulate transport by ABCB1 [164]. Finally, the N- and C- termini of ABCB1 are located in the cytoplasm (Figure 6).

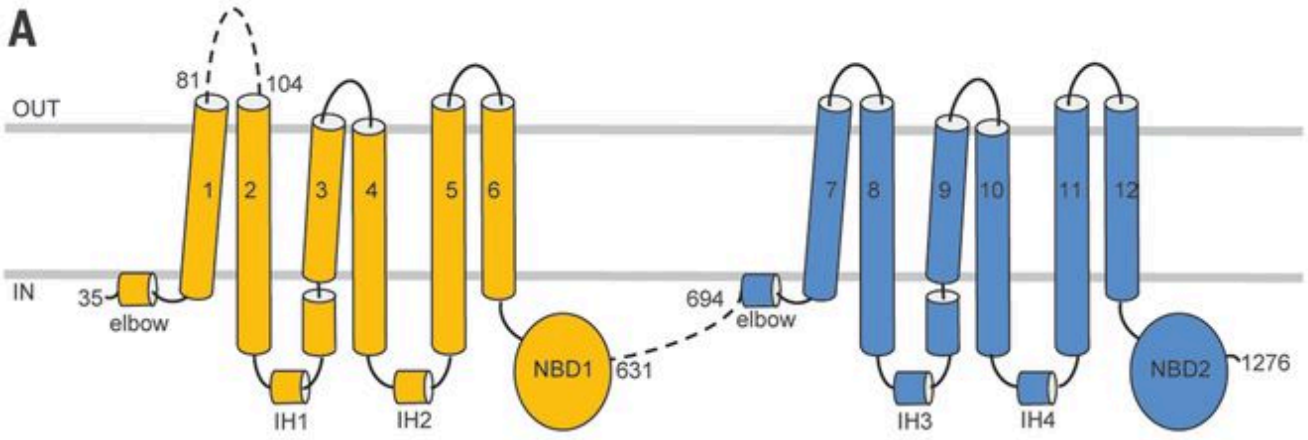

Figure 6. Conformation of ABCB1 in the plasma cell membrane. From Kim, Y.; Chen, J., Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. *Science* **2018,** *359* (6378), 915-919.

N-linked glycosylation of ABCB1 is possible at three distinct sites in the first extra-cellular loop –N91, N94 and N99- and is thought to play a role in proper trafficking of the protein to the surface of the cell membrane, but does not play a role in function [165, 166]. Phosphorylation of the ABCB1 linker region by protein kinases PKA and PKC is possible at S661, S667, S671, S683 [167], as depicted in Figure 7. Several different kinase inhibitors have been shown to be substrates or inhibitors of ABCB1, but the catalytic function of ABCB1 is unaffected by mutations at some of the phosphorylation sites [168]. On the other hand, ABCB1 contains a Pim-1 phosphorylation consensus sequence (QDRKLS); phosphorylation of ABCB1 by Pim-1 is said to protect the pre-

glycosylated form of the protein from proteosomal degradation and allow for glycosylation and eventual cell-surface expression [169, 170].

While early topological work was done using the MRK-16 monoclonal antibody against ABCB1 [171], 3D structure of ABCB1 comes from studying the X-ray crystal structures of mouse [172] and *C. elegans* [173] ABCB1, since the crystal structure of human ABCB1 remains unresolved to this day.

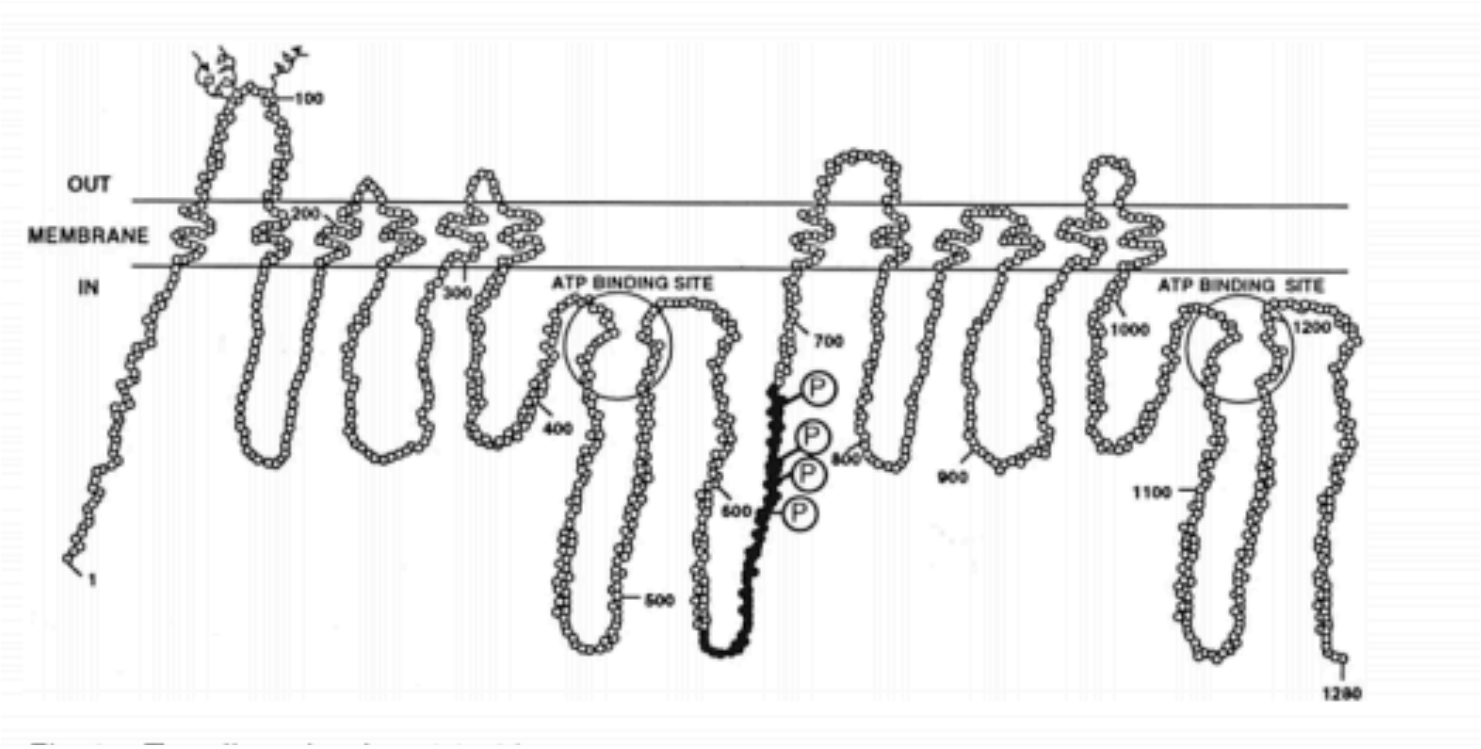

Figure 7. Amino acid conformation of ABCB1 in the plasma membrane. From Germann, U. A.; Chambers, T. C.; Ambudkar, S. V.; Pastan, I.; Gottesman, M. M., Effects of phosphorylation of P-glycoprotein on multidrug resistance. *Journal of bioenergetics and biomembranes* **1995,** *27* (1), 53-61.

3.4. Substrates and polyspecificity

The remarkable ability of ABCB1 to transport hundreds of different substrates, including anticancer drugs, tyrosine kinase inhibitors, antibiotics, calcium channel blockers and fluorescent dyes remains mystifying to comprehend. ABCB1 substrates are mainly hydrophobic or amphipathic compounds, which correlates with the presence of a variety of different nonpolar amino acids in the central cavity, such as leucine, isoleucine, phenylalanine, valine, as well as residues capable of acting as hydrogen bond donors such as tyrosine and glutamine [174]. There are no charged residues in the substrate binding pockets of the TMDs.

Decades of research established that there are likely three major binding sites in the ABCB1 binding pocket: the R (TM 1, 2, 3, 6 and 11), H (TM 4, 5, 6, 7, 8) and P sites [175]. Kinetic studies on CHO cells ABCB1 showed that Hoechst33342 and Rho123, both transport substrates of ABCB1, bind different sites for active transport [176, 177]. The P site on the other hand was determined to be an allosteric binding site for progesterone, and did not lead to transport of the substrate by ABCB1 [176-178]. While positive cooperation was established between the H and R sites, the P site was deemed to be mostly inhibitory, as shown by the fact that Tariquidar, a known ABCB1 inhibitor, does not bind in the H or R sites, but rather binds near TMs 4 and 9, and is not transported by ABCB1 [175]. Important roles in TM helices 5, 6, 11 and 12 have been implicated in substrate binding, as these have been involved in conformational rotations following ATP hydrolysis [179].

Binding-activity relationships between *activators* of ABCB1, measured by increased transport activities of known substrates, have also been shown to have specific binding

sites different from the transport substrates. These activators, including verapamil and modified benzimidazoles, were thought to stimulate ABCB1-mediated transport by increasing ATP hydrolysis or increasing the affinity of ATP for the NBDs [180].

It is probable that ABCB1 has overlapping sites for different compounds in its drug-binding pocket, as the latter is large and flexible by biochemical standards. In addition, ABCB1 is also able to bind certain drugs at secondary sites when the primary binding site is otherwise occupied or chemically modified [181].

3.5. Mechanisms of up-regulation or over-expression

Transcriptional regulation of ABCB1 has been observed, with two major mechanisms: an increase in ABCB1 mRNA or gene copy (gene amplification), or an increase in ABCB1 promoter activity. For example, some studies have shown while *p53* up-regulation does not increase *MDR1* expression, mutations in the *p53* gene have been associated with stimulation in the *MDR1* promoter activity [182]. Other factors affecting MDR1 promoter activity include chemotherapeutic agents [183-185], steroid hormones [186, 187], UV light [188], serum starvation and heat shock [189], suggesting a stress-related up-regulation of MDR1 that seems to be dependent on the presence of YB-1, a DNA binding protein. In addition, protein kinase pathways PKA [190] and PKC [191] have also been implicated in the increase of *MDR1* promoter activity, though it is not directly related to the phosphorylation of ABCB1, but more likely due to the downstream effects by PKC of NF-kB translocation into the nuclei of cells, up-regulating gene expression [192]. In addition, increases of *MDR1* mRNA by chemotherapeutic agents, both substrates and non-substrates of ABCB1, has been extensively discussed

[184, 193]. Epigenetic modulation may also regulate MDR1 expression, as both inhibition of histone acetylation [194] and DNA methylation have been implicated in the up-regulation of MDR1 [195], though these have been disputed [196].

In addition to regulation at the mRNA level, post-translational modifications may also be involved in the regulation of ABCB1 turnover and expression. As mentioned previously, phosphorylation by Pim-1 protects ABCB1 from the proteosomal degradation pathway, though phosphorylation of ABCB1 is not essential for function [169, 170].

Lastly, it is important to note that several other genes on the ABCB1 amplicon seem to be upregulated with *MDR1*. The human ABCB1 gene is found in the 7q21.1 chromosomal region [197]. It is therefore no surprise that several other resistance-associated genes on the same amplicon arm have been shown to be affected by *ABCB1* gene amplification and activation, including *sri* (sorcin) [198, 199], *ADAM22* [200-202], *DBF4* [200], *SLC25A40 [200, 203], RUNDC3B* [204], *ABCB4* (MDR3) [205, 206], *CROT* [200], *TP53TG1 (*down regulation) [207], TMEM243 [207] and DMTF1 (DMP1) [200].

3.6. Function

It has been well established thus far that ABCB1 plays a major role in multidrug resistance in cancer and several other diseases and disorders. And although the exact function and native substrate of ABCB1 is unknown, the normal tissue distribution of the protein suggests it plays a protective and excretory role, by excreting exogenous and endogenous compounds from vital tissues and organs (Figure 8 and Figure 9).

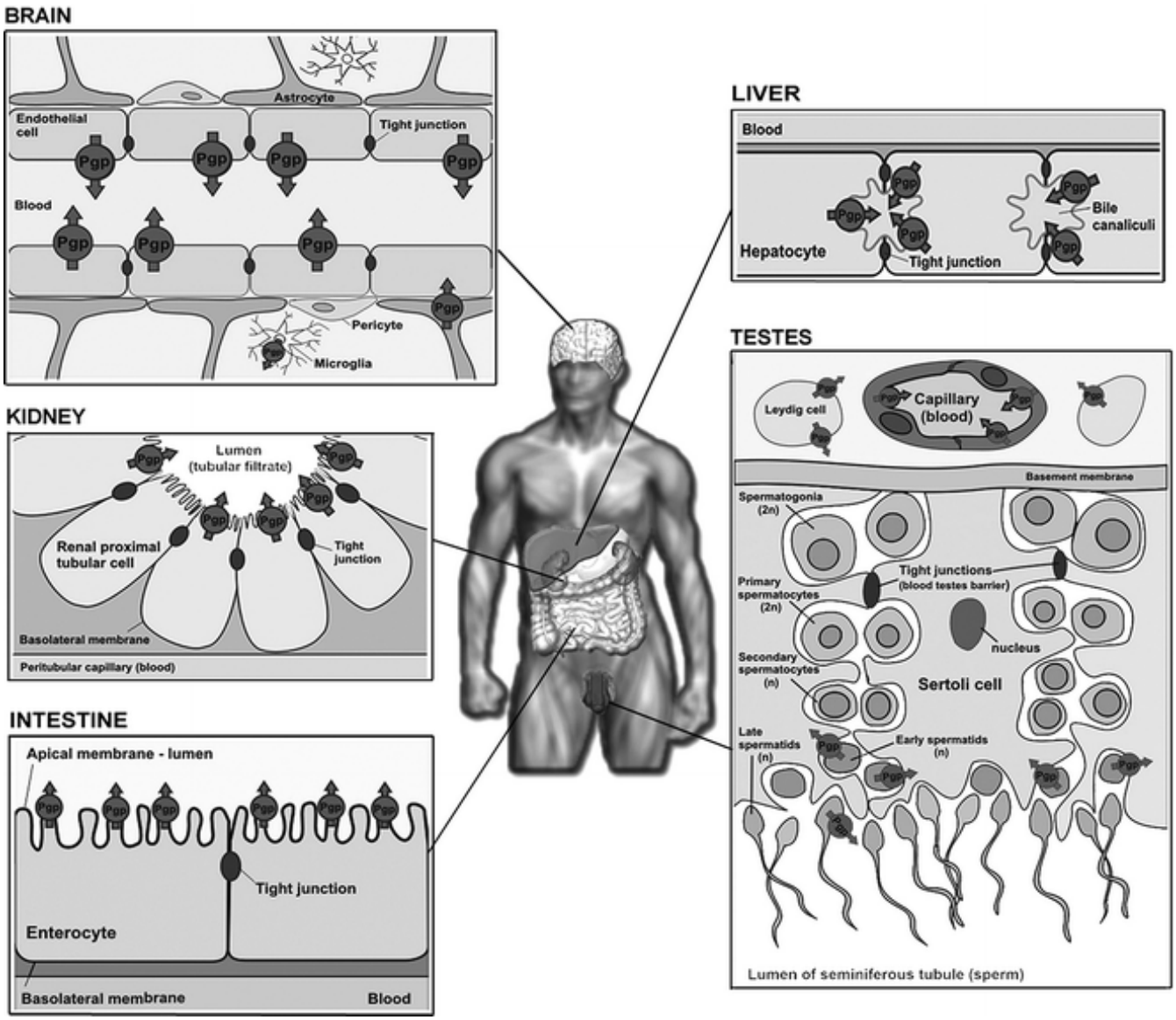

Figure 8. Location and possible functions of ABCB1. From Staud, F.; Ceckova, M.; Micuda, S.; Pavek, P., Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics. In *Multi-Drug Resistance in Cancer*, Springer: **2010**; pp 199-222.

ABCB1 is thought to play a role in drug absorption and excretion due to its significant presence in the small intestine, in the top villi of enterocytes, where it pumps substrates into the intestinal lumen [128]. This, coupled with the fact that many of the substrates for ABCB1 are amphipathic, suggests that the presence of ABCB1 in the small intestine could affect drug absorption. The distribution of MDR1 in the villi is not uniform – expression is highest in columnar cells and lowest in the crypt [208]. Furthermore, ABCB1 is present along the entire gastrointestinal tract, with increasing expression from the stomach to the colon [145]. Many studies on mouse models have shown that oral absorption of drugs is much higher in ABCB1 knockout animals [209], and direct evidence was found for an excretory role, as it expelled injected compounds into the intestinal lumen of mice [210] and humans alike [211]. In addition to its presence in the gastrointestinal system, ABCB1 is also found in the kidney and liver, the main excretory organs of the body. Biliary excretion of drugs is an active transport mechanism that takes place at the apical membrane of hepatocytes, where ABCB1 is highly expressed [208, 212]. A direct role of ABCB1 transport of vincristine was found to take place in the rat liver [213], and it was determined to also be responsible for oral drug elimination by biliary excretion in humans [214, 215]. Lastly, ABCB1 is expressed at the apical side of proximal tubule cells in the kidney, where it contributes to renal drug excretion [216, 217].

ABCB1 is also suspected to play a protective role because it is found in defensive tissues such as the blood-brain barrier (BBB), the blood-testes barrier (BTB) and the placenta. The blood-brain barrier is both a mechanical and active barrier that protects brain tissue from exogenous substances and maintains brain homeostasis [218]. Only

hydrophobic molecules are able to cross the BBB by passive diffusion, but many compounds that fit this description –mostly ABCB1 substrates- do not accumulate in the brain [218]. In fact, ABCB1 is highly expressed in the endothelial cells of the BBB [219], as well as the astrocytes [220, 221] and microglia [222, 223] in the brain. *In vivo* studies on mice and rats showed a direct functional action of ABCB1 efflux of drugs out of the brain [224, 225]. In recent years, a role of ABCB1 in different CNS and neurodegenerative diseases has been suggested. It was first hypothesized that a decrease in ABCB1 expression in the brain associated with aging is responsible for progressive neurodegeneration and Parkinson's disease [226-228]. Studies have also shown that ABCB1 plays a major role in Alzheimer's disease (AD) because it transports β amyloids (the accumulation of which causes AD), and ABCB1 expression in the brain decreases with age [229, 230]. Similarly to the blood-brain barrier, the blood-testes barrier acts as a protective tissue for the sensitive germ line cells found in the seminiferous tubules of the male reproductive organ [218]. ABCB1 was found to be expressed in human testicular capillaries where it readily effluxes toxic compounds such as anticancer drugs [231-233]. In the placenta, drugs must cross the ABCB1-expressing syncytiotrophoblast membrane in order to reach the foetus [234]. Since placental ABCB1 expression in humans is highest in the first trimester and decreases with gestational time [235], it is believed that it helps protect the developing fetus during the most vulnerable stages of embryonic development.

Of importance, the knockout of ABCB1 in mice does not lead to a lethal phenotype, however, the absence of ABCB1 in these animals leads to large differences in drug pharmacodynamics, compared to wild-type counterparts [236, 237]. Additionally, 29

different small nucleotide polymorphisms (SNPs) have been identified in the human *MDR1* gene [238], affecting drug bioavailability [239], drug accumulation [240-242] and causing toxicity in human patients [243-245]. Moreover, SNPs have also been implicated in susceptibilities towards certain disorders such as ulcerative colitis [246], Crohn's disease [246], Parkinson's disease [247-249]. Lastly, many SNPs have been identified as markers of poor prognosis during cancer treatment [250-255]. These findings suggest that while functional ABCB1 does not seem to be vital to survival, certain mutations or complete absences lead to issues in pharmacokinetic properties during treatment, as well as predispositions to certain diseases.

3.7. Methods to overcome MDR

The discovery of ABCB1 and its importance in understanding clinical multidrug-resistance elicited a great deal of effort on trying to reverse or block ABCB1 function. First generation ABCB1 inhibitors, like verapamil, were developed to inhibit the efflux function of ABCB1. Verapamil was tested in clinical trials but was found to not be effective in all cases [256], and it produced high toxicity at doses capable of inhibiting the transporter [257]. Other examples of first generation inhibitors include quinidine, which did not show increased survival [258], and cylosporin A, which at first seemed to positively alter interactions with other chemotherapeutic agents such as etoposide [259], but was ultimately found to be ineffective [260]. Second generation ABCB1 inhibitors such as valspodar were projected to help first generation inhibitors by enhancing their effects and reducing associated toxicities. However, the use of valspodar as a second-generation inhibitor was halted due to its ability to change the

pharmacokinetics of other relevant chemotherapeutic agents [261, 262] and its inability to improve the quality of life of patients [263]. Third generation ABCB1 inhibitors were created to decrease the pharmacokinetic interactions from the second-generation inhibitors, but did not show increased survival. Tariquidar, for example, showed promise as a drug because it successfully blocked ABCB1 function with lower levels of toxicity in healthy patients [264] but did not improve clinical outcome [265]. Clinical trials on ABCB1 inhibitors dwindled largely due to unacceptable toxicities in patients, unanticipated pharmacokinetic interactions with other drugs and inadequate improvement of patient survival and quality of life.

Alternative approaches to attempt to circumvent clinical multidrug-resistance include the use of siRNA to target ABCB1 mRNA [266-268], antibodies such as MRK16 [269], transcriptional regulation by modulating PKA and PKC, drug encapsulation to increase delivery [270-273], the use of ultrasound to prevent drug efflux [274-277], nano-based drug delivery systems [278], photodynamic therapy [279], and collateral sensitivity drugs, also known as paradoxical sensitivity.

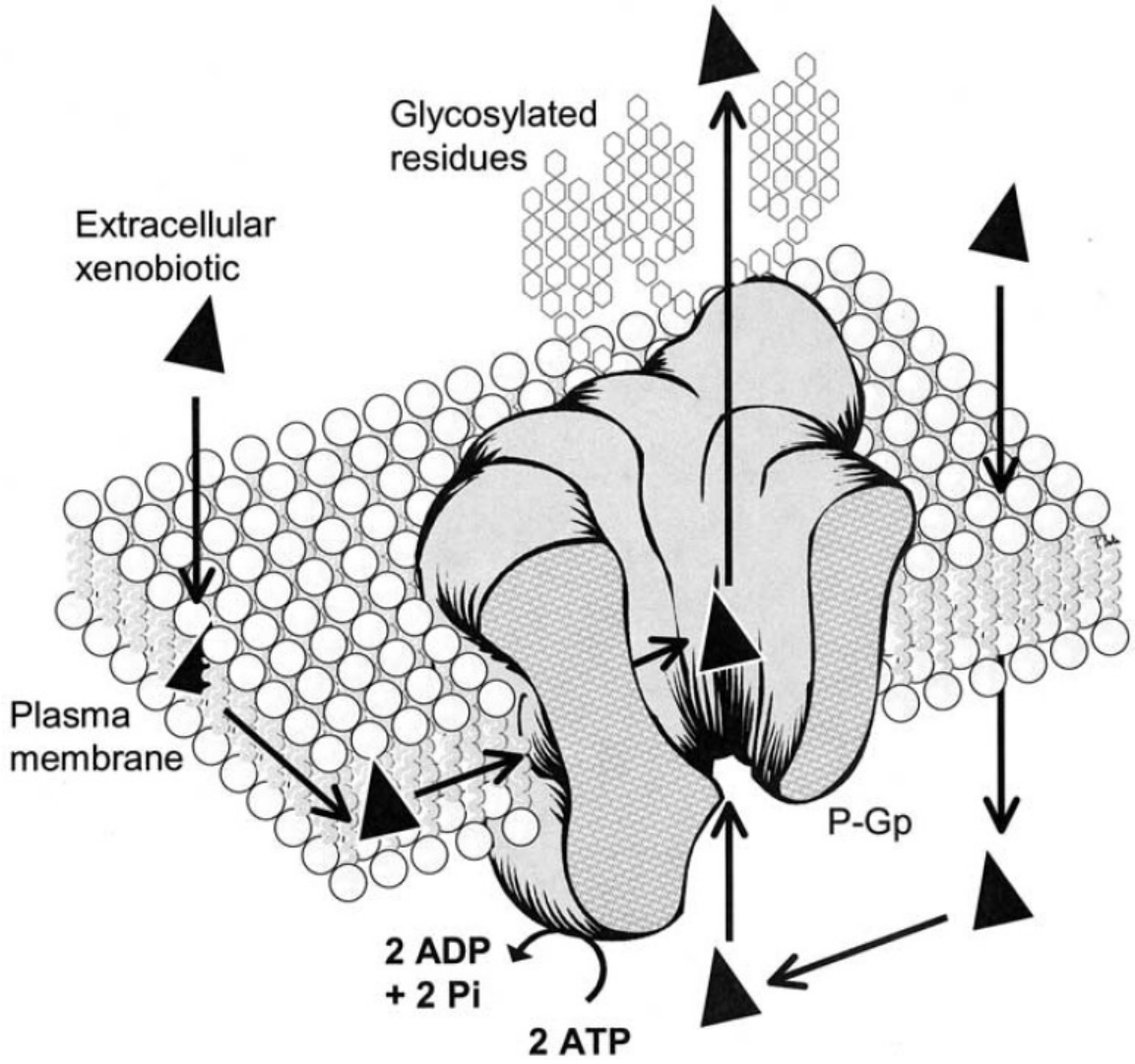

Figure 9. ABCB1 acts as an ATP-driven drug efflux pump on the cell membrane. Marzolini, C.; Paus, E.; Buclin, T.; Kim, R. B., Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. *Clinical Pharmacology & Therapeutics* **2004,** *75* (1), 13-33.

4. Collateral Sensitivity

4.1. Early historical observations

In one of the earliest mentions of collateral sensitivity (CS) by Waclaw Szybalski and Vernon Bryson, they describe the phenomenon by which drug-resistant bacterial cells, which are cross-resistant to several antibiotics, become more sensitive to other agents [280]. Collateral sensitivity in ABCB1 expressing MDR cells was first described by Bech-Hansen *et al*. in their study of colchicine-resistant Chinese hamster ovary cells [281]. These MDR cells were found to be hypersensitive towards certain analgesic compounds such as procaine, tetracaine and xylocaine, as well as hormones such as deoxycorticosterone, 1-dehydrotestosterone and 5β pregnan-3,20-dione, compared with the parental, drug-sensitive cell lines [281]. ABCB1-mediated collateral sensitivity is described as the phenomenon by which multidrug-resistant cells are more sensitive to certain compounds than are the parental, drug-sensitive cell lines from which they are derived, in an ABCB1-dependent manner.

4.2. Verapamil

Verapamil, a phenylalkylamine L-type calcium channel blocker, has been used for the treatment of cardiac irregularities [282-285] since 1982 [286]. Its relevance in the interest of cancer treatment came about after it was discovered that it enhanced cytotoxicity of vincristine against a leukemia cell line in vitro and in vivo [287]. Vincristine, an anti-mitotic chemotherapeutic agent [288, 289], was commonly being used in the treatment of lymphoma [290] and especially acute childhood leukemia [291] starting in the early 1960s. Once resistance to the drug surfaced [292, 293], the

discovery of alternative agents of treatment or methods to overcome this resistance became a priority. Early observations showed that the addition of verapamil increased the cytotoxicity of vincristine to resistant cells and tumors, as in the case of the adriamycin-resistant cell line P388/ADM that became sensitive to vincristine and the vincristine-resistant cell line P388/VCR that was sensitized to adriamycin [287, 294]. A 3- to 4- fold increase of vincristine cytotoxicity was also observed in inherently-resistant Lewis lung carcinoma, B16 melanoma and colon adenocarcinoma cells upon the addition of verapamil [295], showing that the drug could be used on cell lines that expressed endogenous levels of ABCB1. A 90-fold increase in vincristine cytotoxicity was also observed in a human glioblastoma cell line in the presence of verapamil [296]. This promoted interest in the *in vitro* study of the calcium channel blocker as a potentiator of vincristine resistance [297-299].

The breakthrough discovery of ABCB1 [137] and the link between its expression and multidrug resistance [138, 139] steered cancer and resistance research in a new direction: *in vitro* and clinical reversal studies linking verapamil to ABCB1 inhibition swept the field in the late 1980s and early 1990s. It was discovered that membrane vesicles from the multidrug resistant carcinoma cell line KB-C4 and KB-V1 bound 10- to 20- fold more to verapamil than did their sensitive counterpart KB-3-1 [300]. Even pilot studies combining verapamil to vincristine and doxorubicin were hopeful that the drug could alleviate the burden of ABCB1-mediated resistance. Such was the case in one study that showed that verapamil increased the concentration of both vincristine and doxorubicin in the malignant cells of patients with end-stage myeloma, which overexpressed ABCB1 [301]. In another clinical study, 18 patients who failed a

doxorubicin/vincristine containing regimen due to lack of response, presumably from the presence of ABCB1, were treated with a simultaneous infusion of verapamil – this lead to a 72% response rate and 28% total remission [302]. In additional studies, the transport of verapamil by ABCB1 enhanced antitumor chemotherapeutic drug accumulation [303-306], which may have been done via the competition of binding sites on the protein [307]. Though verapamil made it to clinical trials, unacceptable toxicity levels and unexpected pharmacokinetic interactions led to the dwindling of the idea of using verapamil as an inhibitor of ABCB1 for the circumvention of MDR1-mediated multidrug resistance [256, 308-311].

The connection between ABCB1 and verapamil *hypersensitivity* was also made around the same time: Chinese hamster ovary cells, which were 100-fold resistant to vincristine, were found to display a hypersensitivity towards verapamil treatment alone [298, 299]. So although the interest of verapamil as an *inhibitor* of ABCB1 function was all but forgotten, its use as an MDR1 *activator* is what led to the discovery of the concept of collateral sensitivity. Verapamil acts as a ABCB1 inhibitor at concentrations high enough to suppress its ATPase activity, but shows stimulation of the ATPase pump at lower concentrations [312-315]. The consensus of verapamil function on ABCB1 is thus that of a biphasic trend: for MDR cells overexpressing ABCB1, cell survival decreases with increasing drug concentration, up until a peak concentration, and increases again as drug concentration increases, up to a concentration where necrotic cytotoxicity occurs. This is the case for the ABCB1-overexpressing multidrug resistant Chinese hamster ovary cell line, $CHO^{R}C5$. Survival for these cells decreases until the minimum survival at a concentration of 10 μM verapamil (IC_{90}), and then increases to 50 μM until

high levels of the drug (100 μM) are attained again [314]. This correlates with a concurrent increase in ATPase activity until the 10 μM concentration, followed by a decrease at higher levels of verapamil [314] (Figure 10).

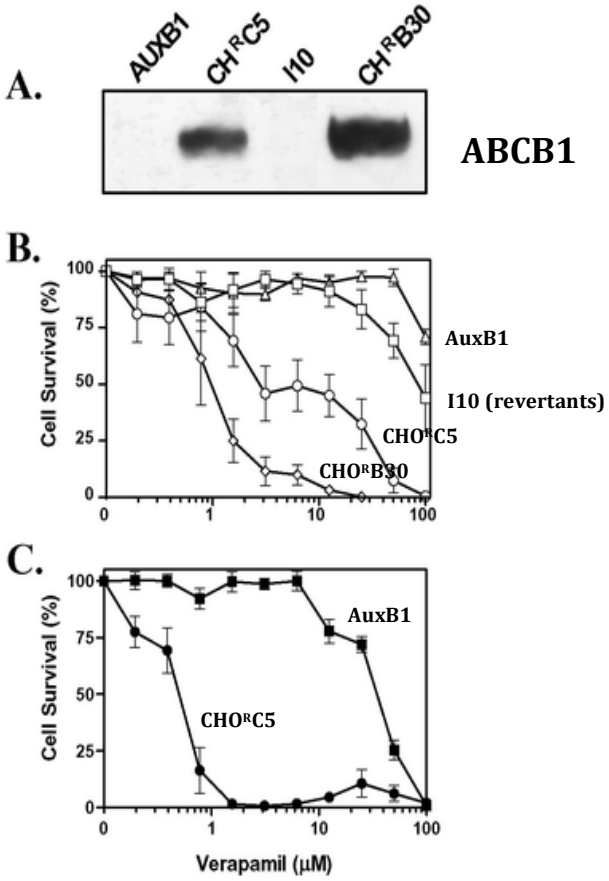

Figure 10. Verapamil preferentially targets ABCB1-expressing MDR cells. Karwatsky, J.; Lincoln, M. C.; Georges, E., A mechanism for P-glycoprotein-mediated apoptosis as revealed by verapamil hypersensitivity. *Biochemistry* **2003,** *42* (42), 12163-12173. **The legend from the original paper is the following:** "Western blot and verapamil dose−response assays for sensitive and multidrug resistant CHO cells. (A) The relative amount of P-gp1 protein is shown by Western blot using the P-gp1 specific mAb, C-219, in the parental AUXB1 cells, the multidrug-resistant CH^RC5 and CH^RB30 cells, and I10 (drug-sensitive revertant derived from CH^RC5). (B) AUXB1 (white squares), CH^RC5 (white circles), CH^RB30 (white diamonds), and I10 (white triangles) were exposed to increasing concentrations of verapamil for 24 h, followed by 4 days of growth in drug-free conditions. The surviving colonies were stained with methylene blue and quantified by spectrophotometry. (C) AUXB1 (black squares) and CH^RC5 (black circles) were exposed to increasing verapamil concentrations for 5 days and survival was quantified as in (B)."

The current accepted mechanism of verapamil-mediated collateral sensitivity involves the cycling of the drug by ABCB1, which stimulates the ATPase of the protein. Therefore, verapamil seems to stimulate, or activate, the ATPase at lower concentrations, and is forcing the cell to rapidly deplete ATP levels in order to efflux the drug. In fact, a reduction of the ATP pool of CHO^{R}C5 cells of up to 50% was seen following treatment with 10 μM verapamil [314], similarly to a MDR human ovarian cell line 2780AD following exposure at 8 μM verapamil [316]. Cells with rapidly declining levels of ATP can undergo oxidative phosphorylation via the mitochondrial electron transport chain (ETC) to produce higher levels of ATP. This consequently generates high levels of reactive oxygen species and induces apoptosis [317, 318] (Figure 12). Levels of glutathione (GSH), a ROS scavenger, decreased in CHO^{R}C5 cells following the addition of verapamil at a concentration of 10 μM [314]. Blocking of the function of ABCB1 with chemical inhibitors such as PSC833 (Valspodar) [314] or knockdown by siRNA [315] successfully obliterated the effect of verapamil as a collateral sensitivity agent, underlining the importance of functional ABCB1 for the effect to take place (Figure 11).

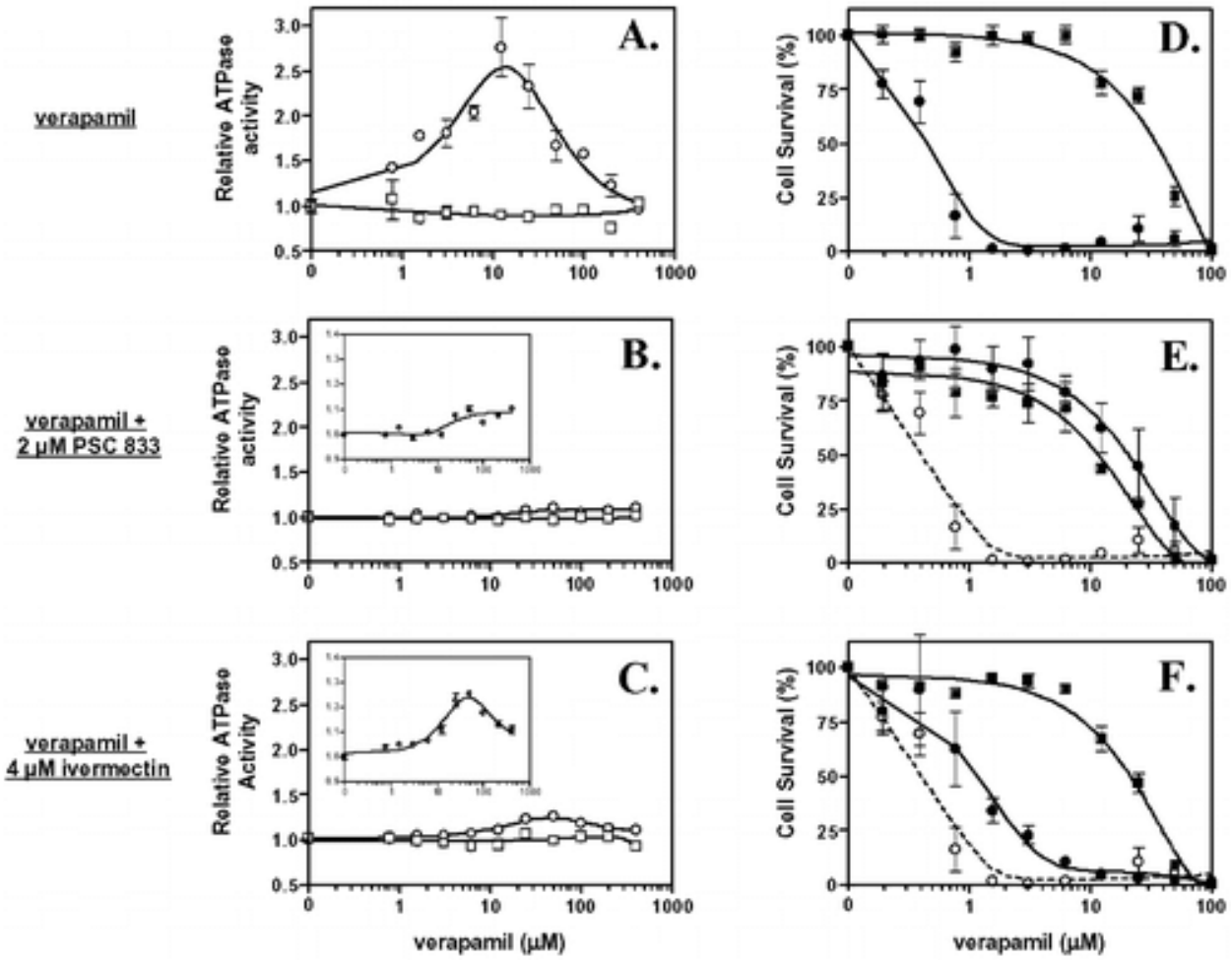

Figure 11. Verapamil increases ATPase activity in ABCB1-expressing MDR cells. Karwatsky, J.; Lincoln, M. C.; Georges, E., A mechanism for P-glycoprotein-mediated apoptosis as revealed by verapamil hypersensitivity. *Biochemistry* **2003,** *42* (42), 12163-12173. **The legend from the original paper is the following:** "Effects of verapamil, PSC 833, and ivermectin on cell survival and P-gp1 ATPase in AUXB1 and CH^RC5. (A−C) ATPase activity of AUXB1 (white squares) and CH^RC5 (white circles) was measured using purified plasma membranes exposed to increasing concentrations of verapamil. In B and C, ATPase activity was measured with an additional pretreatment with 2 µM PSC 833 or 4 µM ivermectin. (D−F) Survival of AUXB1 (black squares) and CH^RC5 (black circles) exposed to increasing concentrations of verapamil was determined by staining colonies with methylene blue and quantified by spectrophotometry. In E and F, cell survival was determined with an additional pretreatment with 2 µM PSC 833 or 4 µM ivermectin. For a comparison, the dashed line in E and F shows the survival curve of CH^RC5 with verapamil alone from D."

One important aspect of verapamil, a feature that separates it from the other attempted reversal agents, is that it does not *inhibit* the function of ABCB1 at concentrations that cause a collateral sensitivity effect to occur in MDR cells, but rather *exploits* it. This is a crucial characteristic of collateral sensitivity drugs, as this will prevent the toxicity brought on by the inhibition of the natural protective function of ABCB1 in biological barriers [319].

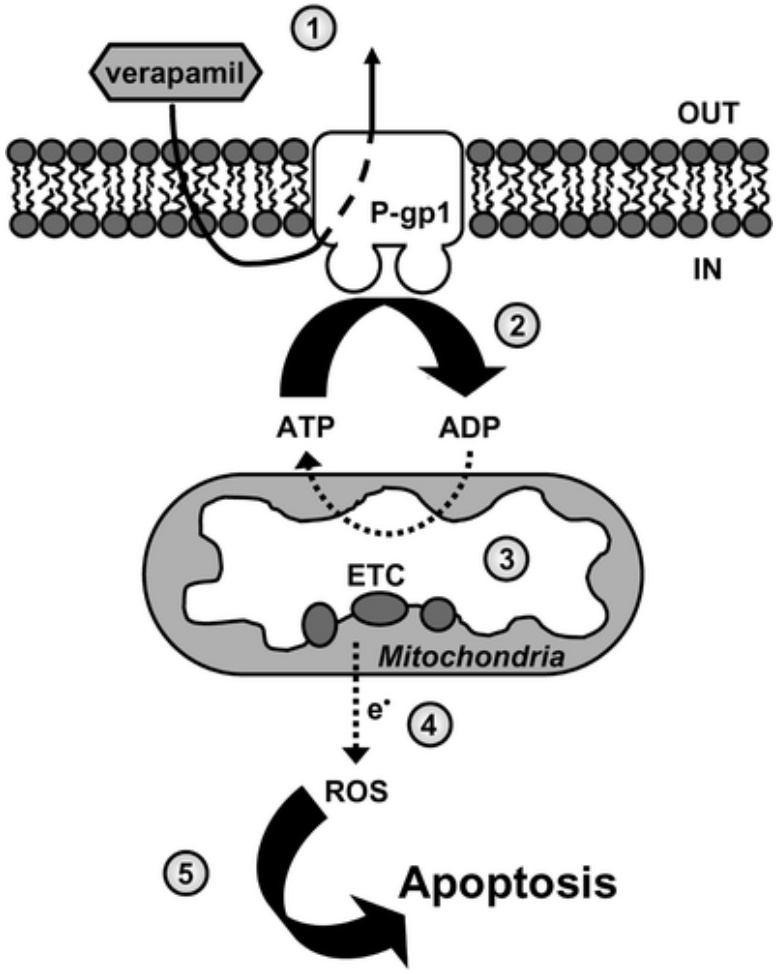

Figure 12. A mechanism for VP-mediated, ATPase-dependent collateral sensitivity. Karwatsky, J.; Lincoln, M. C.; Georges, E., A mechanism for P-glycoprotein-mediated apoptosis as revealed by verapamil hypersensitivity. *Biochemistry* **2003,** *42* (42), 12163-12173.

4.3. Steroid hormones

Though progesterone (PRO) [178, 320] and deoxycorticosterone (DOC) [321] interact with ABCB1, the mechanism by which MDR cells are collaterally sensitive to the drugs is unrelated to the reversal of ABCB1 function. Progesterone increases the ATPase activity of ABCB1 [322], and Laberge *et al.* showed that both PRO and DOC stimulated ATPase activity in MDR1-overexpressing Chinese hamster ovary cells, which lead to a depletion of ATP in the cells, an increase in oxidative phosphorylation and subsequently, ROS, that lead to cell death of the MDR cells by apoptosis [323] much like the mechanism of verapamil.

L1210/VR cells were shown to be collaterally sensitive to both deoxycorticosterone and dexamethasone [324], though the mechanism at the time was unknown. In spite of the fact that the dexamethasone analog 17-Deoxydexamethasone was shown to inhibit ABCB1 function [325], the drug actually stimulated ABCB1 drug efflux activity in a rat brain endothelial primary cell line, GPNT [326]. With the knowledge that we have now about the correlation between an increase in ABCB1 ATPase activity and a rise in ROS, we can infer that the collateral sensitivity mechanism may be ROS-mediated.

More recently, ABCB1-expressing MDR Chinese hamster ovary cell lines $CHO^{R}C5$ and $CHO^{R}B30$ were shown to be collaterally sensitive to N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine (DPPE), a tamoxifen derivative with antihistamine activity [327]. Originally, DPPE was shown to be effective with doxorubicin for late-stage breast cancer patients, as it increased overall survival in a Phase III clinical trial [328, 329]. This prompted the exploration of DPPE as a collateral sensitivity agent. In fact, the IC_{50} values for DPPE were approximately 4.5- and 7.3- fold

lower for CHO^{R}C5 (4.2 μM) and CHO^{R}B30 (2.6 μM) cells, respectively, compared to their drug-sensitive parental cell line, AuxB1 (19 μM) [327]. This is interesting to note, since the cells with higher protein levels of ABCB1 (Ch^{R}B30) were collaterally sensitive to the effects of DPPE than those expressing lower levels of the protein (CHO^{R}C5), underlining the importance of ABCB1 for conferring collateral sensitivity. The mechanism of action of DPPE for the collateral sensitivity effect is similar to that of verapamil, wherein it activates the ABCB1 ATPase and depletes ATP levels, prompting an increase in oxidative phosphorylation and thus increasing the levels of ROS in the cells. However, it is important to note that like progesterone, DPPE is not transported by ABCB1.

4.4. The use of collateral sensitivity for cancer treatment

While the overall purpose of studying ABCB1-mediated drug resistance and collateral sensitivity is to better understand the elusive mechanisms of ABCB1, the ultimate purpose is to determine the feasibility of drug cycling during cancer chemotherapy in order to avoid or decrease the emergence of MDR1-overexpressing multidrug resistant tumors [330-333].

A major caveat of conventional chemotherapy is the emergence of the multidrug-resistant tumor, which arises when drug-sensitive cells are eliminated by chemotherapy but drug-resistant cells continue to proliferate. Whether drug resistance is due to the selection of existing drug-resistant cells in a heterogeneous population, or the drug-induced changes in genes coding for resistance-related proteins, the use of collateral sensitivity drugs to eliminate these cells during the early phases of treatment is an appealing idea. The rudimentary idea would be to eliminate drug-sensitive tumor

cells with standard chemotherapy, and then to treat MDR1-overexpressing MDR cells with collateral sensitivity drugs to either eliminate all the MDR cells completely, or revert them back to the drug-sensitive phenotype (Figure 13). However, the effect of collateral sensitivity drugs on surviving MDR cells has yet to be explored, and is an important aspect in the overall understanding of the collateral sensitivity mechanism.

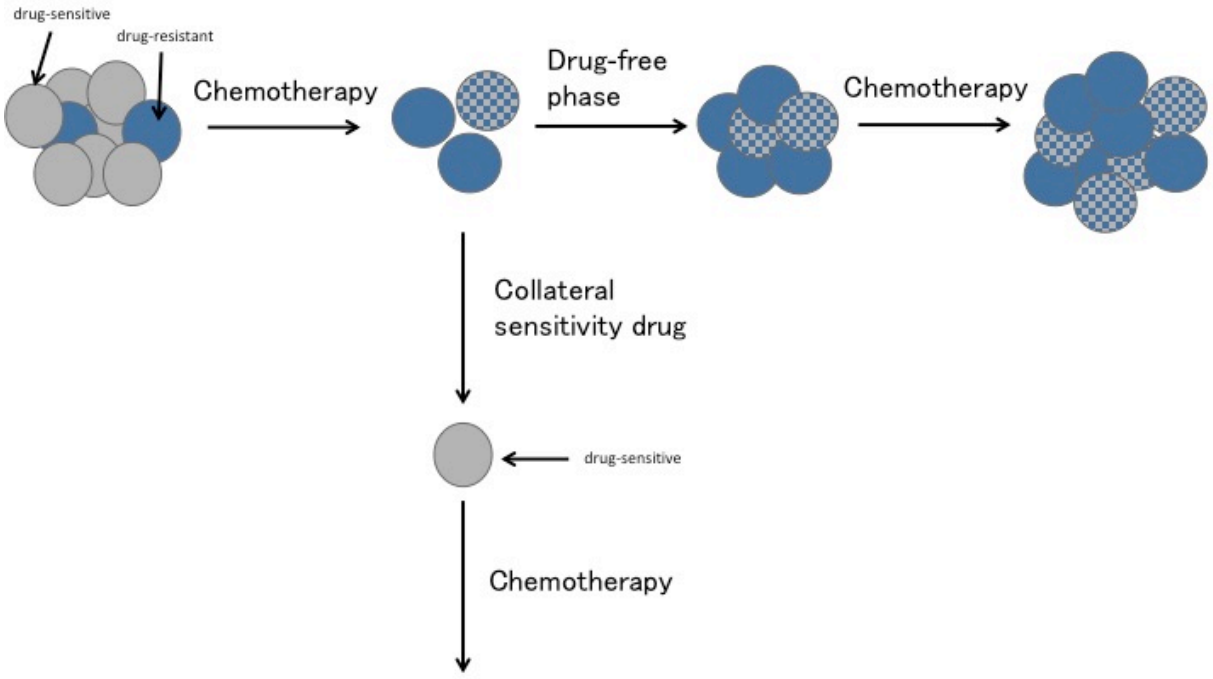

Figure 13. A possible use for collateral sensitivity drugs in the clinical setting. According to the hypothesis of this work, collateral sensitivity drugs have the potential to be used to eliminate or prevent the occurrence of drug resistant tumor cells.

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CONNECTING STATEMENT 1

The previous chapter presented a review of cancer treatment and the setbacks brought on by multidrug resistance. We described that much of the clinical multidrug resistance is caused by the overexpression of ATP binding cassette transporters, especially ABCB1, which efflux chemotherapeutic drugs out of cells. Unfortunately, the inhibition of the efflux function of ABCB1 is not a feasible resolution to this problem, as this causes toxicity in patients who are undergoing chemotherapy. However, earlier works show that some compounds such as verapamil are able to target multidrug resistant cells, in a process called collateral sensitivity. While these compounds preferentially target MDR cells, some survive. In this first manuscript (Chapter 2), we explore the effect of selection by the collateral sensitivity agents verapamil and tamoxifen on surviving drug-resistant clones, the first investigation of its kind.

2

Tamoxifen down-regulates ABCB1 expression and up-regulates enolase I expression in multidrug resistant cells

Contents

Tamoxifen down-regulates ABCB1 expression and up-regulates enolase I expression in multidrug resistant cells

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Key words: P-glycoprotein; Collateral Sensitivity; Multidrug Resistance; ABCB1; enolase; and Tamoxifen.

The authors disclose no potential conflicts of interest.

COMMON ABBREVIATIONS

MDR: multidrug resistance or multidrug resistant
ABCB1: ATP-binding cassette transporter B1 (MDR1/P-gp)
TAM: tamoxifen
VP: verapamil
ROS: reactive oxygen species
GSH: glutathione
CS: collateral sensitivity
DPPE: N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethamine

ABSTRACT

The emergence of drug resistance remains an obstacle in the clinical treatment of cancer. One of the most efficient mediators of drug resistance is the presence of ATP-binding cassette transporters (ABC) such as MDR1 (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2), which act as drug efflux pumps to extrude chemotherapy out of cancer cells. Recent developments in the studies of drug resistance have identified compounds such as verapamil and DPPE that specifically target ABCB1-expressing multidrug resistant cells, through an ATP-dependent ROS-generating mechanism. This sensitivity phenotype of drug-resistant cells to certain drugs was referred to as "*collateral sensitivity*". Tamoxifen is an estrogen-receptor antagonist commonly used to prevent the recurrence of estrogen receptor-positive breast cancer. In this report, we demonstrate that treatment of $CHO^{R}C5$ cells and clones with concentrations of tamoxifen or verapamil that affect ABCB1-positive cells but do not kill ABCB1-negative cells down-regulates ABCB1 protein and mRNA expression in these MDR clones. This change in ABCB1 expression results in an increased sensitivity of the MDR cell clones to the chemotherapeutic agents colchicine and doxorubicin. Additionally, cross-resistance between verapamil and tamoxifen seen in selected clones suggests a shared mechanism of collateral sensitivity between the two compounds. Importantly, we show for the first time that down-regulation of ABCB1 expression expression leads to up-regulation of α-enolase (enolase I) protein levels and activity. These findings demonstrate a possible effect of ABCB1 expression on the metabolic homeostasis of MDR cells.

INTRODUCTION

Clinical multidrug resistance, intrinsic or acquired, remains an obstacle in the treatment of cancer patients with chemotherapeutic drugs. P-glycoprotein (P-gp, MDR1) is a member of the B-subfamily (ABCB1) of the ATP binding cassette (ABC) family of transporters and known marker of poor prognosis and clinical drug resistance in many different types of cancer, including those of the bladder [1], cervix [2], lung [3], breast [4] and gastrointestinal system [5, 6]. Like other members of the ABC family, ABCB1 relies on energy derived from ATP hydrolysis to cause drug efflux. While ABCB1 is expressed normally in tissues where it is thought to have a protective function [7-9], this ATP-dependent drug efflux pump mediates the transport of a variety of different compounds, including chemotherapeutic drugs, hormones, cholesterol and other xenobiotics [10, 11]. Efforts to overcome ABCB1-mediated clinical drug resistance have led to the development of progressively more specific and effective inhibitors (i.e., 3rd generation MDR-reversing drugs) that unfortunately failed to provide significant clinical benefits due to unacceptable toxicity levels in patients [12-16]. Consequently, the use of ABCB1-inhibiting compounds for clinical benefit remains challenging.

Earlier work suggesting that verapamil, a known competitive inhibitor of the ABCB1 drug efflux pump, seemed to target vincristine-resistant cells [17] preceded the discovery that ABCB1-overexpressing multidrug-resistant (MDR) cells were hypersensitive, or collaterally sensitive, to verapamil at sub-inhibitory concentrations [18]. The proposed mechanism of this collateral sensitivity effect is that ABCB1-positive cells require higher levels of ATP hydrolysis to efflux verapamil across the cell membrane, depleting levels of ATP in MDR cells but not in cells that have less ABCB1. This depletion of ATP pools

putatively prompts increased levels of oxidative phosphorylation, which in turn increase the levels of reactive oxygen species produced by the cells at higher rates than normal, and leads to apoptotic cell death [19, 20]. Other drugs known to induce a collateral sensitivity effect in ABCB1 overexpressing drug-resistant cells include NSC73306 [21], 2-Deoxy-D-glucose [22], steroid hormones such as progesterone and deoxycorticosterone [23], KP772 [24] and N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethamine [25] (DPPE, a tamoxifen derivative). Given that ABCB1 remains an important target of clinically resistant tumor cells, it was of interest to examine the effects of collateral sensitivity drugs, at clinically achievable concentrations, on ABCB1 expression in MDR cells. Earlier work on non-clonal, heterogeneous populations of cells has shown that verapamil decreases ABCB1 levels [26]. In this present study, we evaluated the effects of verapamil and tamoxifen pressure on individual cell clones of multidrug-resistant cell lines on ABCB1 expression and cell metabolism. [27-30]

MATERIALS AND METHODS

Chemicals and reagents - Methylene blue hydrate (M4159), sulforhodamine B sodium salt (S9012), trichloroacetic acid (T6399), bovine serum albumin (A2153), tamoxifen (T5648), doxorubicin hydrochloride (D1515), colchicine (C9754) and verapamil (V4629) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of the highest commercially available grade.

Cell Culture and clonal selection - Chinese hamster ovary (AuxB1 and $CHO^{R}C5$) and human triple negative breast cancer cells (MDA-MB-231 and MDA-$Doxo^{400}$) were cultured in αMEM or DMEM media supplemented with 8%-10% fetal bovine serum (Wisent 080-150) at 37°C in 5% CO_2. $CHO^{R}C5$ cells were cultured in 5 μg/mL colchicine prior to selection of clones. $CHO^{R}C5$ cells were trypsinized and counted, and a serial dilution in 96-well plates was carried out to a calculated value of 0.5 cells per well in colchicine-free media. Once cells adhered, colchicine-containing media was added to each of the wells to a final concentration of 5 μg/ml. Cells were allowed to proliferate to approximately 50 cells prior to transferring colonies to a 24-well plate where they were allowed to proliferate in colchicine-containing media. Clones (A6 and A10) were then cultured in T-25 flasks in colchicine-containing media to approximately 70% confluency. Colchicine was removed, and cells were cultured as usual for 21 days in the presence of 0 μM (referred to as untreated), 1 μM or 5 μM tamoxifen to establish tamoxifen-pressured cell clones. For verapamil, cells were cultured for 21 days in the presence of 0 μM (referred to as untreated), 10 μM or 25 μM verapamil to establish verapamil-pressured clones. MDA-MB-231 cells resistant to 400 nM Doxo (MDA-$Doxo^{400}$) were pressured for 21 days in the

presence of 0 μM, 2.5 μM or 10 μM tamoxifen. The CRISPR knockout protocol of the ABCB1 CHO cell lines (AuxB1, $CHO^{R}C5$) and their characterization is described elsewhere (Limniatis and Georges, ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells shows complete reversal of drug resistance and collateral sensitivity phenotypes 2020; manuscript in preparation).

For the 20-hour selection in 5 μM tamoxifen, clones A6 and A10 were pressured in 5 μM tamoxifen for 20 hours, followed by mRNA extraction, cDNA synthesis and qPCR protocol, as described below.

Protein extraction and Western immunoblotting - Cells were harvested, without the use of trypsin, with phosphate-buffered saline (PBS) solution containing 5 mM ethylenediaminetetraacetic acid (EDTA). Pelleting of the cells was done by centrifugation, and a series of cold washes with PBS was followed by cell lysis in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25 % sodium deoxycholate, 1% NP-40, pH = 7.4) containing protease inhibitors (Thermo Scientific 78429), followed by protein quantification using the Pierce™ BCA Protein Assay Kit (Thermo Scientific 23225). For Western immunoblotting, cell lysates (20 μg) were loaded onto 8% or 10% SDS-PAGE gels without heating the samples, and transferred onto Immobilon-P® PVDF membranes (Sigma Aldrich IPVH) for 3 hours at 400 mA. Membranes were probed for ABCB1 (1:1000 v/v; C494 mouse monoclonal antibody), anti α-enolase (1:5000 v/v; rabbit polyclonal from Santa Cruz Biotechnology) or anti-α-tubulin (1:1000 v/v; mouse monoclonal antibody from NeoMarkers) overnight at 4°C. Membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibodies, anti-rabbit (1:5000 v/v; from BioRad) or

anti-mouse (1:1000 v/v; from Thermo Scientific) for 90 minutes at room temperature, and detection was carried out using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific).

Cytotoxicity assays - Cell growth assays were carried out using the sulforhodamine B assay or clonogenic method. For the clonogenic assay, cells were seeded at between 5-15% confluency in a 48-well plate, and compounds were added 24h later at applicable concentrations. Cells were allowed to proliferate until visible colonies formed and covered approximately 70% of the plate, between 7-10 days later. CHO and MDA colonies were stained in 0.5% and 1% methylene blue solution, respectively, and washed. The stain was solubilized using a 0.1% w/v solution of SDS/PBS and read at 660 nm using the Synergy™ H4 plate reader (BioTek). For the sulforhodamine B assays, the method was followed as previously described [31]. Briefly, cells were seeded at 60% confluency in a 96-well plate, and compounds were added 24h later at applicable concentrations. Cells were allowed to grow for 3 days, after which the assay was stopped by the addition of 100 µL ice-cold 10% (w/v) TCA and incubated at 4^{o}C for 1 hour. The plates were rinsed with water and SRB stain was added (0.057% (w/v) SRB in 1% (v/v) acetic acid) for 30 minutes. The plates were rinsed with 1% acetic acid, dried and solubilized in 10 mM Tris buffer pH 10.5, and absorbance was read at 510 nm using the Synergy™ H4 plate reader (BioTek). Data analysis was done using GraphPad Prism®, version 6.0.

mRNA isolation and cDNA synthesis - Cells were cultured as usual to 70% confluency, collected via trypsinization and pelleted. Pellets were resuspended in 1 ml TRIzol reagent

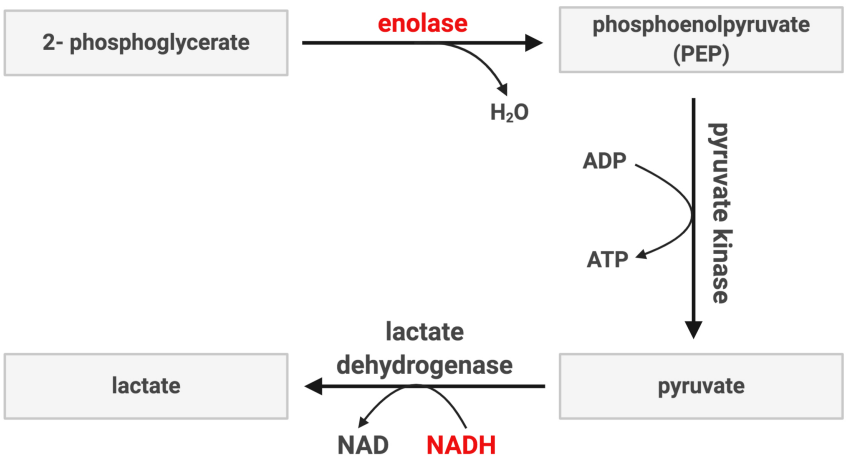
(ThermoFisher 15596026), incubated at room temperature for 5 minutes and gently mixed. Phase separation was achieved by adding 200 µL chloroform, vortexing for 15 seconds and incubating at room temperature for 3 minutes. Samples were centrifuged for 18 000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh 1.5 mL tube, and RNA precipitation was done by adding 500 µL 2-propanol and incubating samples at room temperature for 10 minutes and centrifuged at 18 000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed 3 times with 75% ethanol followed by spinning at 10 500 x g for 5 minutes at 4°C. All ethanol was removed and the pellet was resuspended in 30 µL of RNAse- and DNAse-free water. cDNA synthesis was carried out using the SuperScript® IV Reverse Transcriptase kit by Invitrogen (ThermoFisher 18090010).

<u>*RT-qPCR*</u> - Primers were designed to cover exon-exon junctions on *ABCB1* and *β-actin*.

	ABCB1	***β-ACTIN***
Forward	CTC TGG ATA AGG CTA GAG AAG GC	CCT TCC TGG GTA TGG AAT CCT G
Reverse	CCT GCT GTC TGT GTC ATG ACA AG	CAG GAG GAG CAA TGA TCT TGA TCT TC

The StepOne ™ Real-Time PCR System (ThermoFisher Scientific) was used to carry out the qPCR experiment. Primers efficiencies were assayed via a melting curve with serial dilutions. The FastStart Universal SYBR Green Master (Rox) from Millipore Sigma was used as the master mix. All manufacturers' protocols were followed. Relative mRNA was established by comparing samples to parental cell lines.

Enolase activity assay - Adapted from Fukano et al., [32]. Enolase activity is measured indirectly in cell lysates, quantified by the decrease in NADH fluorescence per sample per minute, as a measure of NADH conversion to NAD, based on the following reactions:



Thus, higher enolase activity results in a greater decrease in fluorescence ($360_{ex}/460_{em}$) per minute in each sample, as a measure of NADH converted to NAD per minute.

Cells were grown to 80% confluency, collected by scraping and pelleted. The pellet was washed three times with ice-cold PBS and the cells were lysed using the same RIPA buffer as for protein extractions and centrifuged at 18 000 x g for 20 minutes. Protein quantification was done using the Pierce™ BCA Protein Assay Kit, and all samples were equalized for 1 µg/µL protein using the RIPA buffer as diluent. Samples were kept on ice for the duration of the preparation steps for the experiment, until ready to be placed in the plates for the kinetic enzymatic assay.

Assay buffer A was prepared as follows: 100 mM triethanolamine (Thermofisher T407), 0.2 mM β-NADH (Sigma Aldrich N8129), 30 mM magnesium sulfate (JT Baker 2500), 120 mM potassium chloride (Sigma Aldrich P3911), 1.75 mM ADP (Sigma Aldrich A2754), 20 U PK/ 30 U LDH (Sigma Aldrich P0294). Assay buffer B was prepared the same way as buffer A, with the addition of 4.5 mM 2-phosphoglycerate (Sigma Aldrich 79470).

Twenty-fold dilutions of protein lysates were prepared in sample buffer A and 50 µL was put in a clear bottom black-well plate (in triplicate). NADH fluoresces at $360_{ex}/460_{em}$, so baseline fluorescence was measured, using the Synergy™ H4 plate reader. A kinetic reading protocol was set up for reads at $360_{ex}/460_{em}$ every 45 seconds for 12 minutes. In quick succession, 50 µL of buffer B was added to each well and the kinetic reading was started. The average decrease of NADH fluorescence per minute was calculated for each sample.

Statistical analysis **-** Data is represented as a mean ± standard deviation, and statistical significance was computed with GraphPad Prism version 6.0 to 8.0 using one-way ANOVA. Probability (P) values of less than 0.05 were considered statistically significant.

RESULTS

Collateral sensitivity of ABCB1-overexpressing multidrug-resistant cells towards tamoxifen - Tamoxifen has been used as a chemopreventative agent for women who have recovered from estrogen-positive (ER+) breast cancer, or those who are at high risk of developing breast cancer.

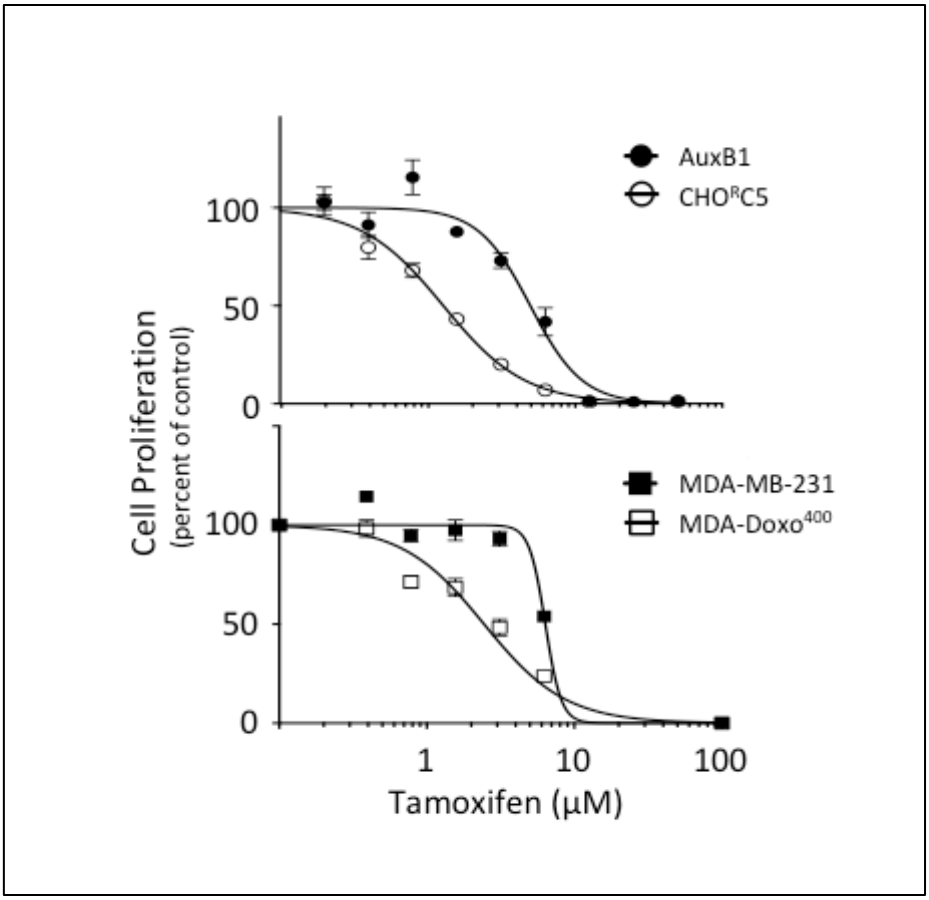


Figure 1. Effect of tamoxifen on growth of drug-resistant and drug-sensitive cells. Drug-sensitive AuxB1 and MDA-MB-231 cells and drug-resistant CHO^{R}C5 and MDA-Doxo^{400} cells were treated with a range of concentrations of tamoxifen for 7 days. Cell growth was estimated using the clonogenic assay and methylene blue dye. Cell growth was plotted as a percentage of cells treated with carrier solvent. Graphs represent the mean ± standard deviation for three independent experiments done in triplicate. CHO^{R}C5 and MDA-Doxo^{400} cells, which are both drug resistant and over-express ABCB1, are collaterally sensitive to tamoxifen than the drug-sensitive parental cell lines.

The results in figure 1 show that the drug-resistant cells CHO^{R}C5 and MDA-Doxo^{400} were more sensitive, or collaterally sensitive, to tamoxifen (1.18 ± 0.09 μM and 2.97 ± 0.52 μM, respectively), compared to the drug sensitive cell lines AuxB1 and MDA-MB-231 (5.44 ± 0.67 μM and 6.64 ± 0.27 μM, respectively). Collateral sensitivity (CS) drugs are compounds that selectively target ABCB1-overexpressing multidrug resistant cells. While the mechanism of tamoxifen-mediated collateral sensitivity on MDR cells has not yet been fully resolved, its preference for ABCB1-expressing MDR cells made it an interesting candidate for studying the effects of selection of CS drugs on MDR cells. In addition, verapamil is a well established CS drug, preferentially targeting ABCB1 overexpressing CHO^{R}C5 compared to the parental cell line AuxB1, in a ABCB1-dependent ATPase stimulating mechanism that leads to increased ROS production and apoptosis [18, 20]. Verapamil at a concentration of 15 μM decreases ABCB1 expression in approximately 72 hours [26]. We therefore used verapamil as control to verify and further elucidate the mechanism of ABCB1 downregulation in clonal cells.

Effect of tamoxifen on ABCB1 expressing MDR cells. - It was of interest to investigate whether cells surviving in the presence of the collateral sensitivity drug tamoxifen were doing so by the downregulation of ABCB1 expression at the protein level, or whether a selection of clones naturally expressing lower levels of ABCB1 was taking place in a heterogeneous population of cells. To investigate this, we used clonal populations of CHO^{R}C5 cells as a starting point for selection, CHO^{R}C5 clones A6 and A10. The latter clones were then pressured in 1 μM (corresponding to approximately 50% survival) or 5 μM tamoxifen (corresponding to approximately 10% survival) for 21 days and checked for

ABCB1 expression by Western blot. As shown in figure 2A, clones A6 and A10 selected in 5 μM tamoxifen, and to a lesser level in 1 μM, showed a marked decrease in ABCB1 expression.

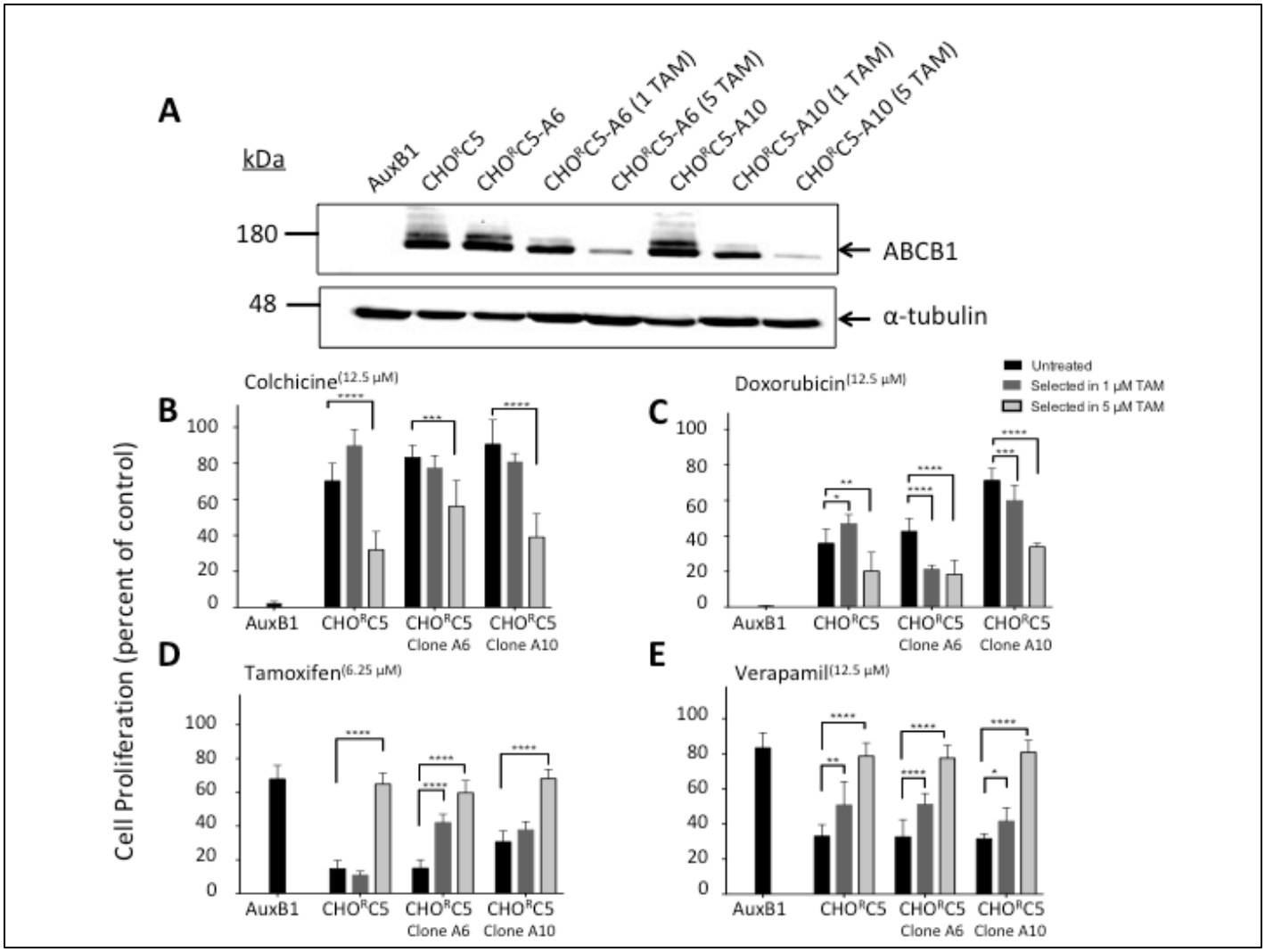


Figure 2. Effect of tamoxifen on multidrug-resistant cells and clones. CHOᴿC5 cells were diluted in order to grow clonal populations of cells. Clones A6 and A10 were pressured in 1 μM and 5 μM tamoxifen for 21 days. (A) A western blot was done using the C494 anti-ABCB1 mAb and anti-α-tubulin was used as a loading control. Results show that ABCB1 was down-regulated when MDR cells were selected in 5 μM tamoxifen. This decreased expression of ABCB1 led to a subsequently higher sensitivity towards (B) colchicine and (C) doxorubicin, and higher resistance towards (D) tamoxifen and (E) verapamil. Cell growth was plotted as a percentage of cells treated with carrier solvent and assessed using the SRB survival assay (Sulforhodamine B dye). Graphs represent the mean ± standard deviation for three independent experiments done in triplicate.

To establish whether a decrease in ABCB1 expression was associated with a decrease in drug resistance, cytotoxicity assays were carried out on the same CHO^{R}C5 cells and clones that were selected in 1 μM and 5 μM tamoxifen. As seen in figure 2B, in the presence of 12.5 μM colchicine, CHO^{R}C5 population cells had 73.39 (± 10.03) % survival, while population cells selected in 1μM and 5 μM tamoxifen had 77.63 (± 15.00) % and 35.97 (± 6.49) % survival, respectively. Similarly, untreated CHO^{R}C5 clones A6 had a survival of 84.18 (± 8.85) % and clones selected in 1 μM and 5 μM tamoxifen had 77.40 (± 7.24) % and 63.69 (± 4.91) % survival, correspondingly. Clone A10 had a survival of 94.51 (± 10.10) % in 12.5 μM colchicine, and A10 clones selected in the same concentrations of tamoxifen had survival of 80.71 (± 4.74) % and 45.45 (± 6.42) %. Varying amounts of survival between clones was expected. Selection in 5 μM tamoxifen significantly sensitized MDR cells to colchicine.

Comparably, as shown in figure 2C, CHO^{R}C5 cells had a survival of 45.13 (± 10.13) % in the presence of doxorubicin, and decreased to 24.24 (± 4.02) % when cell lines selected in 5 μM tamoxifen were used. Clones A6 had a survival of 45.34 (± 8.74) % when untreated, 21.11 (± 2.45) % when selected in 1 μM tamoxifen and 21.60 (± 3.50) % for clones selected in 5 μM tamoxifen. Similarly, untreated clones A10 exhibited a survival of 64.55 (± 9.02) %, which decreased to 59.85 (± 8.71) % after selection in 1 μM tamoxifen and further decreased to 33.89 (± 2.14) % when clones selected in 5 μM tamoxifen were used. Selection of MDR clones in 5 μM tamoxifen sensitized the MDR CHO^{R}C5 clones to doxorubicin as well. Verapamil was shown to act as a collateral sensitivity agent targeting MDR cells in an ABCB1-dependant mechanism, similarly to tamoxifen [18, 20]. It was therefore of interest

to investigate whether cells selected in tamoxifen, with lower ABCB1 expression, were less sensitive to the effects of verapamil. CHO^{R}C5 cells are collaterally sensitive to verapamil, with a survival of 30.49 (± 6.72) % in 12.5 μM verapamil compared to the drug-sensitive parental cell line AuxB1, with a survival of 70.39 (± 8.30) %, as seen in figure 2E. However, CHO^{R}C5 population cells selected in 1 μM and 5 μM tamoxifen had survivals of 50.72 (± 13.43) % and 78.65 (± 7.61) %, respectively, showing that selection in tamoxifen and subsequent downregulation of ABCB1 made these cells more resilient to the effects of verapamil. Analogously, CHO^{R}C5 clones A6 and A10 both showed a significant increase in survival in verapamil following selection in 5 μM tamoxifen, as survival for A6 clones increased from 28.84 (± 9.45) % to 77.50 (± 7.36) % following selection **(fig. 2E)**. CHO^{R}C5 clone A10 followed a similar trend; with untreated cells surviving at 28.51 (± 5.77) % in 12.5 μM verapamil, while 5 μM-selected cells had a survival of 80.86 (± 6.77) % **(fig. 2E)**. Supplemental table 1A summarizes the changes in % survival of CHO^{R}C5 cells and clones selected in tamoxifen.

Effect of verapamil on P-gp expressing MDR cells. - As previously shown by Karwatsky *et al.*,[19], ABCB1-expressing MDR CHO^{R}C5 cells are collaterally sensitive to verapamil compared to the drug-sensitive parental cell line, AuxB1, in a biphasic trend, wherein lower concentrations stimulate the ATPase and kill MDR cells, and higher concentrations inhibited ATPase activity [19]. It was therefore of interest to determine whether selection in verapamil for 21 days at concentrations equal to approximately 50% survival (25 μM) and 10 % survival (10 μM) would decrease the expression of ABCB1 at the protein level, similarly to tamoxifen. Multidrug resistant CHO^{R}C5 population cells selected in 10 μM

verapamil, and less so at 25 μM verapamil, showed a marked decrease in ABCB1 expression at the protein level **(supplemental fig. 1)**. To resolve whether this decrease in expression was due to a selection of cells with naturally lower levels of ABCB1 or a downregulation of the protein, clones A6 and A10 were also selected in 10 μM and 25 μM verapamil for 21 days. Both clones showed a decrease in ABCB1 after selection in 10 μM verapamil **(fig. 3A)**. Because $CHO^{R}C5$ population cells, as well as clones A6 and A10, showed a decrease in ABCB1 expression following selection in 10 μM verapamil, cytotoxicity assays were carried out to determine whether this downregulation sensitized the MDR cells to colchicine and doxorubicin. Untreated $CHO^{R}C5$ population cells had a 73.39 (± 10.03) % survival in 12.5 μM colchicine, while cells selected in 10 μM verapamil had 45.64 (± 5.51) % survival **(fig. 3B)**. Similarly, $CHO^{R}C5$ clones A6 selected in 10 μM verapamil had 39.60 (± 8.38) % survival in colchicine, down from 84.18 (± 8.85) % survival for unselected cells **(fig. 3B)**. Likewise, clone A10 cells selected in 10 μM verapamil had a survival decrease from 94.51 (± 10.10) % down to 40.87 (± 10.64) % in the presence of 12.5 μM colchicine when previously selected in 10 μM verapamil **(fig. 3B)**.

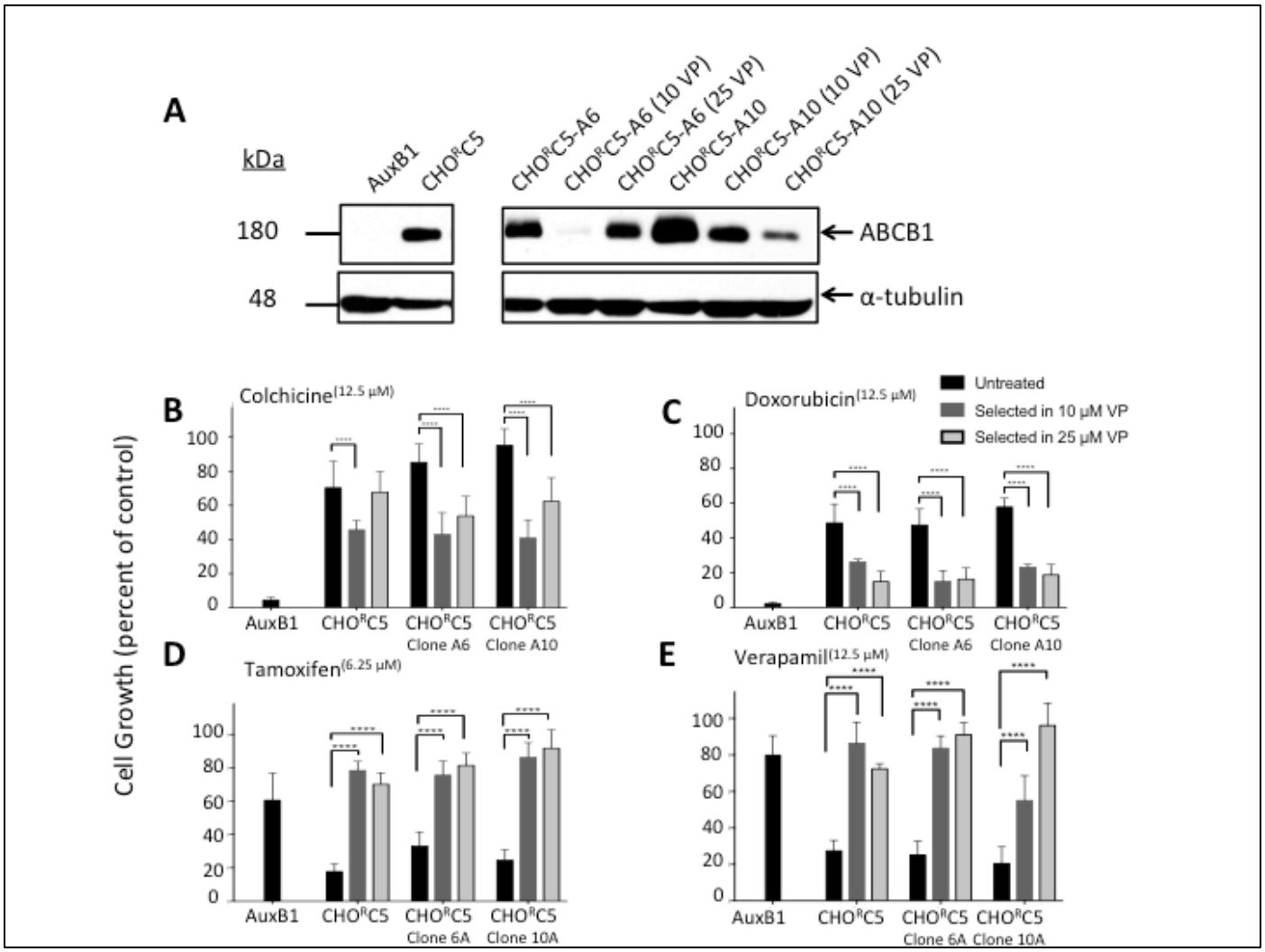


Figure 3. Effect of verapamil on multidrug-resistant cells and clones. CHO^{R}C5 cells and clones A6 and A10 were selected in 10 μM and 25 μM verapamil for 21 days. (A) A western blot was done using the C494 anti-ABCB1 mAb and anti-α-tubulin was used as a loading control. Results show that ABCB1 expression decreased when the drug-resistant cells were selected in 10 and 25 μM verapamil, which subsequently led to increased sensitivity towards (B) colchicine and (C) doxorubicin, but decreased sensitivity in the presence of (D) tamoxifen and (E) verapamil. Cell growth was plotted as a percentage of cells treated with carrier solvent and assessed using the SRB survival assay (sulforhodamine B dye). Graphs represent the mean ± standard deviation for three independent experiments done in triplicate.

Analogously, similar trends were observed when CHO^{R}C5 cells and clones were exposed to 12.5 μM doxorubicin. Untreated CHO^{R}C5 population cells survived at 45.13 (± 10.13) %, and decreased to 26.26 (± 1.51) % for the cells resistant to 10 μM verapamil **(fig. 3C)**. Untreated CHO^{R}C5 clone A6 had survivals of 45.34 (± 8.74) % which dropped down to 14.92 (± 6.35) when cells had been pressured in 10 μM verapamil **(fig. 3C)**. Similarly, CHO^{R}C5 clone A10 had a survival of 64.55 (± 9.02) %, in 12.5 μM doxorubicin, which decreased to 23.20 (± 1.64) % after 10 μM VP selection. Selection in 10 μM verapamil, and the subsequent downregulation of ABCB1, made the MDR CHO^{R}C5 clones more vulnerable to both colchicine and doxorubicin. It is interesting to note that cells selected in 25 μM verapamil were also more sensitive to colchicine and doxorubicin as well, but not always to the extent or significance as those selected in 10 μM verapamil.

It was of interest to determine whether MDR CHO^{R}C5 clones selected in verapamil were less sensitive to tamoxifen, since both verapamil and tamoxifen are thought to act as collateral sensitivity agents with a similar mechanism, in that they both target ABCB1-overexpressing cells, deplete their ATP pools and increase ROS production. Interestingly, as previously mentioned, cells selected in tamoxifen became resistant to both tamoxifen and verapamil. Remarkably, cells selected in verapamil also showed resistance to tamoxifen; whereas multidrug-resistant CHO^{R}C5 cells are collaterally sensitive to 6.25 μM tamoxifen, with a survival of 17.76 (± 4.80) %, compared to AuxB1 cells which survived at 68.46 (± 7.91) % **(fig. 3D)**, CHO^{R}C5 cells selected in 10 μM verapamil had an increase of survival to 78.34 (± 5.45) % **(fig. 3D)**. Likewise, CHO^{R}C5 clones A6 and A10 were collaterally sensitive to tamoxifen, with survivals of 28.35 (± 9.79) % and 27.61 (± 7.00) %, respectively,

compared to the clones selected in 10 μM verapamil: A6 clones selected in 10 μM verapamil had a survival of 75.50 (± 8.54) % in 6.25 μM tamoxifen, while A10 clones survived at 86.23 (± 8.82) %. Once again, cells selected in 10 μM and 25 μM verapamil were better able to survive in the presence of tamoxifen. Supplemental table 1B summarizes the changes in % survival of $CHO^{R}C5$ cells and clones selected in verapamil.

Mechanism of downregulation. - Verapamil was previously shown to modulate ABCB1 expression [26]. However, the mechanism of downregulation has not yet been fully elucidated, as only Northern blots suggested some transcriptional regulation, showing an approximate 2-fold decrease in mRNA in population cells selected in verapamil [26]. It was therefore of interest to determine whether or not the decrease in ABCB1 expression seen in our verapamil-selected clones was due to a downregulation of ABCB1 mRNA. Additionally, tamoxifen had never been shown to modulate ABCB1 expression. In order to characterize the different aspects of these collateral sensitivity compounds on ABCB1 expression, RT-qPCR was used to see whether a common mechanism was responsible for the decrease in ABCB1 protein expression in isolated cell clones. Figure 4 shows that drug-resistant ABCB1 overexpressing $CHO^{R}C5$ cells and clones A6 and A10 have significantly more ABCB1 mRNA (85.441 ± 23.2 fold more, 95.35 ± 13.23 fold more and 118.13 ± 31.66 fold more, respectively) compared to the drug-sensitive parental cell line AuxB1. Clone A6 selected in 10 μM and 25 μM verapamil showed a significant decrease in ABCB1 mRNA, corresponding to a drop of approximately 63.99% and 58.97% of ABCB1 mRNA, respectively **(fig. 4A and supplemental table 2)**. Furthermore, clone A10 selected in 10 μM and 25 μM verapamil also showed a decrease in ABCB1 mRNA, of 55.43% and 57.50%, respectively **(fig. 4B and**

supplemental table 2). Though tamoxifen is thought to be transported by ABCB1, it does stimulate the ATPase, and selection in 5 μM also had an effect on ABCB1 mRNA in both CHO^{R}C5 clones, resulting in approximate decreases of 42.67% ABCB1 mRNA for clone A6 **(fig. 4A and supplemental table 2)** and 50.49% ABCB1 mRNA for clone A10 **(fig. 4B and supplemental table 2)**.

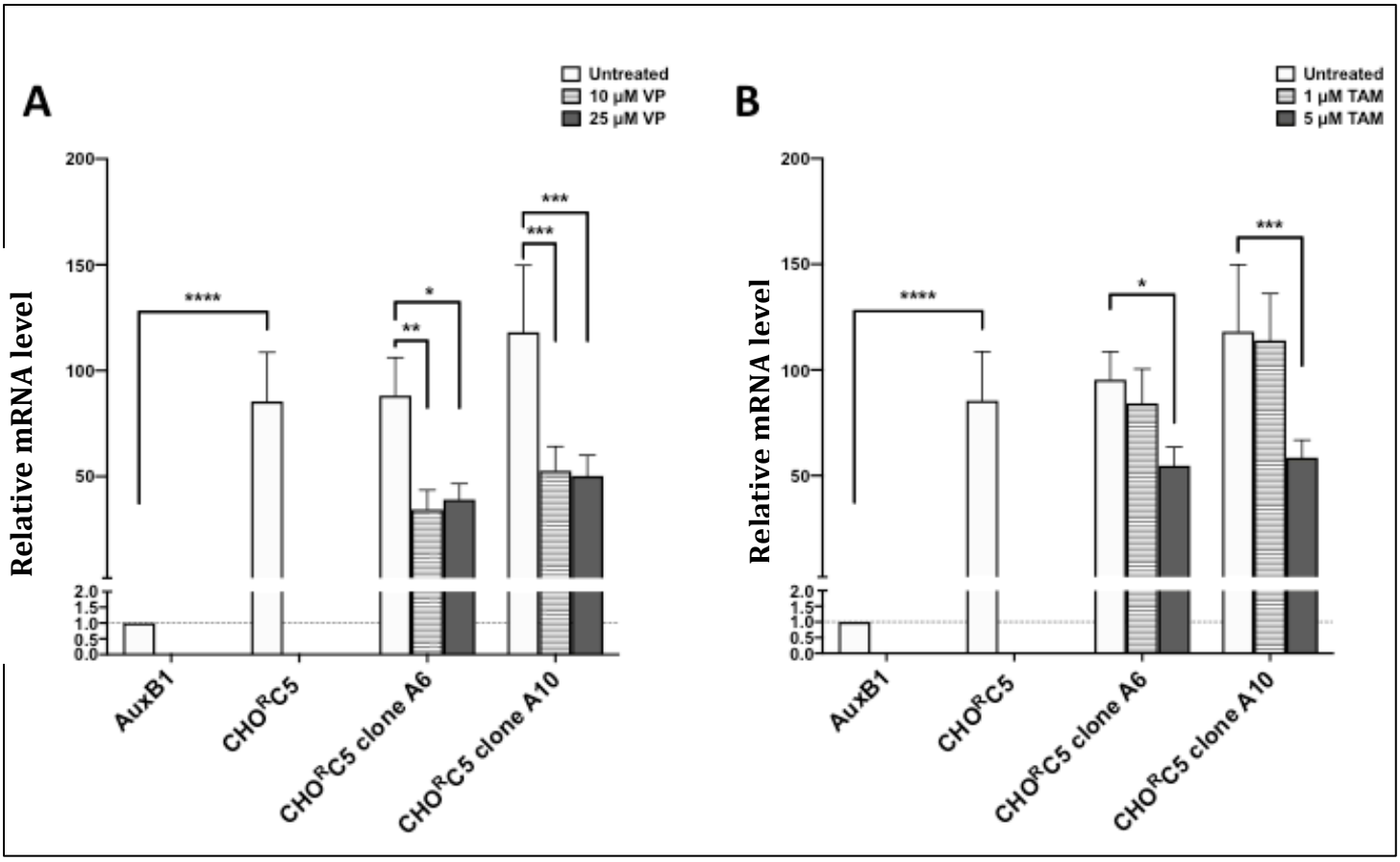


Figure 4. Treatment of CHO^{R}C5 cells with collateral sensitivity drugs decreases ABCB1 mRNA expression. Multidrug-resistant CHO^{R}C5 cells were selected in 10 μM and 25 μM verapamil, and 1 μM and 5 μM tamoxifen. (A) Drug resistant CHO^{R}C5 cells have approximately 80-fold more ABCB1 mRNA compared to the drug-sensitive parental AuxB1 cells. When clones were selected in verapamil, ABCB1 mRNA decreased by about half. (B) Similarly, selection of clones in 5 μM tamoxifen also decreased ABCB1 mRNA levels. Graphs represent the mean ± standard deviation for at least one representative, independent experiment done in triplicate.

In order to differentiate downregulation of ABCB1 mRNA from selection of clones expressing lower levels of ABCB1 in a heterogeneous population, clones A6 and A10 were pressured in 5 μM tamoxifen for 20 hours, followed by mRNA extraction, cDNA synthesis and qPCR using the same primers as previously. Figure 5 shows a significant decrease in ABCB1 mRNA in both clones after treatment with 5 μM tamoxifen. The levels of ABCB1 mRNA expression are compared relative to AuxB1, low-ABCB1-expressing population cells, several years apart, which could explain discrepancies between previous results.

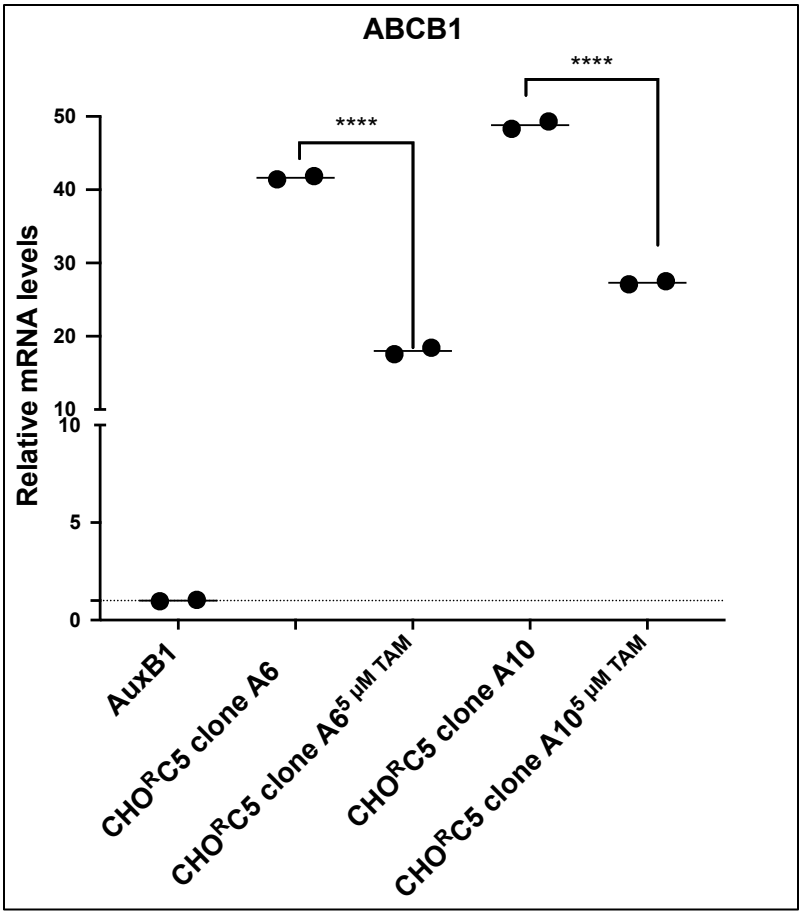


Figure 5. Tamoxifen treatment of CHO^{R}C5 cells (20 hours) decreases ABCB1 expression. Multidrug-resistant CHO^{R}C5 clones were pressured in 5 μM tamoxifen. Both clones A6 and A10 showed a significant ($p < 0.001$) decrease in ABCB1 mRNA following drug treatment.

Tamoxifen decreases ABCB1 expression in MDA-MB-231 $^{400\ nM\ Doxo}$ cells. -

To determine if the effect of tamoxifen pressure on ABCB1-expressing CHO cells is applicable to other MDR cell lines, we examined tamoxifen drug pressure on the human MDR cell line, MDA-Doxo^{400}, selected with doxorubicin and overexpressing ABCB1, relative to its parental triple negative human breast cancer cells **(fig. 6A).** The results in figure 6 show the expression of ABCB1 in MDA-Doxo^{400} following 2.5 μM or 10 μM tamoxifen pressure that kill approximately 50% and 90% of the cells. MDA-Doxo^{400} cells were pressured for 21 days in both 2.5 μM and 10 μM tamoxifen to render them resistant to the drug at those concentrations. MDA-Doxo^{400} cells express lower intrinsic levels of ABCB1 than CHO^{R}C5 cells, making them slightly less susceptible to tamoxifen.

Cells pressured in 10 μM tamoxifen showed a drastic decrease in ABCB1 levels **(fig. 6A)**. MDA-MB-231 parental cells are sensitive to doxorubicin, with a survival of 4.64% in 2.5 μM drug. On the other hand, MDA-Doxo^{400} cells show resistance towards doxorubicin, with a survival of 61.92 (± 2.09) %. However, MDR MDA-Doxo^{400} cells treated with 10 μM tamoxifen, which showed a downregulation of ABCB1 at the protein level, had increased sensitivity in the presence of 2.5 μM doxorubicin, at a survival of 43.27 (± 5.70)% **(fig. 6C)**, showing that treatment with tamoxifen and subsequent downregulation of ABCB1 decreases the level of resistance of the MDR cells towards the conventionally-used chemotherapeutic agent doxorubicin.

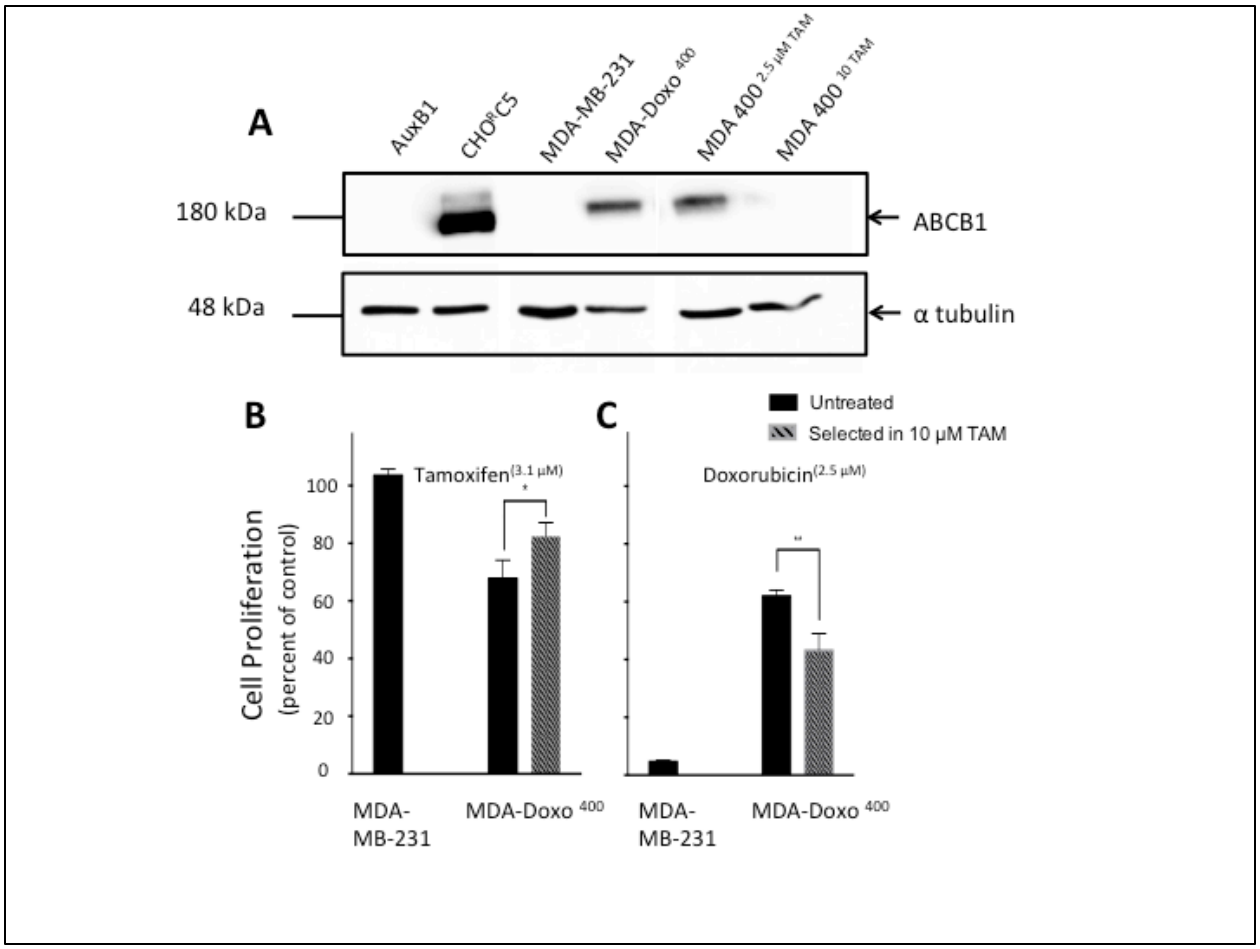


Figure 6. Tamoxifen treatment of MDA-MB-231 cells decreases ABCB1 expression. Multidrug-resistant MDA-MB-231 cells selected with 400 nM Doxo (MDA-Doxo^{400}) were pressured in 2.5 μM and 10 μM tamoxifen. (A) MDA-Doxo^{400} cells treated with 10 μM tamoxifen had decreased ABCB1 expression, and (B) have increased resistance towards tamoxifen and (C) increased sensitivity to doxorubicin compared to the parental cell line. Graphs represent the mean ± standard deviation for at least one representative, independent experiment done in triplicate.

Expression of α-enolase increases following treatment with tamoxifen. - Earlier studies have demonstrated a correlation between tamoxifen resistance and α-enolase expression in human breast cancer cells and poor prognosis, with greater tumor size and a short disease-free interval [33]. Moreover, given our working hypothesis with respect to collateral sensitivity whereby drugs that stimulate ABCB1-ATPase activity affect cellular ATP levels, and enolase's role as a glycolytic enzyme, it was of interest to examine the levels of α-enolase (ENO1 or Enolase I) expression in drug-sensitive and -resistant cells prior to and following tamoxifen drug pressure.

Figure 7A shows α-enolase expression in drug-sensitive parental AuxB1 cells compared to drug-resistant $CHO^{R}C5$ cells and clones by Western blot. Surprisingly, α-enolase expression and activity in $CHO^{R}C5$ cells was considerably reduced relative to the parental drug sensitive AuxB1 cells **(fig. 7A & 7B).** Treatment with 1 μM and 5 μM tamoxifen led to an increase in the expression of α-enolase in both $CHO^{R}C5$ clones A6 and A10 **(fig. 7A).** Similarly, treatment with 10 μM and 25 μM verapamil also increased α-enolase expression in $CHO^{R}C5$ clones A6 and A10 **(fig. 7A)**.

In order to better assess α-enolase activity in selected cells and clones, an enolase activity assay was designed based on the principle of NADH oxidation by lactate dehydrogenase following the conversion of 2-phosphoglycerate to phosphoenolpyruvate by α-enolase. NADH was chosen as an indicator of enolase activity because it fluoresces ($360_{em}/460_{ex}$) and decreases in proportion to the amount of active α-enolase present in a sample. Therefore, samples containing more active α-enolase will show a greater decrease in NADH fluorescence per minute of reaction, representative of NADH conversion to NAD. Figure 6B shows that clones A6 and A10 grown in 1 μM tamoxifen also showed a larger decrease in

enolase activity compared to untreated clones; from 924 (fU/min) to 1110 fU/min for clone A6, and 520 fU/min to 686 fU/min for clone A10. Treatment with 5 μM tamoxifen only showed a significant ($p<0.001$) increase in enolase activity for clone A10, from 520 fU/min to 1233 fU/min. Similarly, clones A6 and A10 treated with 10 μM verapamil showed higher enolase activity compared to untreated clones; from 924 fluorescence units per minute (fU/min) to 1542 fU/min for clone A6, and 520 fU/min to 1324 fU/min for clone A10 **(fig 7B)**.

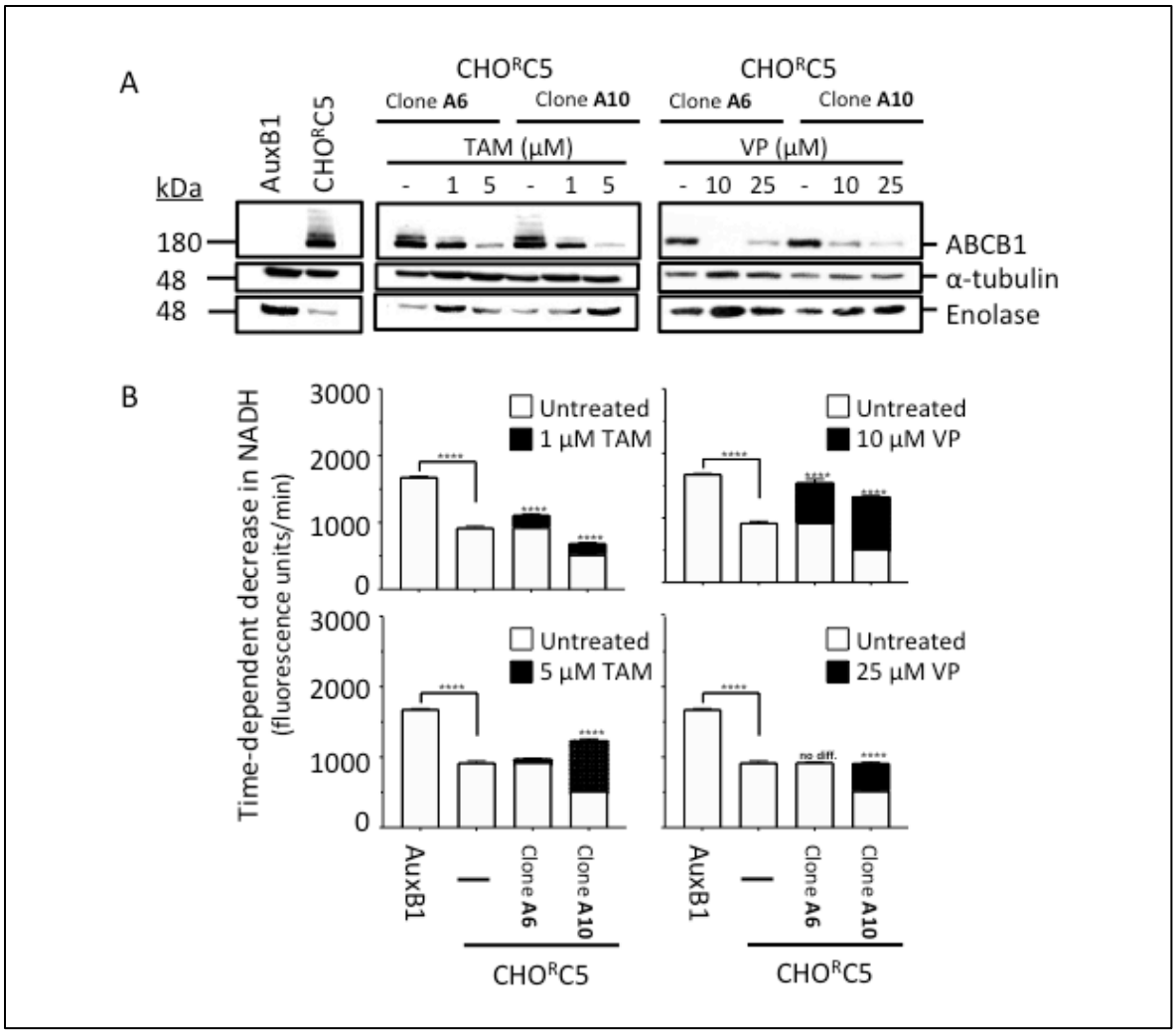


Figure 7. Enolase activity in CHO^{R}C5 cells increases following treatment with verapamil and tamoxifen. (A) Multidrug-resistant CHO^{R}C5 cells and clones showed a decreased expression of α-enolase compared to AuxB1 cells. Following treatment with 10 μM and 25 μM verapamil, and 1 μM and 5 μM tamoxifen, α-enolase expression increases. (B) The relative time-defendant decrease of NADH in cell lysates, measured in fluorescence units per minute, reflective of α-enolase activity. Graphs represent the mean ± standard deviation for at least one representative, independent experiment done in triplicate.

DISCUSSION

In this report, we demonstrate that drug-pressuring ABCB1 expressing, multidrug-resistant $CHO^{R}C5$ clones in the collateral sensitivity drugs tamoxifen and verapamil decreases ABCB1 protein expression and *MDR1* mRNA, while upregulating α-enolase expression levels and activity.

Tamoxifen, which interacts with ABCB1 but is not a transported substrate [36], is a well tolerated drug that is used as a chemopreventative agent for women who have been treated for estrogen receptor positive breast cancer, or who are at high risk of developing breast cancer [37]. Our findings demonstrate that tamoxifen preferentially targets ABCB1 expressing, ER-negative MDR cell lines $CHO^{R}C5$ and MDA-$Doxo^{400}$. The use of estrogen analogues, including tamoxifen, in combination with chemotherapy has been previously shown to reverse drug resistance in ABCB1 positive cancer cell lines [36, 38-40], however this is the first report of using tamoxifen alone as a collateral sensitivity agent in a strategy to circumvent multidrug resistance or specifically targeting ABCB1-overexpressing tumor cells. Other hormonal analogues have been shown to act as collateral sensitivity agents in ABCB1 positive MDR cells, including the tamoxifen derivative N, N-Diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine (DPPE) [25], progesterone and deoxycorticosterone [25]. Estrogen-mediated post-transcriptional downregulation of ABCB1 has been reported in MDR1-transfected cell lines [41], yet this is the first time the anti-estrogen analogue tamoxifen has been shown to modulate endogenous ABCB1 expression. The estrogen receptor negative cell lines CHO and MDA-MB-231 were used to show that the effect of tamoxifen on ABCB1 expression is independent of its function as an estrogen receptor antagonist. In our study, tamoxifen alone targeted ABCB1 expressing MDR cells. Twenty-

one-day pressure in the drug decreased expression of the protein and associated mRNA, and significantly sensitized the clones to chemotherapeutic agents colchicine and doxorubicin alone, in the absence of any other ABCB1 modulator. Together, these results demonstrate that tamoxifen can modulate ABCB1 protein expression and *MDR1* mRNA independently of its effects as an estrogen receptor antagonist, and this downregulation leads to significant sensitization of resistant cells to chemotherapeutic agents.

Verapamil is an ABCB1 efflux substrate and inhibitor that has been shown to target MDR cells in an ABCB1-mediated, ROS-dependent mechanism, even in its inactive form [18, 20]. Verapamil has been tested in clinical trials as a combination agent for modulation of ABCB1 in multidrug resistant cancers such as advanced non-small cell lung cancer [42], lymphoma [43, 44], leukemia [45], ovarian cancer [46] and myeloma [47], with no conclusive therapeutic advantages. To this day, verapamil has not been clinically tested as a stand-alone modulator of MDR1-mediated multidrug resistance. Although verapamil alone has been shown to decrease ABCB1 expression in MDR cell lines [26], there are no reports studying whether this is due to an effect of selection in a heterogeneously ABCB1-expressing population of cells. Thus, it was of interest for us to examine the effects of verapamil treatment on ABCB1-expressing, multidrug resistant clones. For the first time, it is shown that verapamil causes a decrease in ABCB1 mRNA and protein expression in clonal cell populations, and this decrease in ABCB1 significantly sensitizes cells to the chemotherapeutic agents colchicine and doxorubicin. Both tamoxifen and verapamil are clinically approved drugs used to treat hormonal cancer and cardiac hypertension, respectively. Achievable serum concentrations for tamoxifen in the treatment of breast cancer are in the range of 0.20 μM – 0.80 μM [48], whereas in the breast they are in the

range of 0.56 μM – 6.88 μM [49]. For verapamil, serum concentrations vary between 0.30 μM to 2.0 μM [50, 51]. While one limitation of the use of verapamil as a treatment for multidrug-resistant cancers is the lack of evidence showing achievable serum concentrations in the range of 10 μM, we show that clinically relevant concentrations of tamoxifen (1 μM and 5 μM) selectively target ABCB1 expressing drug-resistant cells, lower expression of the MDR protein in the surviving cells and sensitize them to chemotherapy. While verapamil is a transported ABCB1 substrate and tamoxifen may not be, both interact with the protein, and cells selected for resistance to one compound also show resistance to the other, suggesting a shared aspect in the mechanism of collateral sensitivity between the drugs. Collateral sensitivity as a therapy for MDR diseases or infections has been suggested and extrapolated theoretically in a protocol called collateral sensitivity cycling, in which drugs with compatible collateral sensitivity profiles are used in sequence or in combination to treat infections and select against the growth of drug resistant clones [52]. Many ABCB1-dependent collateral sensitivity drugs have been identified in cancer cells *in vitro*, including NSC73306 [21], KP772 [24] and 2-Deoxy-D-glucose [22, 53], but clinical trials have yet to be explored.

The commonly accepted mechanism for ABCB1-dependent collateral sensitivity compounds that stimulate the ABCB1 ATPase, such as verapamil and DPPE, is that they bind to ABCB1 and are effluxed at high rates, which requires higher levels of ATP hydrolysis and oxidative phosphorylation to replenish ATP levels for the demands of the cell [19, 20, 23]. This increase in oxidative phosphorylation in MDR cells that express ABCB1 leads to higher than normal production of reactive oxygen species, depletion of cellular GSH and increased apoptosis [19, 20, 23]. It is well known that many cancer types

use aerobic glycolysis as a preferred method of ATP synthesis [27-29, 35], however previous works show that CHO^{R}C5 cells do have stimulated ATPase activities in the presence of collateral sensitivity drugs, and there is a substantial increase in the production of ROS shortly thereafter [19, 20, 23], suggesting a role by OxPhos and the preferential use of the electron transport chain for energy metabolism in ABCB1 expressing MDR cells such as CHO^{R}C5. This correlates with our findings that CHO^{R}C5 cells express less of the glycolytic enzyme α-enolase compared to drug-sensitive AuxB1 cells, supported by the results showing lower enolase activity levels in the resistant cell line. Our study shows that clones pressured in tamoxifen, with decreased expression of ABCB1, had higher levels and activities of enolase compared to untreated cells. This finding is directly supported by previous evidence showing that α-enolase expression increases in tamoxifen-resistant breast cancer cells [30]. Furthermore, we are the first to show that treatment with verapamil also increases α-enolase activity in the MDR clones. This suggests that MDR cells pressured in collateral sensitivity drugs have less ABCB1 and may therefore be reverting to aerobic glycolysis instead of oxidative phosphorylation, and should be the subject of further study. This, in addition to the fact that increased α-enolase levels have been associated with other forms of drug resistance [54-56], underlines the need for broadened research in the field of collateral sensitivity**.** Cancer cells are well known to favor the use of aerobic glycolysis for ATP synthesis [28, 29, 34, 35], but the large demand for ATP in ABCB1-expressing MDR cells may push them to preferentially use oxidative phosphorylation instead, which could mean a decrease in active glycolytic enzymes such as α-enolase. On the other hand, cells with decreased levels of ABCB1 require less ATP in the

presence of drug, and may therefore revert back to aerobic glycolysis, which has many benefits on the tumor microenvironment of malignant cells [34, 35].

In summary, our novel findings showed that two collateral sensitivity agents, tamoxifen and verapamil, selectively target ABCB1 expressing MDR cells, and downregulate ABCB1 protein expression and mRNA in the surviving cells and clones in a mechanism that is not dependent on selection in a heterogeneous population of cells. This decrease in ABCB1 sensitizes clones to colchicine and doxorubicin, and seemingly affects the metabolic profile of the cells. It remains unclear whether there is a direct correlation between energy metabolism and ABCB1-mediated drug resistance, and this is the subject of ongoing studies.

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Conflicts of Interest – There are no conflicts of interest to report from either of the authors.

	AuxB1	CHO-C5			CHO-C5 clone A6			CHO-C5 clone A10		
		Untreated	1 µM TAM	5 µM TAM	Untreated	1 µM TAM	5 µM TAM	Untreated	1 µM TAM	5 µM TAM
Colchicine (12.5 µM)	3.98 (± 1.73) %	73.39 (± 10.03) %	77.63 (± 15.00) %	35.97 (± 6.49) %	84.18 (± 8.85) %	77.40 (± 7.24) %	63.69 (± 4.91) %	94.51 (± 10.10) %	80.71 (± 4.74) %	45.45 (± 6.42) %
Doxorubicin (12.5 µM)	1.74 (± 1.08) %	45.13 (± 10.13) %	46.90 (± 5.30) %	24.24 (± 4.02) %	45.34 (± 8.74) %	21.11 (± 2.45) %	21.60 (± 3.50) %	64.55 (± 9.02) %	59.85 (± 8.71) %	33.89 (± 2.14) %
Tamoxifen (6.25 µM)	68.46 (± 7.91) %	15.77 (± 4.99) %	10.85 (± 2.40) %	64.87 (± 6.57) %	28.35 (± 9.79) %	42.00 (± 5.06) %	59.67 (± 7.30) %	27.61 (± 7.00) %	37.65 (± 5.00) %	68.07 (± 5.53) %
Verapamil (12.5 µM)	70.39 (± 8.30) %	30.49 (± 6.72) %	50.72 (± 13.43) %	78.65 (± 7.61) %	28.84 (± 9.45) %	51.10 (± 6.24) %	77.50 (± 7.36) %	20.44 (± 9.12) %	41.57 (± 7.63) %	80.86 (± 6.77) %

	AuxB1	CHO-C5			CHO-C5 clone A6			CHO-C5 clone A10		
		Untreated	10 µM VP	25 µM VP	Untreated	10 µM VP	25 µM VP	Untreated	10 µM VP	25 µM VP
Colchicine (12.5 µM)	3.98 (± 1.73) %	73.39 (± 10.03) %	45.64 (± 5.51) %	67.74 (± 11.99) %	84.18 (± 8.85) %	39.60 (± 8.38) %	53.72 (± 11.43) %	94.51 (± 10.10) %	40.87 (± 10.64) %	62.35 (± 13.85) %
Doxorubicin (12.5 µM)	1.74 (± 1.08) %	45.13 (± 10.13) %	26.26 (± 1.51) %	17.23 (± 4.69) %	45.34 (± 8.74) %	14.92 (± 6.35) %	19.74 (± 1.35) %	64.55 (± 9.02) %	23.20 (± 1.64) %	18.72 (± 6.02) %
Tamoxifen (6.25 µM)	68.46 (± 7.91) %	15.77 (± 4.99) %	78.34 (± 5.45) %	70.07 (± 6.92) %	28.35 (± 9.79) %	75.50 (± 8.54) %	81.36 (± 7.49) %	27.61 (± 7.00) %	86.23 (± 8.82) %	91.71 (± 11.08) %
Verapamil (12.5 µM)	70.39 (± 8.30) %	30.49 (± 6.72) %	86.29 (± 11.66) %	72.28 (± 2.88) %	28.84 (± 9.45) %	83.60 (± 6.80) %	91.05 (± 6.65) %	20.44 (± 9.12) %	54.97 (± 13.60) %	96.10 (± 12.29) %

Most sensitive Most resistant

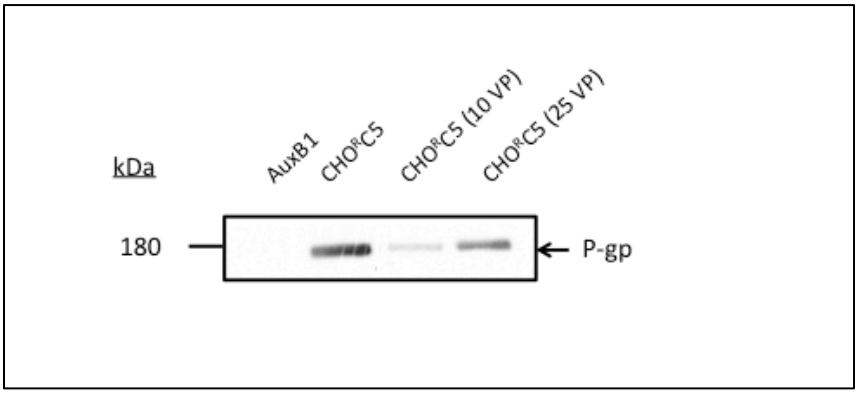
Supplemental Table 1. Changes in survival for multidrug resistant cells and clones pressured in verapamil and tamoxifen exposed to conventional chemotherapeutic agents. Cells were exposed to different levels of conventional chemotherapeutic drugs such as colchicine and doxorubicin, and collateral sensitivity drugs tamoxifen and verapamil. The top table summarizes the % survival of drug-sensitive and multidrug-resistant cells and clones treated with tamoxifen. The bottom table summarizes the % survival of drug-sensitive and multidrug-resistant cells and clones pressured in verapamil.

Cell Line	Untreated			10 µM verapamil			25 µM verapamil		
	Mean relative mRNA*	% ctrl**	Decrease in %	Mean relative mRNA*	% ctrl**	Decrease in %	Mean relative mRNA*	% ctrl**	Decrease in %
AuxB1	1								
CHO^RC5	85.44 ± 23.21								
CHO^RC5 clone A6	93.35 ± 13.23			34.31 ± 9.17	36.01	63.99	39.12 ± 7.49	41.03	58.97
CHO^RC5 clone A10	118.13 ± 31.66			52.66 ± 11.49	44.57	55.43	50.21 ± 9.87	42.5	57.5

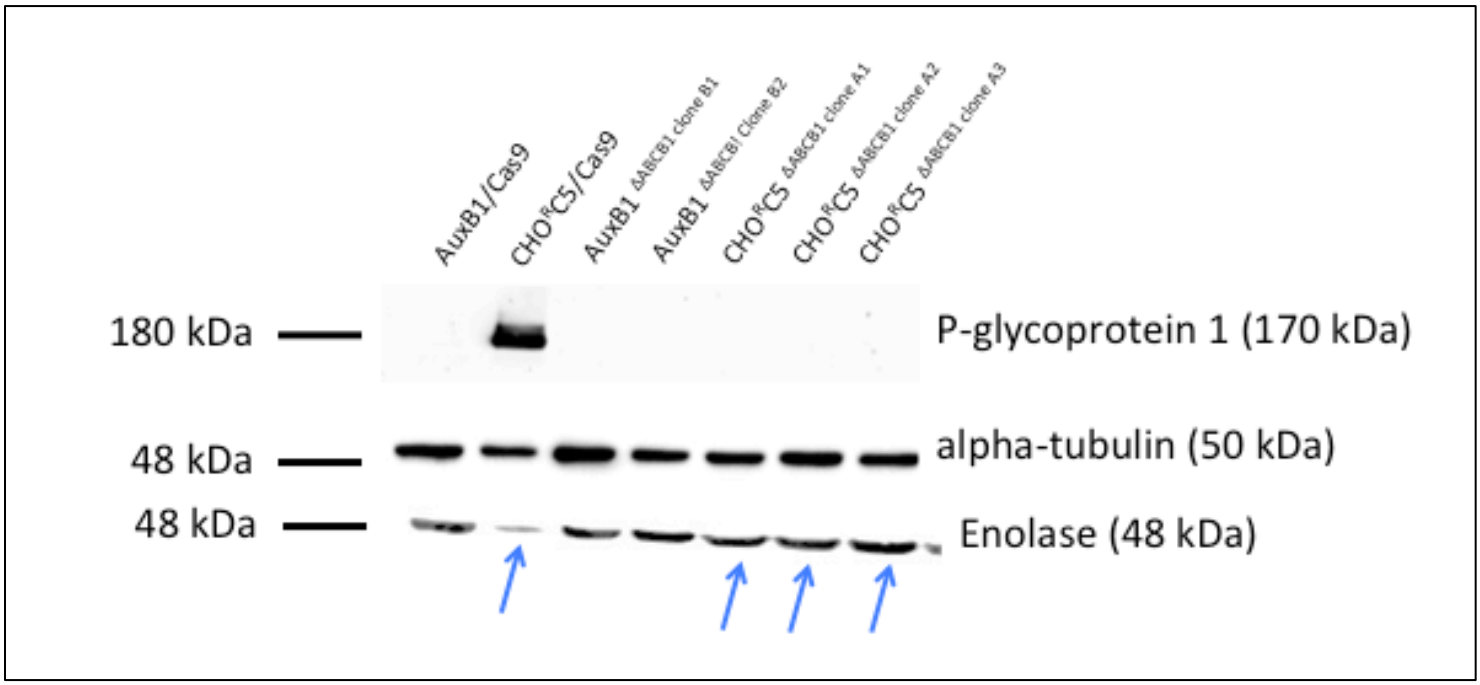
Cell Line	Untreated			1 µM tamoxifen			5 µM tamoxifen		
	Mean relative mRNA*	% ctrl**	Decrease in %	Mean relative mRNA*	% ctrl**	Decrease in %	Mean relative mRNA*	% ctrl**	Decrease in %
AuxB1	1								
CHO^RC5	85.44 ± 23.21								
CHO^RC5 clone A6	93.35 ± 13.23			84.17 ± 16.26	90.17	9.83	54.66 ± 8.82	58.55	41.45
CHO^RC5 clone A10	118.13 ± 31.66			113.93 ± 22.19	96.44	3.56	58.49 ± 8.31	49.51	50.49

* compared to AuxB1
** % of untreated cell line

Supplemental Table 2. Percent decrease in ABCB1 mRNA after treatment with collateral sensitivity drugs. This table shows the calculations used to establish the percent decrease in ABCB1 mRNA in the VP- and TAM- treated clones.



Supplemental Figure 1. Treatment with 10 μM verapamil decreases ABCB1 expression in an MDR cell population. This is a Western blot showing a decrease in ABCB1 expression in a population of CHO^{R}C5 cells.



Supplemental Figure 2. Enolase expression in ABCB1 knockouts. This is a Western blot showing an increase in enolase expression in our ABCB1 CHO^{R}C5 knockouts, which are further characterized in the next section of this thesis.

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CONNECTING STATEMENT 2

In the first manuscript (Chapter 2), we showed that treatment with the collateral sensitivity drugs tamoxifen and verapamil downregulates ABCB1 expression and, in the process, sensitizes multidrug resistant cells to chemotherapy. Though verapamil is a transported substrate of ABCB1 and tamoxifen interacts with ABCB1 but is not transported, cells treated with tamoxifen display cross-resistance to verapamil and vice versa, suggesting a shared mechanism of collateral sensitivity between the two. In order to better understand this mechanism and to extrapolate possible uses for collateral sensitivity drugs in the clinic, the role of ABCB1 in collateral sensitivity must be further studied. Thus far, only ABCB1 inhibitors or siRNA have been used to show the necessity of ABCB1 expression in the mechanism of collateral sensitivity. Therefore, in order to clarify the mechanism of collateral sensitivity and create a tool that can be used efficiently and practically in the laboratory setting, we created and characterized the $CHO^{R}C5$ ABCB1 knockout cell line, as described in this second manuscript (Chapter 3). We use two collateral sensitivity drugs, verapamil and progesterone, as well as substrates of ABCB1 such as Rh123, colchicine and doxorubicin, to show that ABCB1 expression is absolutely necessary for the drug resistance and collateral sensitivity mechanisms.

3

ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells completely reverses the drug resistance and collateral sensitivity phenotypes

Contents

ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells completely reverses the drug resistance and collateral sensitivity phenotypes

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Key words: P-glycoprotein; CRISPR-Knockout; Collateral sensitivity; Multidrug Resistance; and ABCB1.

The authors disclose no potential conflicts of interest.

ABSTRACT

ATP binding cassette (ABC) transporters are significant contributors to clinical multidrug resistance (MDR) in the treatment of several diseases, including cancer. P-glycoprotein (P-gp, ABCB1, MDR1), the B1 member of the ABC transporter family, is a prevalent marker of poor prognosis in cancer treatment due to its ability to efflux most anti-cancer drugs. Several generations of ABCB1 inhibitors were tried in combination chemotherapy but all failed to provide any clinical benefit, so ABCB1 inhibition was all but abandoned as a therapy for overcoming MDR. However, MDR cells were also found to be collaterally sensitive to certain compounds, including calcium channel blockers, steroid hormones and metal chelators, in ABCB1-dependant mechanisms. The collateral sensitivity mechanism of verapamil and progesterone, a calcium channel blocker and steroid hormone, respectively, are ROS-mediated in response to ABCB1 ATPase stimulation. In this report, we use the ABCB1-expressing multidrug resistance cell line $CHO^{R}C5$ to show that ABCB1 expression is necessary for the ROS-mediated mechanism of collateral sensitivity to occur, by underlining the lack of ROS production or decrease in cellular thiol levels in ABCB1 knockouts. For the first time, the CRISPR/Cas9 system is used to support the ROS-mediated collateral sensitivity mechanism, as well as the multidrug-resistance phenotype, in MDR Chinese hamster ovary cells. Additionally, we show differences in accumulation of fluorescent dyes in ABCB1 knockouts, which may be explained by the presence of an MDR-associated cationic transporter. We show that despite other cellular changes associated with the drug-resistance phenotype, ABCB1 expression is necessary for both drug resistance and collateral sensitivity to occur.

ABBREVIATIONS

ABCB1= P-gp = MDR1
P-gp: P-glycoprotein
MDR1: Multidrug Resistance Protein 1
ABCB1: ATP Binding Cassette Transporter B1
MDR: Multidrug Resistance
VP: Verapamil
PRO: Progesterone
CS: Collateral Sensitivity
ROS: Reactive Oxygen Species
DPPE: N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethamine
Rh123: Rhodamine 123

INTRODUCTION

Clinical multidrug resistance (MDR) is one of the major causes of cancer chemotherapy failure [1-3]. One of the key contributors to MDR is P-glycoprotein (P-gp, MDR1), a member of the B-subfamily of ATP binding cassette (ABC) transporters (i.e., ABCB1); [4]. MDR cells overexpressing ABCB1 efflux chemotherapeutic compounds through an ATP-dependent mechanism [5] and ABCB1 overexpression in patients' tumors correlated with poor treatment prognosis. Moreover, an increase in ABCB1 expression post-treatment has been described in different malignancies, including breast, neuroblastoma and lung cancers [6]. Efforts to inhibit ABCB1 using highly specific MDR-reversing drugs, including 3rd generation drugs (i.e., cyclopropyldibenzosuberane LY 335979; acridonecarboxamide GF 120918; [7]) were largely unsuccessful due to unacceptable toxicity levels seen in the patients when reversal agents were used in combination with other anti-cancer drugs [8-12]. Consequently, the strategy to block ABCB1 drug efflux function to overcome ABCB1-mediated clinical multidrug resistance has been abandoned.

Earlier findings [13] that ABCB1-expressing MDR Chinese hamster ovary (CHO) cells were hypersensitive to certain local anesthetics such as procaine and xylocaine as well as steroid hormones such as corticosterone, were intriguing but were not elaborated until recently [14]. Verapamil, an ABCB1 inhibitor previously used in attempts to treat drug-resistant cancers [15-18], was shown to act as a collateral sensitivity drug at low concentrations in an ABCB1-dependant, ATPase-mediated mechanism [14, 19]. Other compounds such as progesterone, deoxycorticosterone and N,N-diethyl-2-(4-phenylmethyl)ethanamine (DPPE) have been shown to induce collateral sensitivity in MDR

cells by the same mechanism, whereby they are able to stimulate the ABCB1 ATPase, increasing ATP hydrolysis with consequent rise in reactive oxygen species and oxidative cell death [20, 21]. One study indicated that DPPE, an ABCB1 inhibitor, combined with doxorubicin significantly improved overall breast cancer patient survival without the increase in cardiotoxicity seen with verapamil [22, 23]. Hypothetically, this may be due to DPPE's effect as a collateral sensitivity agent, and not as a reversal agent. Additionally, there exist other ABCB1-dependant collateral sensitivity agents that are not mediated by the increase in ATPase activity, but still require functional ABCB1 to take effect. These include NSC73306 [24], Dp44mT [25] and Entinostat [26], which have been suggested to cause collateral sensitivity in MDR cells through heightened ROS or ROS-recycling.

While there are more than 300 different known substrates of ABCB1, including fluorescent dyes, steroid hormones, cytotoxic compounds, calcium channel blockers and narcotics [27], the native function and substrate(s) of ABCB1 remain unknown, though it is believed to have a protective function in certain tissues [28-30]. Previous studies using inhibitors such as PSC833 (Valspodar) [31-35] or MDR1 siRNA [36-40] to study the inhibition of function or the absence of functional ABCB1 in relation to its normal functions and biology are associated with off-target effects and are transient in nature.

In this report, we have used the CRISPR/Cas9 system to knock out ABCB1 in a multidrug-resistant model cell line, $CHO^{R}C5$, to study the importance of ABCB1 on drug resistance and collateral sensitivity. Moreover, the availability of $CHO^{R}C5$ ABCB1-knockout cells as an important new tool for studying MDR should: a) establish the role of ABCB1 in the broad MDR phenotype relative to other cellular changes that may occur during the drug selection process of MDR cell lines, b) determine if cellular changes associated with ABCB1

overexpression in CHO^{R}C5 cells are regulated by ABCB1 expression and c) address possible normal substrates of ABCB1.

MATERIALS AND METHODS

Tissue culture and cell proliferation assay - Chinese hamster ovary (CHO) cell lines were maintained in αMEM medium (ThermoFisher 12571-063) supplemented with 8% FBS (Wisent 098-150), maintained at 37 °C with 5% CO_2 and ambient O_2. To measure the effects of drugs on the proliferation of drug sensitive (AuxB1) and resistant (CHO^RC5) cells, a clonogenic growth study was used; AuxB1 and CHO^RC5 cells (300-700 cells) in 200 µL medium supplemented with 8% FBS were seeded per well of a 48-well plate. After 24 hours of incubation, drug dilutions were added, at 2X concentration in 200 µL medium. The plates were allowed to incubate for a total of 7 days, after which the medium was removed and cells were stained with 0.1% methylene blue (1:1 EtOH:H_2O) for 20 minutes. The dye was washed out and the plates set out to air dry. The cell bound dye was dissolved using 500 µL of 0.1% SDS in PBS, and absorbance was read at 660 nm using the Synergy™ H4 plate reader (BioTek). To establish the percent cell growth for each cell line and drug treatment, each growth condition was compared to 100% cell growth in solvent control (<2% solvent).

Cell proliferation assay (Incucyte) - The IncuCyte® ZOOM system from Essen Bioscience was used to compare growth curves between wild-type AuxB1 or CHO^RC5 cells, and cells transiently transfected with Cas9. Briefly, 350 000 cells were seeded in a 6-well plate in 2 mL αMEM medium supplemented with 8% FBS, and placed into the IncuCyte for readings every 6 hours. The Basic Analyzer tool was used for analysis, wherein a confluence mask was placed on the individual cells, and cell confluency was measured over time, up to 60 hours.

Protein extraction and Western blotting - Cells were harvested with phosphate-buffered saline (PBS) solution containing 5 mM ethylenediaminetetraacetic acid (EDTA), no trypsin. Cells were washed in cold PBS several times prior to extraction with RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA; 0.25 % sodium deoxycholate; 1% NP-40, pH 7.4), containing protease inhibitors (Thermo Scientific 78429). Proteins were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific 23225). For Western immunoblotting, 20 µg of each cell lysate was loaded onto 10% SDS-PAGE gels [41] and transferred onto Immobilon-P® PVDF membranes (Sigma Aldrich IPVH) for 3 hours at 400 mA [42]. Membranes were probed using ABCB1-specific monoclonal antibody (mAb) (C494 mAb, a gift from V. Ling, 1:1000 (v/v)), anti-HSP60 mAb (MA3-013, from ThermoFisher), anti sorcin (1:500, C-20 from Santa Cruz) or anti-α-tubulin mAb (1:200, mouse hybridoma 12G10, deposited to the DSHB by Frankel, J. / Nelsen, E.M. (DSHB Hybridoma Product 12G10 anti-alpha-tubulin)) overnight at 4°C. Following several washes in PBS/Tween (0.05%), membranes were probed with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000; from Thermo Scientific) for 90 minutes at room temperature. Specific antibody binding signals were detected using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific).

CRISPR/Cas9 knockout of ABCB1 in CHO cell lines - eSpCas9(1.1) was a gift from Feng Zhang (Addgene plasmid #71814; http://n2t.net/addgene:71814 ;RRID :Addgene_71814). The following guideRNA sequences were designed against TMD1 of *Cricetulus griseus* ABCB1 :

Name	Sequence
GL CRISPR ABCB1 Cho guideRNA #1 FW:	CACC GCTTATAGTTGCCTACATTC
GL CRISPR ABCB1 Cho guideRNA #1 RV	AAAC GAATGTAGGCAACTATAAGC

The constructs were transformed into TOP10 cells, and empty plasmid, or plasmid containing the guideRNA was isolated and transiently transfected into the AuxB1 or $CHO^{R}C5$ cells using the Lipofectamine 2000 kit (Invitrogen 11668027). Populations of the transfected cells were grown and knockout clones were isolated via serial dilution method. The absence of P-glycoprotein was verified using a Western blot. Genetic validation was confirmed by Sanger sequencing of the genomic DNA for each clone.

Measurement of total reduced thiol – Reduced cellular thiol levels, including reduced glutathione (GSH), were measured in cell lysates as a reflection of cell oxidative status. Briefly, 50 000 cells were seeded per well in a 48-well plate (8 wells per cell line) in 200 µL medium supplemented with 8% FBS. For accurate and comparative measurement, 5 wells were used to assess thiol levels and 3 identically treated wells were used to normalize the measurements with protein (SRB assay). After 24 hours, appropriate solvent or drug concentrations were added at 2X in 200 µL medium, and the plates were incubated for another 48 hours. For reduced thiol measurement, media was removed, wells were washed once with PBS and 250 µL RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 0.5% (w/v) sodium deoxycholate; 1% (v/v) NP-40; 0.1% (w/v) SDS, pH 8) was added to each well. Immediately before quantification, 10 µL of freshly prepared 50 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent/DTNB) was added to each well and the plates were read at 412 nm in the Synergy™ H4 plate reader (BioTek) at 0 and 5 minutes of incubation. Sulforhodamine B (SRB) was used to normalize the absorbance in thiol levels with the amount of total protein for each sample, for equal comparisons. In the wells to be treated with SRB, 200 µL of ice-cold 10% trichloroacetic acid (TCA) was added and the plates were

incubated at 4^{0}C for 1 hour. Wells were rinsed with water and 200 µL of 0.057% (w/v) SRB (in 1% v/v acetic acid) was added to each well and incubated for 30 minutes at room temperature to allow the dye to bind to protein. Wells were rinsed with 1% acetic acid and dried overnight. To dissolve the protein-bound dye, 500 µL of 10 mM Tris base pH 10.5 was added to each well, and the plate was read at 510 nm using the Synergy™ H4 plate reader (BioTek). Absorbance values from the thiol measurements were normalized using the average absorbance of SRB for each cell line, and compared to relevant solvent control to establish percent of reduced thiols relative to control.

Measurement of Reactive Oxygen Species - Cells (5×10^{4}) were seeded in 100 µL of phenol red-free medium (ThermoFisher 41061-029) supplemented with 8% FBS and allowed to attach. Following this, 50 µL of 150 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was added for a final concentration of 50 µM, and left to incubate for 45 minutes. The medium was removed and wells were washed with ice-cold HBSS, after which 100 µL of phenol red-free medium was added into each well. Cells were allowed to incubate for 30 minutes, and 100 µL of appropriate 2X drug concentrations were added to each well. Plate fluorescence at $492_{ex}/528_{em}$ was read 28 hours later, and each drug reading was compared to the appropriate solvent control for the cell line in question, to measure the percentage of ROS compared to control.

Accumulation of fluorescent dyes - For Rhodamine 123 (Rh123), cells were detached using a trypsin-free removal buffer (0.5 mM EDTA in PBS). A total of 5×10^{6} cells of each cell line were pelleted and resuspended in 1 mL of pre-warmed 10 µM Rh123 (in HBSS buffer) and

allowed to incubate for 1 hour. The cells were pelleted once and washed with ice-cold DPBS (2.7 mM KCl; 1.5 mM KH_2PO_4; 136.9 mM NaCl; 8.9 mM Na_2HPO_4 ⋅ $7H_2O$) and allowed to sediment through an ice-cold 20% sucrose cushion by centrifugation. The cells were then lysed using 2% Trixon X-100 and the lysate was read in a black-welled plate at $485_{ex}/535_{em}$ using the Synergy™ H4 plate reader (BioTek). The readings were compared to a standard curve measuring known concentrations of Rh123 as it is autofluorescent.

For Hoechst 33342, the same protocol for accumulation and lysis was followed (with more cells), using 10 μg/mL Hoechst 33342 in PBS. Fluorescence reading was done at $350_{ex}/460_{em}$ using the Synergy™ H4 plate reader. Total well fluorescence was normalized to protein absorbance in the appropriate wells.

Fluorescence confocal microscopy - Cells were seeded in a suitable clear-bottomed 96-well plate at 10% confluency and incubated for 48 hours to allow for propagation. Media was removed, and 100 μL of the appropriate dye was added (5 μM Rh123 or 10 μg/mL Hoechst 33342 or 5 μM doxorubicin or 250 nM MitoTracker™ Red from ThermoFisher) and incubated at 37 ^{o}C for 30 minutes. Dyes were washed out and phenol red-free αMEM medium was added, and cells were allowed to incubate for 30 more minutes. Images were acquired using the Zeiss LSM710 with Airyscan and the appropriate program-integrated fluorescence wavelengths.

Resazurin fluorescence - A total of 5 x 10^5 cells were seeded per well in a 24-well plate in 1 mL medium supplemented with 8% FBS and left to adhere for 1 hour. A final concentration of 0.00125% w/v resazurin was used, and plates were left to incubate for 6 hours.

Fluorescence was measured at $530_{ex}/590_{em}$, and the fluorescence for each cell line was compared to the non-transfected parental control to establish changes in metabolism of resazurin.

mRNA isolation and cDNA synthesis **-** Cells were cultured to 70% confluency, collected via trypsinization and pelleted. Cell pellets were resuspended in 1 ml TRIzol reagent (ThermoFisher 15596026) and incubated at room temperature (RT) for 5 minutes. Gentle mixing and then phase separation was achieved by adding 200 µL chloroform, vortexing for 15 seconds and incubating at RT for 3 minutes. Samples were centrifuged at 18 000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean 1.5 mL tube, and RNA precipitation was completed by adding 500 µL 2-propanol and incubating samples at room temperature for 10 minutes followed by a centrifugation at 18 000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed 3 times with 75% ethanol followed by spinning at 10 500 x g for 5 minutes at 4°C. Ethanol was removed and the pellet was resuspended in 30 µL of RNAse- and DNAse-free water. cDNA synthesis was carried out using the SuperScript® IV Reverse Transcriptase kit by Invitrogen (ThermoFisher 18090010) and following the manufacturer's protocol.

OCTN1 qPCR **-** Primers were designed to cover exon-exon junctions on *OCTN1* and *β-actin*.

	OCTN1	***β-ACTIN***
Forward	CCA TCG TGA CTG AGT GGA ATC TGG TG	CCT TCC TGG GTA TGG AAT CCT G
Reverse	CAC GAA GCT ATA ATC TTC AGG TAC CAC TTG G	CAG GAG GAG CAA TGA TCT TGA TCT TC

The StepOne ™ Real-Time PCR System (ThermoFisher Scientific) was used to accomplish the qPCR experiment. Primers efficiencies were assessed via a melting curve with serial dilutions. The FastStart Universal SYBR Green Master (Rox) from Millipore Sigma was used and all manufacturers' protocols were followed. Relative mRNA was calculated by comparing normalized Ct values of samples to parental cell lines.

Statistical analysis **-** Data is represented as a mean ± standard deviation, and statistical significance was computed with GraphPad Prism version 6.0 to 8.0 using one-way ANOVA. Probability (P) values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

CRISPR/Cas9-mediated knockout of ABCB1 in CHO cells - We used the CRISPR/Cas9 system containing guideRNA encoding sequences from TMD1 to knock out functional ABCB1 from MDR cells by transient transfection. Cells were transiently transfected with empty Cas9 vector or Cas9-ABCB1-specific guide RNA, followed by the isolation of two clones for AuxB1 (AuxB1$^{\Delta ABCB1}$clones B1 and B2) and three clones for CHO^{R}C5-ABCB1 knockout cell (CHO^{R}C5$^{\Delta ABCB1}$clones A1-A3), respectively. The rationale for isolating ABCB1-knockout clones for AuxB1 drug sensitive cells was based on the fact that these cells expressed very low levels of ABCB1 [43] which could influence their drug metabolism or ABCB1 protein interactions, in addition to providing better background control. To demonstrate the successful knockout of ABCB1 in AuxB1 and CHO^{R}C5 cells, total cell lysates were extracted from Cas/9 transfectants and ABCB1 knockout AuxB1 and CHO^{R}C5 cells, respectively, and the Western blots containing the latter cell lysates were probed for ABCB1 expression using an ABCB1-specific monoclonal antibody [44]. The results in figure 1A show strong ABCB1 expression in cell lysate from CHO^{R}C5/Cas9 cells, but not in three ABCB1-knockout clones of CHO^{R}C5 cells (i.e., CHO^{R}C5$^{\Delta ABCB1}$ A1 – A3). No ABCB1 polypeptide was detected in AuxB1/Cas9 or AuxB1$^{\Delta ABCB1}$clones B1 and B2 **(fig. 1A)**. To confirm the Western results in figure 1A, with respect to the absence of ABCB1 expression in CHO^{R}C5$^{\Delta ABCB1}$ A1 – A3 clones, genomic DNA sequencing was performed on each clone, which demonstrated the chosen clones all contained an indel mutation or the introduction of an in-frame stop codon. Moreover, the transient transfection of the plasmid (empty Cas9 vector) itself had no discernible effect on cell growth, as revealed from the proliferation

curves using the IncuCyte system to monitor cell division over 60 hours between parental and transiently transfected cell lines **(supplemental figure 1)**.

Multidrug resistance to doxorubicin and colchicine is completely reversed in CHO ΔABCB1 clones – To confirm the role of ABCB1 on the MDR phenotype of CHO^{R}C5 cells, we compared the sensitivity of the latter cells to CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3 to colchicine and doxorubicin. Figure 1B shows the proliferation of AuxB1, AuxB1$^{\Delta ABCB1}$ clones B1 and B2, CHO^{R}C5, and CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3 cells in the presence of increasing concentrations of colchicine or doxorubicin. AuxB1 cells, which do not express ABCB1, in addition to the AuxB1$^{\Delta ABCB1}$ clones B1 and B2, exhibited sensitivity to both colchicine and doxorubicin compared to CHO^{R}C5 cells **(fig. 1B)**. The ABCB1-expressing CHO^{R}C5/Cas9 cells are derived from CHO^{R}C5 cells, which were originally selected in colchicine and are resistant to the drug, and display cross-resistance to doxorubicin [13]. This cross-resistant phenotype is well known to be mediated by the drug efflux mechanism of ABCB1. The CHO^{R}C5$^{\Delta ABCB1}$ clones A1, A2 and A3 all show complete reversal of resistance to both colchicine and doxorubicin, confirming the importance of ABCB1 in drug resistance **(fig. 1B)**. The slight variation in the sensitivity between the different CHO^{R}C5$^{\Delta ABCB1}$ clones towards colchicine and doxorubicin **(fig. 1B)** is interesting and could be due to differences in the expression of other MDR-associated proteins in CHO^{R}C5 cells. Specifically, CHO^{R}C5$^{\Delta ABCB1}$ clones A1 and A3 were slightly more resistant to colchicine and doxorubicin than CHO^{R}C5$^{\Delta ABCB1}$ clone A2 **(fig. 1B)**. Early reports examining gene amplification in CHO^{R}C5 cells had demonstrated the amplification of five genes (*ABCB4, SRI, DBF4, TMEM243, and RUNDC3B*) within the *ABCB1* amplicon [45, 46]. Of the latter five genes, *SRI,* encoding the low molecular weight

calcium binding protein sorcin, has been associated with low level multidrug resistance phenotypes [47-49], including direct binding to doxorubicin [50], in tumor cell lines. Hence, it was of interest to examine the expression levels of sorcin in CHO^{R}C5 and their ABCB1-knockout clones.

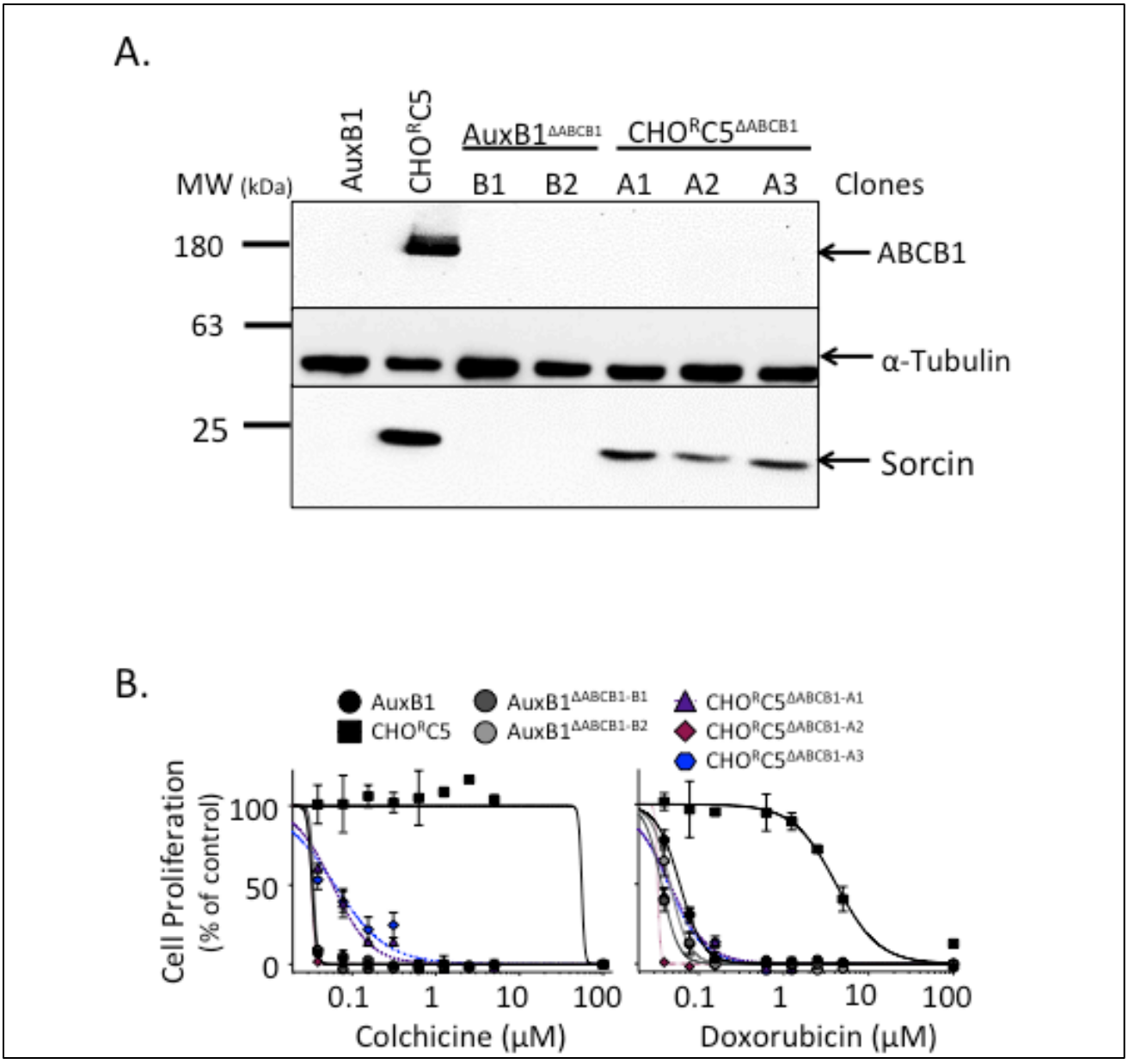

Figure 1. CRISPR/Cas9-mediated knockout of ABCB1 in drug-sensitive and –resistant CHO cells. Panel A shows ABCB1 expression in CHO cells before and following CRISPR/Cas9 knockout. Total protein cell extracts from drug-sensitive (AuxB1) and -resistant (CHO^RC5) cells transiently transfected with vector alone (AuxB1/Cas9 and CHO^RC5/Cas9, respectively) or vector plus ABCB1-guide RNA ($AuxB1^{\Delta ABCB1\ clone\ B1\text{-}2}$, and $CHO^RC5^{\Delta ABCB1\ clone\ A1\text{-}3}$) were resolved on SDS-PAGE, transferred to PVDF membrane and probed with ABCB1-specific monoclonal antibody (C494 mAb) and α-tubulin specific monoclonal antibody (University of Iowa hybridoma bank – 12G10) for loading control. The positions of the molecular weight markers are indicated on the left of the figure. Panel B shows growth curves for the drug-sensitive (AuxB1) and -resistant (CHO^RC5) cells transfected with vector alone (AuxB1/Cas9 and CHO^RC5/Cas9, respectively) or vector plus ABCB1-guide RNA ($AuxB1^{\Delta ABCB1\ clone\ B1\text{-}2}$, and $CHO^RC5^{\Delta ABCB1\ clone\ A1\text{-}3}$) in the presence of increasing concentrations of colchicine and doxorubicin. The ABCB1-overexpressing MDR cells CHO^RC5 are resistant to the cytotoxic drugs doxorubicin and colchicine. The parental, ABCB1-negative cell line AuxB1 is sensitive to both drugs. Additionally, the AuxB1 and CHO^RC5 ABCB1 knockouts are sensitive to both doxorubicin and colchicine, and the removal of functional ABCB1 from CHO^RC5 cells abolished their ability to survive in both the presence of both compounds. Graphs represent the mean ± standard deviation for a representative independent experiment done in triplicate. Sorcin is a resistance-associated protein that is present in the MDR CHO^RC5 cells but not in drug-sensitive AuxB1 cells. Knockdown of ABCB1 from the CHO^RC5 cells did not abolish sorcin expression, though it does seem to be overall lower in the CHO^RC5 ΔABCB1 clones. Direct comparison of sorcin expression between clones, or before vs. after knockout, is difficult because clones were selected from a population of CHO^RC5 ΔABCB1 cells, as is the standard for all CRISPR/Cas9-mediated knockouts. Nevertheless, sorcin expression appears to be higher in CHO^RC5 ΔABCB1 clones A1 and A3, compared to A2.

Figure 1A shows the results of a Western blot probed with sorcin-specific monoclonal antibody. The results in figure 1A show, as expected, high levels of sorcin expression in CHO^RC5 cells that was decreased in CHO^RC5-ABCB1 knockout clones, suggesting for the first time a direct correlation between ABCB1 expression and sorcin in CHO^RC5 cells. Moreover, a comparison in sorcin levels between the three CHO^RC5-ABCB1 knockout

clones shows CHO^{R}C5$^{\Delta ABCB1}$ clone A2 to express lower levels of sorcin, relative to CHO^{R}C5$^{\Delta ABCB1}$ clones A1 and A3, respectively **(fig. 1A)**. The latter findings suggest that the increased sensitivity observed for CHO^{R}C5$^{\Delta ABCB1}$ clone A2 towards colchicine and doxorubicin, relative to CHO^{R}C5$^{\Delta ABCB1}$ clones A1 and A3, is consistent with differences in sorcin expression **(fig. 1A versus fig. 1B)**. Based on these results, it would be of interest to examine the nature of ABCB1-sorcin interactions, and to determine the spectrum of drug resistance associated with sorcin expression. Furthermore, future studies will focus on the effects of ABCB1 knockout on the expression levels and interactions with the other four gene products (i.e., *ABCB4, DBF4, TMEM243, and RUNDC3B*) within the *ABCB1* amplicon [45, 46].

Collateral sensitivity to verapamil, progesterone and NSC73306 is completely reversed in CHO ΔABCB1 clones – Earlier reports, using inhibitors of ABCB1 and ABCB1-specific siRNA, have demonstrated CHO^{R}C5 cells to be collaterally sensitive to several drugs, including verapamil (VP) and progesterone (PRO) [14, 19, 20]. However, in the reports where the ABCB1-ATPase inhibitor PSC-833 was used, it was not possible to rule out off-target effects, while the use of ABCB1-siRNA lead to transient knockdown of ABCB1 expression that did not span the entire assay time. By contrast, the complete knockout of ABCB1 expression bypasses these obstacles and allows the isolation of stable clones. To determine the role of ABCB1 expression and activity in relation to CHO^{R}C5 collateral sensitivity, it was of interest to examine the effects of verapamil and progesterone on the cells and clones. Although both drugs (verapamil and progesterone) interact with and

stimulate ABCB1-ATPase activity, only verapamil is a substrate for the ABCB1-drug efflux pump [20].

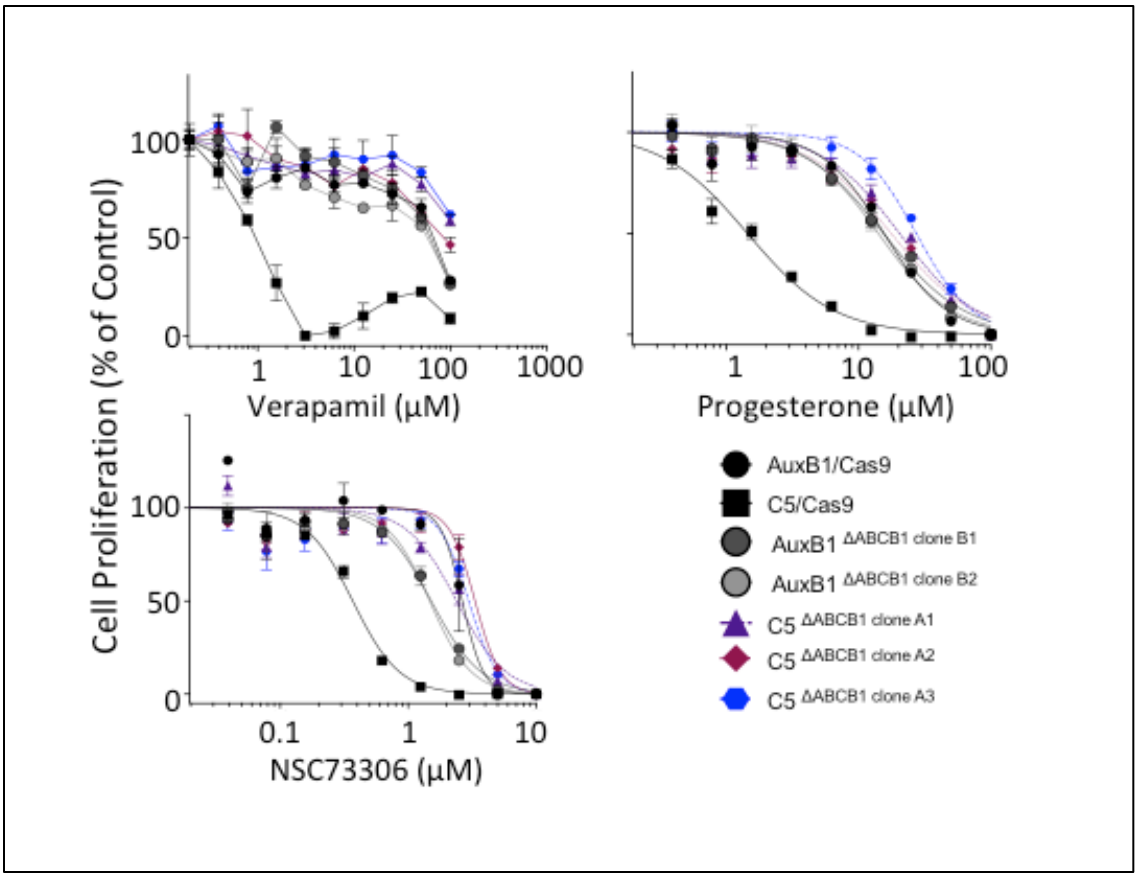

Figure 2. Effects of ABCB1 knockout on collateral sensitivity of verapamil, progesterone and NSC73306 on CHO cells. This figure shows growth curves for the drug-sensitive (AuxB1) and -resistant (CHO^{R}C5) cells transfected with vector alone (AuxB1/Cas9 and CHO^{R}C5/Cas9, respectively) or vector plus ABCB1-guide RNA (AuxB1$^{\Delta ABCB1\ clone\ B1\text{-}2}$, and CHO^{R}C5$^{\Delta ABCB1\ clone\ A1\text{-}3}$) in the absence and the presence of increasing concentrations of verapamil (VP), a transported substrate of ABCB1, progesterone (PRO), a substrate of ABCB1 that is not transported but does bind and stimulate the ATPase, and NSC73306, a non-ABCB1-interacting compound. ABCB1-expressing MDR cells CHO^{R}C5 are collaterally sensitive to VP, PRO and NSC73306 compared to AuxB1 cells. This sensitivity is reversed with the knockout of ABCB1 from the CHO^{R}C5 clones. Graphs represent the mean ± standard deviation for a representative independent experiment done in triplicate.

We also examined the effects of ABCB1-knockout on the collateral sensitivity of CHO^{R}C5 cells to NSC73306, a thiosemicarbazone metal chelator, whose collateral sensitivity activity is dependent on functional ABCB1 but did not interact with or stimulate ABCB1 ATPase activity [24]. Figure 2 shows the proliferation of AuxB1, CHO^{R}C5 and their corresponding ABCB1-knockout clones (AuxB1$^{\Delta ABCB1}$ clones B1-B2 and CHO^{R}C5$^{\Delta ABCB1}$ clone A1-A3, respectively) in the presence of increasing concentrations of verapamil, progesterone and NSC73306. The results in figure 2 show that the collateral sensitivity of CHO^{R}C5 cells to all three drugs was abolished by the removal of functional ABCB1 from the MDR cells, as CHO^{R}C5 ΔABCB1 clones A1, A2 and A3 survive better in the presence of verapamil, progesterone and NSC73306 compared to wild-type CHO^{R}C5 cells. Although there was slight variability between the responses of the different CHO^{R}C5$^{\Delta ABCB1}$ clones to NSC73306 and verapamil, these differences did not correlate with differences in sorcin expression between CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3 and A2 discussed earlier with respect to their sensitivity to doxorubicin and colchicine **(fig. 1B)**. Moreover, the results in figure 2 demonstrated that all examples of collateral sensitivity drugs required functional ABCB1, regardless of whether the drug is a substrate for the ABCB1-efflux pump (e.g. verapamil), not a substrate but stimulates the ABCB1 ATPase (e.g. progesterone) or interacts indirectly with ABCB1 (NSC73306).

ROS and GSH levels in CHO ΔABCB1 clones are comparable to AuxB1 cells - Previous studies on the verapamil- and progesterone- induced collateral sensitivity mechanism have described an increase in ROS production and a drop in GSH levels when CHO^{R}C5 cells were exposed to these compounds [14, 19, 20]. For this study, we used similar concentrations to

those assessed previously to determine whether these effects were present in the CHO^{R}C5 ABCB1-knockout clones. Moreover, as most collateral sensitivity drugs stimulated ABCB1-ATPase activity, drug concentrations that led to peak ABCB1-ATPase activity for verapamil and progesterone (i.e., 2 μM VP and 10 μM PRO) were used to measure their effects on ROS production and reduced total cellular thiols in cells. Figure 3A shows the percent increase in ROS in AuxB1 and CHO^{R}C5 and their respective ABCB1-knockout clones in the presence of verapamil and progesterone, respectively. As previously demonstrated, both verapamil and progesterone caused a significant increase in ROS in CHO^{R}C5 cells, relative to AuxB1 cells **(fig. 3A)**. Neither verapamil nor progesterone caused significant increase in ROS in CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3 or AuxB$^{\Delta ABCB1}$ clones B1 and B2, confirming the role of drug-induced stimulation of ABCB1-ATPase on the production of ROS in MDR cells. Figure 3B shows the percent total cellular reduced thiols in AuxB1, CHO^{R}C5 and their respective ABCB1-knockout clones in response to verapamil and progesterone treatment. The results in figure 3B show the reverse of those in figure 3A, whereby verapamil- and progesterone-induced increases in ROS lead to a significant drop in total cellular reduced thiols that correlated with ABCB1 expression between AuxB1 and CHO^{R}C5, an effect that was not seen in CHO^{R}C5-ABCB1 knockout clones.

Together, these results corroborate with our previous results, which used ABCB1 mediators to demonstrate that functional ABCB1 is required for the ROS-mediated collateral sensitivity mechanism by VP and PRO to occur.

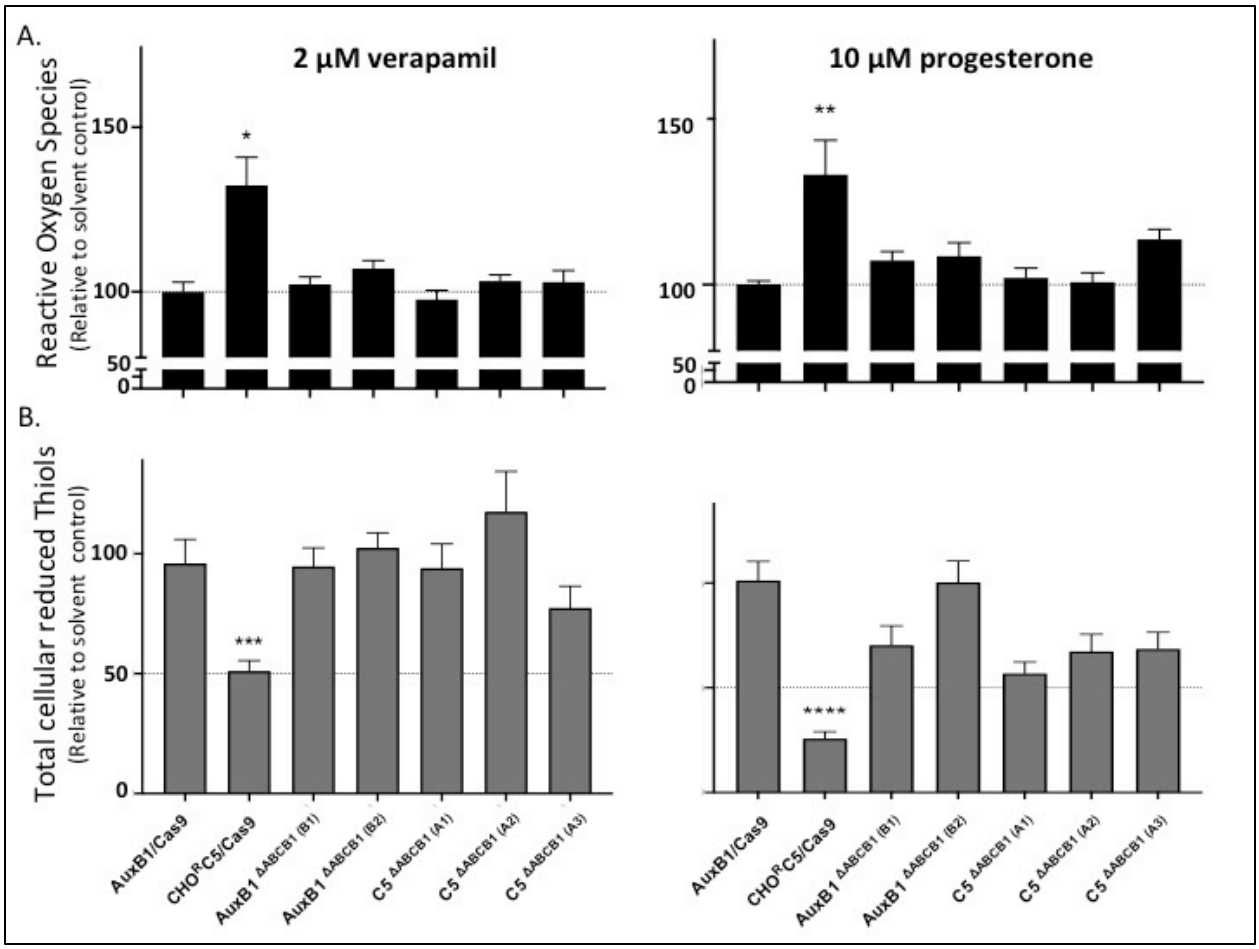

Figure 3. Effects of collateral sensitivity drugs verapamil and progesterone on ROS and GSH levels in CHO cells. Verapamil and progesterone are collateral sensitivity drugs that stimulate ABCB1 ATPase activity, increase the production of ROS and decrease GSH levels in the ABCB1-expressing MDR cells CHO^{R}C5. Graphs with black bars represent % ROS and graphs with grey bars represent % cellular reduced thiols. Panel A shows that 2 µM verapamil significantly increases ROS levels in CHO^{R}C5 cells compared to solvent control, but there is no effect on AuxB1 cells. Additionally, the CHO^{R}C5 ABCB1 knockouts do not have increased ROS levels in the presence of 2 µM verapamil. This increase in ROS is reflected by a decrease in total thiol levels, of which GSH is included, in the CHO^{R}C5 cells but not the knockouts, as seen in panel B. No significant decrease in cellular thiol levels is seen in the AuxB1 cells or any of the CHO knockouts. Comparably, 10 µM progesterone increases ROS levels in the CHO^{R}C5 cells, but not in the AuxB1 cells or the CHO^{R}C5 knockouts, as seen in panel A. Again, total thiol levels decrease in response to 10 µM progesterone in the ABCB1-expressing MDR CHO^{R}C5 cells, but not in any of the ABCB1 knockouts or parental AuxB1 cells. Graphs represent the mean ± standard deviation, representative of three independent experiments with at least 5 replicates.

Rh123 accumulation is increased in CHO^{R}C5 ΔABCB1 clones - Rhodamine 123 (Rh123) is a cell-permeant cationic dye that accumulates in cellular mitochondria and cytoplasm at higher concentrations. Rh123 is also a well-documented transport substrate of ABCB1, and has been used extensively to study ABCB1 transport function in cells in culture as well as in tumors [51-53]. For these reasons, and in an effort to further characterize CHO^{R}C5 ABCB1-knockout cells, we used Rh123 to study and compare differences in accumulation function between CHO^{R}C5 and CHO^{R}C5-ABCB1 knockouts. In agreement with previous reports, higher concentrations of Rh123 were found in the ABCB1 negative cell lines AuxB1 and AuxB1/Cas9, compared to the ABCB1 expressing MDR cells CHO^{R}C5 and CHO^{R}C5/Cas9 **(fig. 4A)**. Based on our previous results studying other substrates of ABCB1, it was expected that the CHO^{R}C5 ΔABCB1 clones A1, A2 and A3 would accumulate more Rh123, to similar levels of the ABCB1-negative cells. However, while we found that the knockouts accumulated significantly higher levels of Rh123 compared to the AuxB1 and CHO^{R}C5 cells, Rh123 accumulation was unexpectedly much higher in the CHO^{R}C5 ΔABCB1 clones compared to the ABCB1 negative cells **(fig. 4A)**. Analogously to the quantitative steady-state Rh123 accumulation studies seen in **(fig. 4A)**, confocal microscopy showed that AuxB1/Cas9 cells and AuxB1 ΔABCB1 clones B1 and B2 accumulated more Rh123 compared to CHO^{R}C5 cells, and the CHO^{R}C5 ΔABCB1 clones accumulated much more Rh123 compared to any of the other cell lines at the same gain **(fig. 4B)**. The accumulation was so high in the CHO^{R}C5 ΔABCB1 clones that cellular structures were not discernible. Together, the quantitative Rh123 accumulation and qualitative confocal microscopy studies show that indeed, ABCB1 function is eliminated in the CHO^{R}C5 knockouts, but we unexpectedly discovered that these cells also accumulate Rh123 at much higher levels than the ABCB1-

negative cell lines. To determine if this increased accumulation of Rh123 in CHO^{R}C5$^{\Delta ABCB1}$ clones was associated with specific cellular site of dye accumulation or associated with an active influx of dyes due to differential overexpression of another membrane transporter, we examined the accumulation of another ABCB1 fluorescent dye.

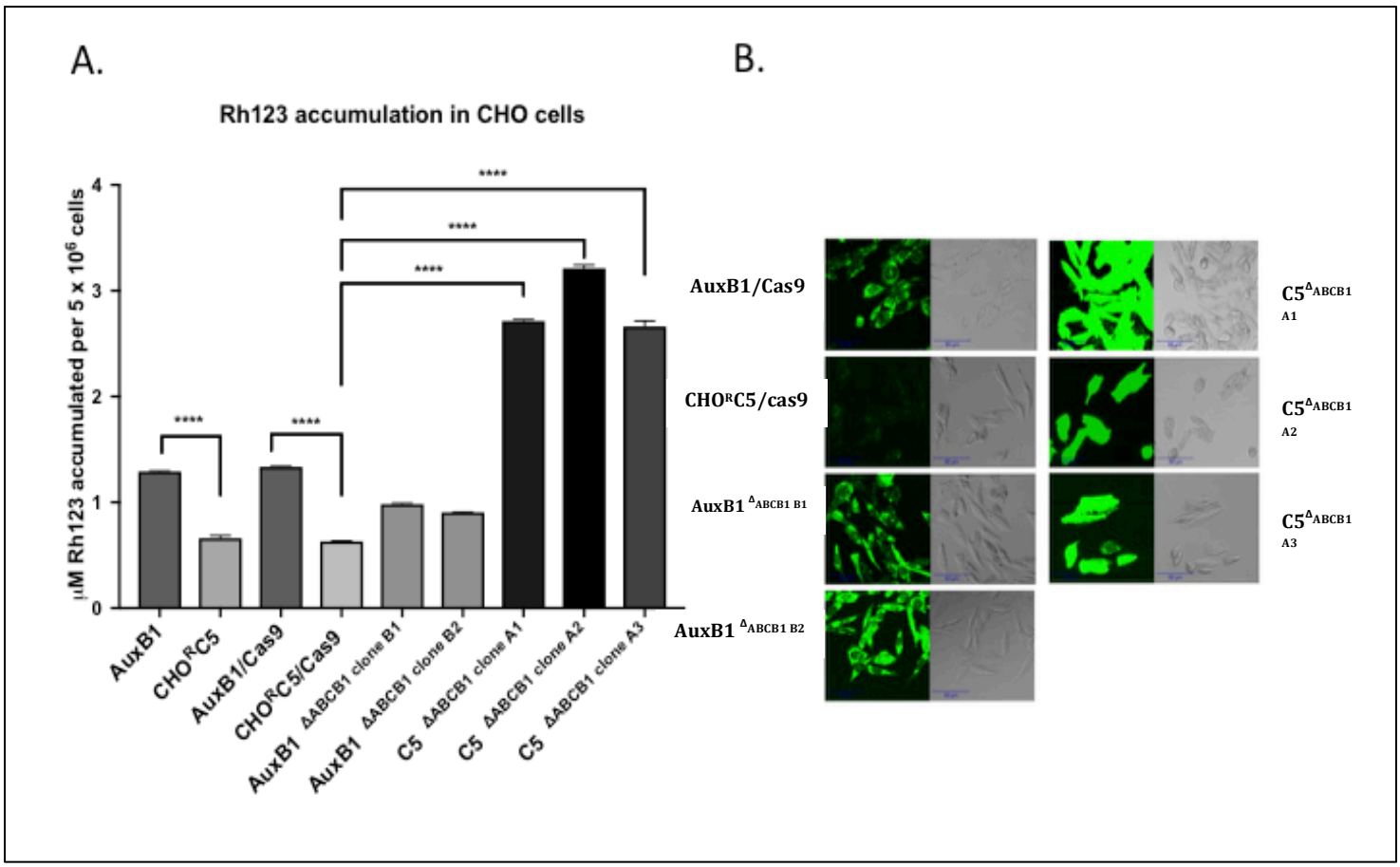

Figure 4. Rh123 accumulation in CHO cells and ABCB1 knockouts. Panel A shows a functional assay for ABCB1. Rh123 enters cells by passive diffusion and accumulates in the cytosol and mitochondria but cannot normally be extruded. There is reduced accumulation of Rh123 in CHO^{R}C5 and CHO^{R}C5/Cas9 cells, as ABCB1 mediates Rh123 efflux out of the cells. AuxB1, AuxB1/Cas9 and AuxB1 ΔABCB1 clones B1 and B2 show comparable accumulation of Rh123. CHO^{R}C5 ΔABCB1 clones A1-A3 show increased accumulation of Rh123 compared to all other cell lines, including those without high endogenous levels of ABCB1. Panel B shows confocal fluorescence microscopy of Rh123 accumulation in CHO cells. In CHO^{R}C5/Cas9 cells, ABCB1 mediated the efflux of Rh123. However, Rh123

accumulates in AuxB1/Cas9 and AuxB1 ΔABCB1 clones B1 and B2, more than in CHO^{R}C5 cells. Rh123 accumulation is visibly increased in the CHO^{R}C5 ΔABCB1 clones A1-A3 compared to all other cell lines for images obtained at the same gain value. Graphs represent the mean ± standard deviation, representative of three independent experiments done in triplicate. One asterisk (*) is indicative of a p value of < 0.05.

Hoechst 33342 is an uncharged, cell-permeant nucleic acid stain that emits fluorescence in the blue range when bound to double-stranded DNA. Hoechst 33342 is another well-characterized transport substrate of ABCB1 that is effluxed out of MDR cells but not the parental ABCB1-negative cell lines [54]. It is important to note that Rh123 and Hoechst 33342 have different binding pockets on ABCB1 [55, 56]. Figure 5A shows AuxB1/Cas9 cells, AuxB1 ΔABCB1 clones and CHO^{R}C5 ΔABCB1 clones accumulated more Hoechst 33342 compared to the ABCB1-expressing CHO^{R}C5 cells. However, dissimilarly to Rh123, the Hoechst 33342 dye did not accumulate more in the CHO^{R}C5-ABCB1 knockout clones than in the AuxB1 or AuxB1$^{\Delta ABCB1}$ clones B1 and B2 **(fig. 5A)**. This was further confirmed by confocal microscopy, whereby dye levels accumulated comparably in AuxB1/Cas9, AuxB1$^{\Delta ABCB1}$ clones and CHO^{R}C5$^{\Delta ABCB1}$ clones **(fig. 5B)**. The accumulation trend for Rh123, wherein the dye accumulates more in ABCB1 knockouts than ABCB1 negative cells, was not upheld for Hoechst 33342.

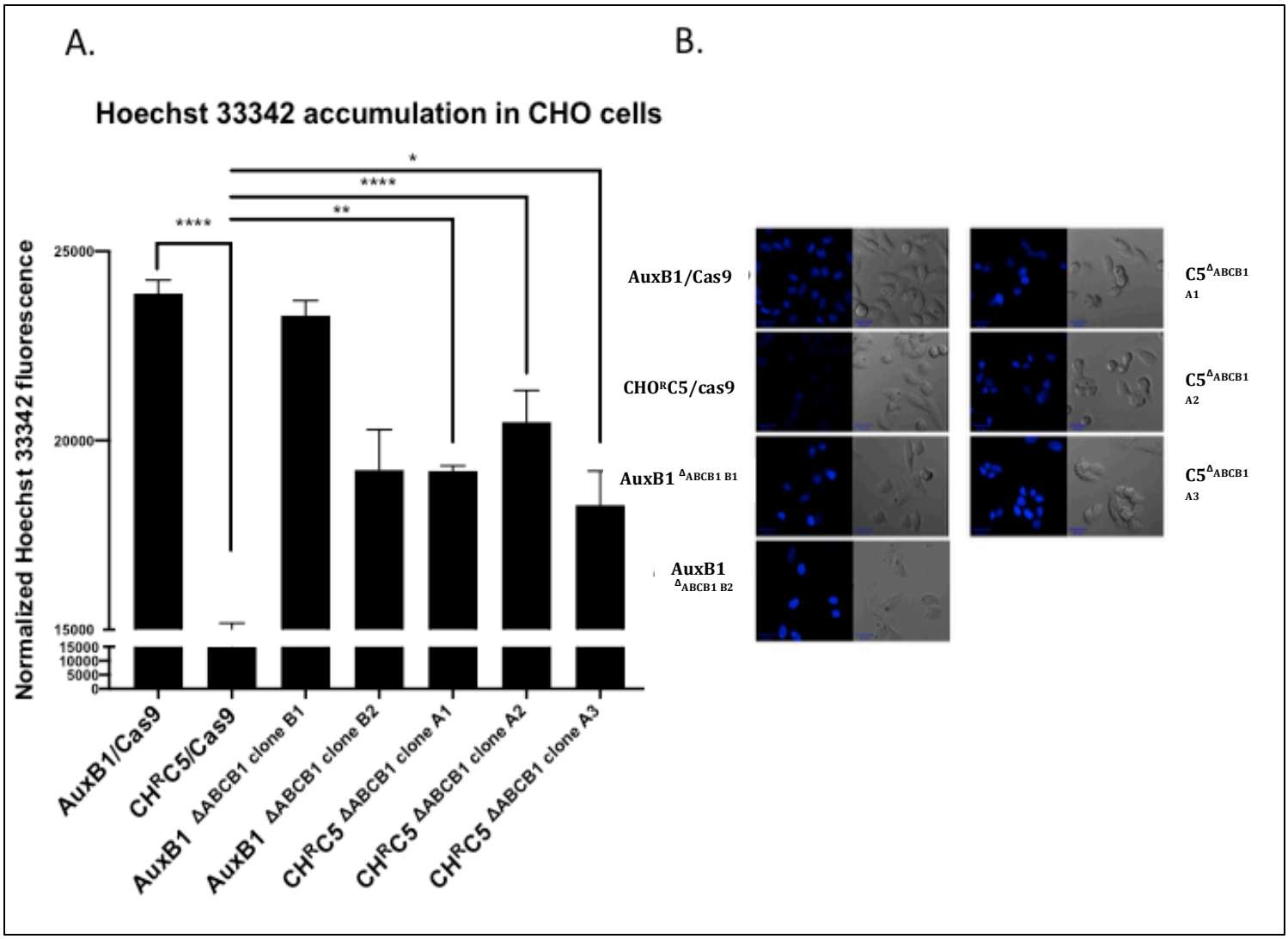

Figure 5. Hoechst 33342 accumulation in CHO cells and ABCB1 knockouts. Panel A shows a functional assay for ABCB1. Hoechst 33342 enters cells by passive diffusion and accumulates in the nucleus but cannot normally be extruded. Because Hoechst dye is a substrate of ABCB1, there is reduced accumulation of Hoechst 33342 in CHO^{R}C5 and CHO^{R}C5/Cas9 cells. AuxB1, AuxB1/Cas9, AuxB1 ΔABCB1 clones B1 and B2 and CHO^{R}C5 ΔABCB1 clones A1-A3 show higher accumulation of Hoechst 33342. Panel B shows confocal fluorescence microscopy of Hoechst accumulation in CHO cells. In CHO^{R}C5/Cas9 cells, Hoechst is effluxed by ABCB1. However, the dye accumulates in AuxB1/Cas9, AuxB1 ΔABCB1 clones B1 and B2 and CHO^{R}C5 ΔABCB1 clones A1-A3 at comparable levels. Graphs represent the mean ± standard deviation, representative of three independent experiments done in triplicate. One asterisk (*) is indicative of a p value of < 0.05.

In an effort to clarify the results obtained in the Rh123 and Hoechst33342 accumulation studies, we decided to examine the accumulation of other fluorescent compounds in the above cell lines as in figures 4 and 5. Doxorubicin is a cationic anthracyline drug used in the treatment of neoplasms, and a well-characterized substrate of ABCB1 [57]. It is known to accumulate in the nucleus and mitochondria of cells that do not express ABCB1 [58, 59], and can also be found in the cytosol at higher concentrations. Confocal microscopy, as shown in supplemental figure 2, shows that doxorubicin accumulated at comparable levels in AuxB1/Cas9 and in AuxB1 ΔABCB1 clones B1 and B2. Very little accumulation was seen in $CHO^{R}C5$/Cas9 cells, due to the presence of ABCB1. Similarly to Rh123, doxorubicin had visibly greater accumulation in the $CHO^{R}C5$ ΔABCB1 clones, especially clones A1 and A2, compared to the ABCB1-negative cell lines. Given these results, it appears that the increased dyes that show preferential accumulation in the cytosol (doxorubicin) and mitochondria (Rh123) show enhanced accumulation in $CHO^{R}C5^{\Delta ABCB1}$ clones relative to AuxB1 and $AuxB1^{\Delta ABCB1}$ clones. Consequently, these results speak to or predict changes at the mitochondria and/or cell membrane level that allows $CHO^{R}C5^{\Delta ABCB1}$ clones to accumulate more dye. To address possible changes in the mitochondria of $CHO^{R}C5^{\Delta ABCB1}$ cells, we examined the accumulation of MitoTracker™ Red, a cationic fluorescent dye derived from Rh123, and designed to accumulate in and stain mitochondria for organelle visualization by fluorescence confocal microscopy. Moreover, it was recently shown to be effluxed by ABCB1 [60]. Confocal microscopy, **(supplemental figure 3)** following steady state accumulation of MitoTracker™ Red shows comparable dye levels in AuxB1/Cas9 and in AuxB1 ΔABCB1 clones B1 and B2, but no accumulation was seen in the ABCB1-positive $CHO^{R}C5$/Cas9 cells. Similarly to Rh123, MitoTracker™ Red had greater accumulation in the

CHO^{R}C5 ΔABCB1 clones compared to the ABCB1-negative cell lines. Thus, given that Rh123, doxorubicin and MitoTracker™ Red all showed greater accumulation in the CHO^{R}C5 ΔABCB1 clones than in ABCB1-negative cells and have been shown to accumulate in the mitochondria it was of interest to assess differences in mitochondrial number or baseline metabolic function in the CHO^{R}C5 cells and CHO^{R}C5 ΔABCB1 clones that could help explain the discrepancy in dye accumulation.

Mitochondrial reduction of resazurin is comparable in all CHO cell lines – Although MitoTracker™ Red is a fluorescent dye that accumulates in the mitochondria; its use on ABCB1 positive cells is futile as it is a substrate for ABCB1. To determine if CHO^{R}C5$^{\Delta ABCB1}$ cells contain higher number of mitochondria or mitochondrial function, we examined the expression levels of a mitochondrial marker protein, HSP60 [61]. Figure 6A show HSP60 levels in AuxB1, CHO^{R}C5 and their respective ABCB1-knockout cell clones by Western blot. The results in figure 6A do not show higher levels of HSP60 in the knockouts, relative to α-tubulin loading control. We subsequently examined the mitochondrial reductive capacity in the above cell lines using the cell-permeable redox dye resazurin, used as an indicator of mitochondrial function, as it fluoresces when it is reduced [62]. Equal numbers of cells from each of the cell lines were exposed to resazurin, and mitochondrial function was assessed by comparing the fluorescence of each cell line relative to its parental cell type, AuxB1 or CHO^{R}C5. No significant differences in mitochondrial function were observed between the CHO^{R}C5 ΔABCB1 clones and any of the other cell lines **(fig. 6B)**. This result, along with the HSP60 levels, indicates that the differences seen in Rh123, doxorubicin and

MitoTracker™ Red accumulation are not likely due to differences in mitochondrial number or metabolic profile.

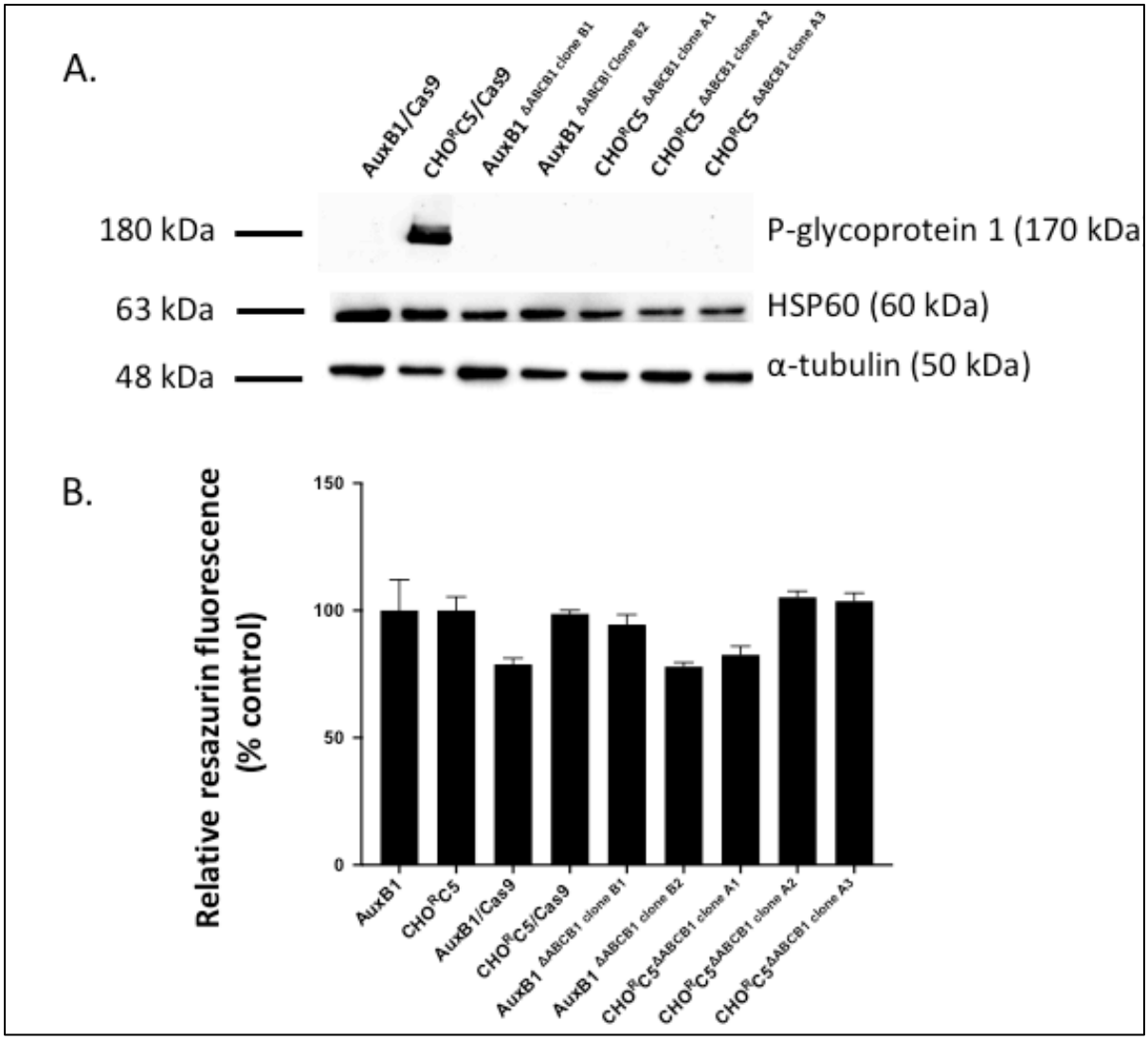

Figure 6. Mitochondrial protein and metabolic abilities in CHO cells. In order to assess whether mitochondrial number was equivalent in the CHO cells and knockouts, the presence of the mitochondrial protein HSP60 was assessed by Western blot in (A). HSP60 bands are comparable between AuxB1/Cas9, CHO^{R}C5/Cas9 and AuxB1 ΔABCB1 clones B1 and B2. The HSP60 band appears to be smaller in the CHO^{R}C5 ΔABCB1 clones A1-A3. Resazurin is a useful tool for measuring mitochondrial activity in live cells, as it is reduced in the mitochondria and fluoresces. Resazurin fluorescence was compared to appropriate parental control lines in (B). No significant difference in resazurin metabolism was noted. Graphs represent the mean ± standard deviation, representative of three independent experiments with at least 3 replicates. One asterisk (*) is indicative of a p value of < 0.05.

In the effort to explain the enhanced Rh123, doxorubicin and Mito-tracker dye accumulation in CHO^{R}C5 ΔABCB1 clones, we examined potential transporters that may recognize these compounds as substrates. We speculated that such a transporter may be upregulated with ABCB1 in the selection of CHO^{R}C5 cells from the parental line AuxB1; however in the presence of high levels of ABCB1, the impact of such a transporter may be negated by ABCB1 overexpression and function **(Fig. 7A-D).**

A number of potential candidates were revealed that shared substrates with ABCB1, notably Rh123, doxorubicin and MitoTracker™ Red. OCT1 (SLC22A1) and OCT2 (SLC22A2) are solute carriers present on cell membranes, and are known to transport Rh123 [63], but do not transport doxorubicin [64]. Another solute carrier protein SLC25A40 has been shown to be upregulated with ABCB1 [38, 65], but a drug substrate for this solute carrier has yet to be elucidated [66]. The organic cation transporter OCTN1 (SLC22A4) is a known cellular importer of doxorubicin mostly localized on the mitochondria and to a certain extent, on the cell membrane [67, 68]. No studies have been conducted on Rh123 or MitoTracker™ Red transport. OCTN1 is an importer responsible for the co-transport of sodium ions and ergothioneine into cells [69] and has recently been associated with a drug-resistance phenotype [70]. Verapamil is a known inhibitor of OCTN1 [71].

The breadth of OCTN1 substrate specificity remains unexplored. Because it was shown to be present on the cell membrane and import doxorubicin, and no studies have been done to confirm the lack of Rh123 transport, we decided to pursue OCTN1 as a candidate.

We assessed OCTN1 mRNA levels in AuxB1 and CHO^{R}C5 cells, and found that there is a significant increase in *OCTN1* mRNA expression in CHO^{R}C5 cells **(fig. 7E)**. OCTN1 may possibly be responsible for the increase in Rh123, doxorubicin and MitoTracker™ Red

accumulation seen in the CHO^{R}C5 ΔABCB1 clones, which may express more OCTN1, compared to ABCB1 negative cells. Following this logic, IC_{50} values for doxorubicin would therefore be expected to be even lower in the ABCB1 knockouts than in the ABCB1-negative cells, as the CHO^{R}C5 ABCB1 knockout clones would accumulate more doxorubicin and therefore be more sensitive to the drug. Preliminary results shown in supplementary Figure 4 corroborate this hypothesis. More investigation on the phenomenon of increased accumulation in CHO^{R}C5 ΔABCB1 clones is required.

In summary, expression of ABCB1 in CHO^{R}C5 cells was shown to be necessary for resistance to doxorubicin and colchicine. In addition, the expression of ABCB1 is essential for the collateral sensitivity effect of verapamil, progesterone and NSC73306, underlining the working mechanism of these drugs on MDR cells in a collateral sensitivity context. Despite other cellular changes associated with the development of the drug resistance phenotype in drug-selected cells, ABCB1 is still necessary and responsible for the collateral sensitivity effect to occur. This was underlined in the ROS and thiol measurement experiments, whereby CHO^{R}C5 ABCB1 knockouts did not have increased ROS or decreased cellular thiol levels that are seen in the wild-type multidrug resistant CHO^{R}C5 cells. During the characterization of the CHO^{R}C5 ABCB1 knockouts, they accumulated more Rh123 compared to the ABCB1-negative cells lines, prompting further investigation. This result is the subject of further study.

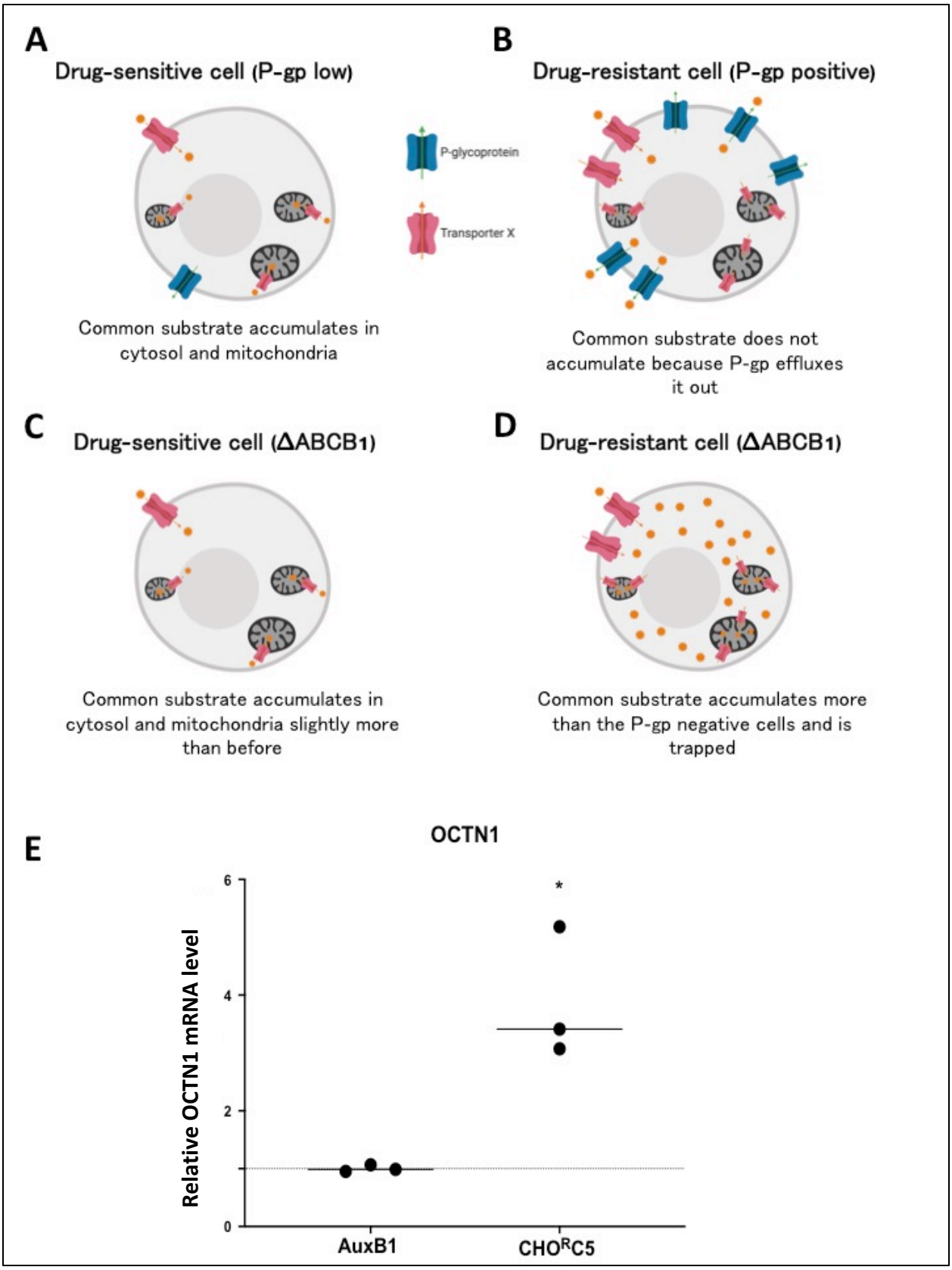
A
Drug-sensitive cell (P-gp low)
P-glycoprotein
Transporter X
Common substrate accumulates in cytosol and mitochondria
B
Drug-resistant cell (P-gp positive)
Common substrate does not accumulate because P-gp effluxes it out
C
Drug-sensitive cell (ΔABCB1)
Common substrate accumulates in cytosol and mitochondria slightly more than before
D
Drug-resistant cell (ΔABCB1)
Common substrate accumulates more than the P-gp negative cells and is trapped
E
OCTN1
Relative OCTN1 mRNA level
6
4
2
0
*
AuxB1
CHORC5

Figure 7. Hypothetical transport protein Transporter X and ABCB1 substrate accumulation in CHO cells. In order to explain the increased accumulation of certain substrates in CHO^{R}C5 ΔABCB1 clones A1-A3, we hypothesized that there exists a transporter with common substrates to ABCB1, on the plasma cell and mitochondrial membranes of CHO cells, and up-regulated in the CHO^{R}C5 cells. (A) A drug-sensitive cell with less ABCB1 and less Transporter X would accumulate the common substrate in the cytosol and mitochondria. (B) A drug-resistant cell with higher levels of expression of ABCB1 and Transporter X would not accumulate the common substrate, as it is effluxed out by ABCB1 before it can accumulate in the cell. (C) A drug-sensitive cell with ABCB1 knocked out and low levels of Transporter X would accumulate the common substrate to comparable levels as the wild-type cells. (D) A drug-resistant cell with ABCB1 knocked out and higher levels of Transporter X would accumulate much more of the common substrate. (E) A qPCR assay was done to compare the mRNA levels of a candidate Transporter X, OCTN1. Results showed increased levels of OCTN1 mRNA in CHO^{R}C5 cells. Each filled circle represents one sample reading. One asterisk is indicative of $p < 0.05$.

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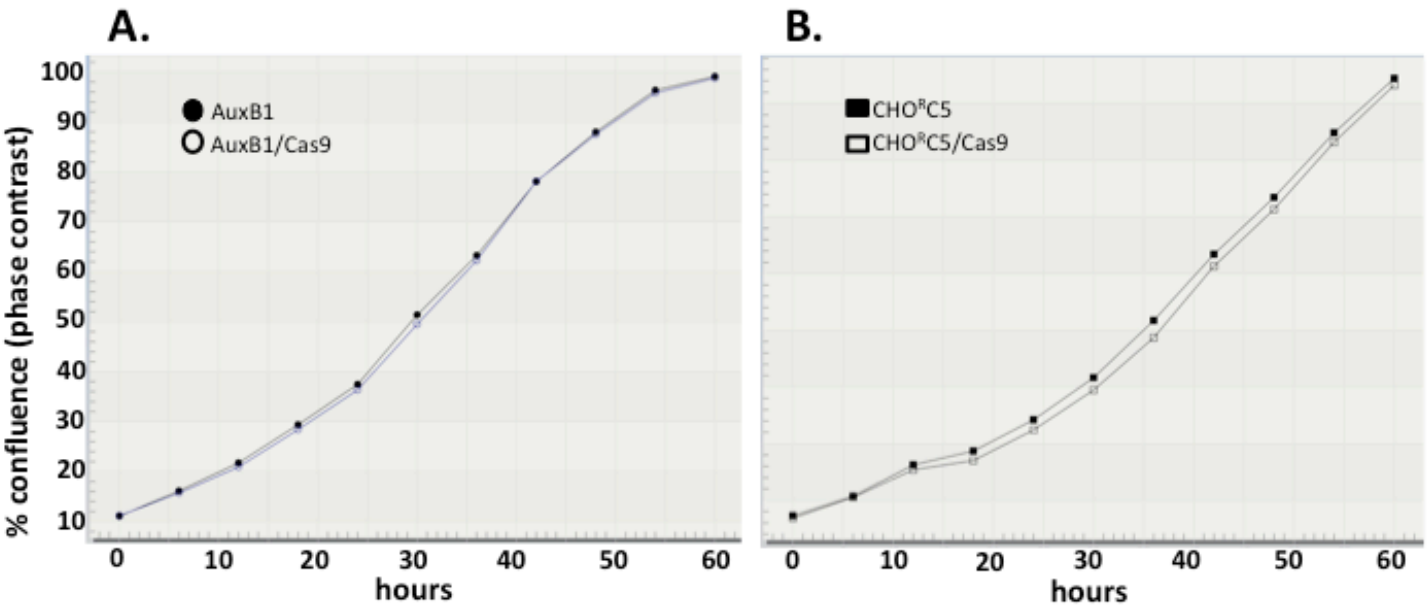
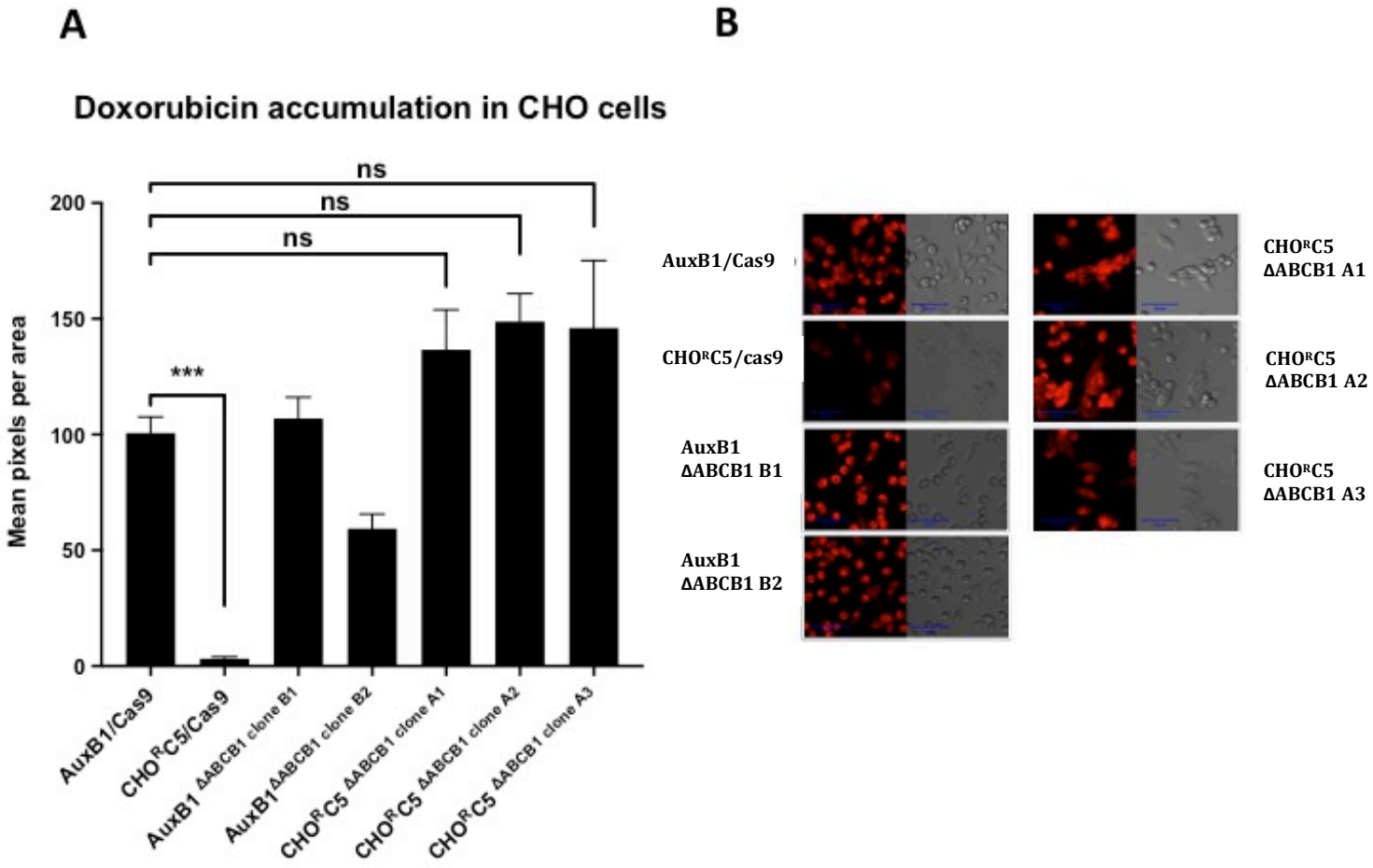
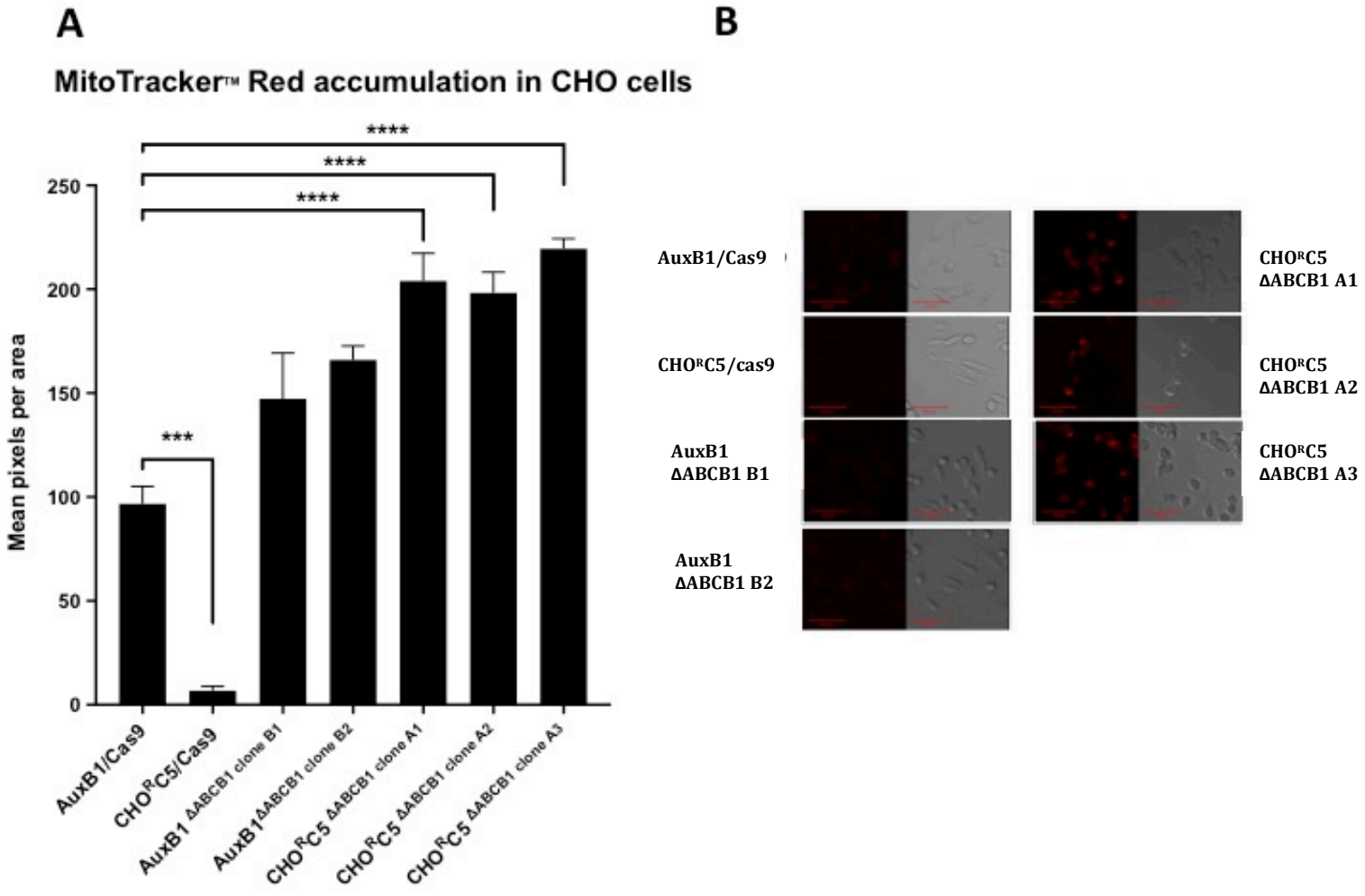
Conflicts of Interest – There are no conflicts of interest to report from either of the authors.



Supplemental Figure 1. Comparing AuxB1 and CHO^{R}C5 wild-type population cells to transiently transfected Cas9 cells. Panel A shows growth curves assessed via the IncuCyte over the course of 60 hours for AuxB1 and AuxB1/Cas9 cells. Panel B shows growth curves assessed via the IncuCyte over the course of 60 hours for CHO^{R}C5 and CHO^{R}C5/Cas9 cells.



Supplemental Figure 2. Confocal images of doxorubicin accumulation in CHO cells. Doxorubicin enters cells by passive diffusion and accumulates in the cytosol, nucleus and mitochondria but cannot normally be extruded. Doxorubicin is a substrate of ABCB1, therefore there is reduced accumulation of the dye in CHO^{R}C5/Cas9 cells. Without any functional ABCB1, AuxB1/Cas9 and AuxB1 ΔABCB1 clones B1 and B2 show comparable accumulation. CHO^{R}C5 ΔABCB1 clones A1-A3 show slightly higher accumulation than AuxB1 cells and AuxB1 ΔABCB1 clones. A semi-quantitative comparison was established by counting mean fluorescent pixels per equal area (minimum of 5 cells) using the ImajeJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.). Calculated pixel differences showed no significant difference.



Supplemental Figure 3. Confocal images of MitoTracker™ Red accumulation in CHO cells. MitoTracker™ Red is a chemical analogue of Rh123. It accumulates in the mitochondria and cytosol of cells by passive diffusion and cannot be extruded. MitoTracker™ is a substrate for ABCB1, so it was no surprise that there is reduced accumulation of the dye in CHO^{R}C5/Cas9 cells. Without any functional ABCB1, AuxB1/Cas9 and AuxB1 ΔABCB1 clones B1 and B2 show comparable accumulation of MitoTracker™. CHO^{R}C5 ΔABCB1 clones A1-A3 show higher accumulation than AuxB1 cells and AuxB1 ΔABCB1 clones. A semi-quantitative comparison was established by counting mean fluorescent pixels per equal area (minimum of 5 cells) using the ImajeJ software. One asterisk is indicative of $P < 0.05$.

	AuxB1/ Cas9	CHO^{R}C5/ Cas9	AuxB1 ΔABCB1 clone B1	AuxB1 ΔABCB1 clone B2	CHO^{R}C5 ΔABCB1 clone A1	CHO^{R}C5 ΔABCB1 clone A2	CHO^{R}C5 ΔABCB1 clone A3
IC_{50} Doxorubicin (μM)	0.06 ± 0.004	3.8 ± 0.016	0.04 ± 0.000	0.05 ± 0.007	0.05 ± 0.002	0.03 ± 0.005	0.04 ± 0.002
IC_{50} Colchicine (μM)	0.03 ± 0.004	55.28 ± 7.06	0.03 ± 0.004	0.03 ± 0.007	0.06 ± 0.007	0.03 ± 0.007	0.06 ± 0.006

Supplemental Table 1. Effect of doxorubicin on CHO^{R}C5 ΔABCB1 clones. We hypothesized that the CHO^{R}C5 ΔABCB1 clones had increased accumulation of doxorubicin compared to ABCB1 negative cells due to the presence of an importer on the cell membrane, with common substrates to ABCB1, that is unaffected by the knockout of the protein. Doxorubicin is a fluorescent drug that accumulated more in the CHO^{R}C5 ΔABCB1 clones, and should therefore have a lower IC_{50} value in those cells compared to the other cell lines, which appeared to be the case based on this very preliminary Sulforhodamine B-based cytotoxicity study. The method for Sulforhodamine B cytotoxicity assays was followed as previously described [72].

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CONNECTING STATEMENT 3

In the first manuscript (Chapter 2), we established that the collateral sensitivity drugs tamoxifen and veramapil target ABCB1-expressing MDR clones, and decrease ABCB1 expression in surviving cells. In the second manuscript (Chapter 3), to better understand the mechanisms involved in collateral sensitivity, we characterized a new tool that may be used to enhance the study and understanding of both drug resistance and collateral sensitivity, the CHO ABCB1 knockout clones, with the ultimate goal of creating collateral sensitivity treatment protocols for clinical use. In this third manuscript (Chapter 3), we use the aforementioned tools to characterize the mechanism of a newly identified collateral sensitivity drug, trifluoperazine. Unlike verapamil, trifluoperazine has no cardiotoxic effects, and it synergizes with tamoxifen.

4

Collateral sensitivity of multidrug resistant cells to the phenothiazine trifluoperazine is dependent on ABCB1 expression and ATPase activity.

Contents

Collateral sensitivity of multidrug resistant cells to the phenothiazine, trifluoperazine, is dependent on ABCB1 expression and ATPase activity

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Keywords: P-glycoprotein, ABCB1, Collateral sensitivity, trifluoperazine, oxidative stress, ATPase, Drug resistance

ABBREVIATIONS

MDR: multidrug resistance or multidrug resistant
ABCB1: ATP-binding cassette transporter B1 (MDR1/P-gp)
TFP: trifluoperazine
ROS: reactive oxygen species
NAC: N-acetyl-L-cysteine
GSH: glutathione
PI: propidium iodide
CDDP: cis-platinum
PARP: poly (ADP-ribose) polymerase

ABSTRACT

The expression of certain ATP-dependent drug transporters in patient tumors post-chemotherapeutic drug treatment has been linked to poor treatment outcome and the multidrug resistance phenotype. The over-expression of P-glycoprotein, a member of the B-subfamily of the ATP-binding cassette (ABC) superfamily (e.g. ABCB1), has been demonstrated to confer resistance to several clinically relevant anticancer drugs in tumor cells. The same protein was shown to confer hypersensitivity or collateral sensitivity to certain drugs. In this report, we examine the capacity of trifluoperazine (TFP), a calmodulin inhibitor previously shown to interact with ABCB1 and stimulate its ATPase activity, to induce the collateral sensitivity of ABCB1-overexpressing multidrug resistance cells. Our results show that TFP-induced collateral sensitivity is linked to ABCB1 expression and ATPase activity, whereby ABCB1 CRISPR knockout cells ($CHO^{R}C5^{\Delta ABCB1}$ clones A1-A3) or inhibitors of ABCB1 ATPase led to the complete reversal of TFP-mediated collateral sensitivity. Moreover, similar to other collateral sensitivity drugs, TFP caused a rise in reactive oxygen species (ROS) and a large drop in reduced thiol levels in ABCB1-expressing cells that were reversed with a specific inhibitor of ABCB1-ATPase activity (e.g., PSC-833) and N-acetyl cysteine (NAC), respectively. In addition, we demonstrate that TFP-induced collateral sensitivity is mediated by apoptotic cell death, likely due to enhanced oxidative stress. Moreover, we show that TFP synergizes with another collateral sensitivity drug, tamoxifen, to induce further collateral sensitivity. Taken together, the findings in this study show for first time the use of TFP as a collateral sensitivity drug, at clinically achievable concentrations, in ABCB1-overexpressing drug resistant cells.

INTRODUCTION

Cancer cells in malignant tumors with intrinsic or acquired mechanisms of multidrug resistance (MDR) remain an obstacle in the treatment of patients with chemotherapeutic drugs [1]. Among the various mechanisms of MDR [2], the P-glycoprotein drug efflux mechanism has been the most studied and prevalent in clinically resistant tumor samples [3]. P-glycoprotein is a surface membrane transporter encoding twelve transmembrane helices and two cytoplasmic domains, each containing an ATP-binding Cassette (ABC) signature sequence [4]. In mammalian cells, the ABC superfamily consists of 49 members divided into 8 subfamilies (ABCA-H), with P-glycoprotein part of subfamily B (i.e., ABCB1) [5]. Using purified ABCB1 reconstituted into liposomes, studies have confirmed the function of ABCB1 as an ATP-dependent drug efflux transporter [6, 7], and as such ABCB1 overexpressing cells have been shown to display resistance to a vast list of cytotoxic compounds, including most chemotherapeutic anticancer drugs and clinically relevant tyrosine kinase inhibitors [8, 9]. Moreover, ABCB1 expression in normal tissues (e.g., blood brain barrier, liver, intestine, placenta, adrenal and kidney) is consistent with a protective detoxification mechanism [10].

ABCB1-interacting drugs have been broadly divided into three groups [11]: a) efflux substrates (e.g., colchicine, paclitaxel); b) drug substrates that stimulate ATPase activity (e.g., verapamil, quinidine); c) Non-substrate drugs that stimulate or inhibit ATPase activity (e.g., progesterone and cyclosporine A, respectively). Among the ABCB1 interacting drugs, certain non-toxic drugs (e.g., verapamil and cyclosporine A) were shown to reverse the resistance of tumor cells by competitive and non-competitive inhibition of its drug efflux mechanism, respectively [12]. The development of 3rd generation ABCB1-specific inhibitors

(e.g. Zosuquidar, Elacridar and Tariquidar), led to several phase III clinical trials whereby ABCB1 inhibitors were combined with cytotoxic anti-cancer drugs with the aim of targeting drug-resistant tumors in cancer patients. Unfortunately, all clinical trials were terminated due to intolerable toxicity caused by altered pharmaco-kinetics of cytotoxic drugs in tissues and organs with normal ABCB1 expression [13].

Earlier studies have revealed that ABCB1-overexpressing MDR cells also exhibited "hypersensitivity or collateral sensitivity" to certain drugs at typically non-toxic concentrations, including nonionic detergents and calcium channel blockers [14, 15]. Later studies by our group revealed the mechanism of the collateral sensitivity drugs on MDR cells, which correlated with their ability to stimulate ABCB1 ATPase activity [16, 17]. The efficiency of most collateral sensitivity drugs appears to correlate with the level of ABCB1 expression and the ability of such compounds to stimulate the ABCB1 ATPase. Hence, exposure of MDR cells to collateral sensitivity drugs was shown to result in increased levels of reactive oxygen species (ROS) and subsequent oxidative cell death [17, 18]. Consistent with the latter proposed mechanism of collateral sensitivity, inhibiting ABCB1-ATPase activity or knockdown of ABCB1 expression reversed the collateral sensitivity of MDR cells [18]. However, certain collateral sensitivity compounds (e.g. thiosemicarbazone) shown to induce collateral sensitivity of MDR cells did not interact with, or stimulate ABCB1 ATPase, but required the overexpression of an active ABCB1 in MDR cells [19]. More recently, Al-Akra *et. al.* [20] have suggested that MDR cells are collaterally sensitive to thiosemicarbazone due to the presence of external stress that leads to ROS and internalization of ABCB1 which actively accumulates copper bound-thiosemicarbazone into lysosomal compartments leading to autophagic cell death. Although it was not shown

how increased ROS induced ABCB1 internalization, cytoplasmic vesicles containing functional ABCB1 have been described in certain MDR cells [21]. Therefore, in an effort to resolve ABCB1-mediated collateral sensitivity, and to repurpose clinically safe drugs as collateral sensitivity drugs, we investigated the effects of trifluoperazine (TFP), a calmodulin inhibitor, previously shown to interact with ABCB1 and stimulate its ATPase activity at clinically achievable concentrations [22, 23], as a collateral sensitivity drug.

MATERIALS AND METHODS

Materials - The trifluoperazine dihydrochloride, Dimethylsulfoxide, 2',7'-dichlorofluorescin diacetate, 5,5'-dithio-bis (2-nitrobenzoic acid), doxorubicin, valspodar and Tween20 (P9416) were all purchased from Sigma Aldrich. The eSpCas9(1.1) was a gift from Feng Zhang (Addgene plasmid; http://n2t.net/addgene; RRID: Addgene). Lipofectamine 2000, the microBCA kit and the Pierce™ ECL Western Blotting Substrate Kit were all products of Thermo Fisher. Propidium iodide and the HRP-conjugated goat anti-mouse were purchased from Invitrogen, and the IncuCyte® is a product of Essen BioScience.

ABCB1 CRISPR knockout and cell proliferation assay - AuxB1, $CHO^{R}C5$, MDA-MB-231 and MDA-MB-231 400 nM doxo cells were cultured in Minimum Essential Medium α (αMEM) supplemented with 8% Fetal Bovine Serum (FBS) or DMEM, respectively. The CRISPR knockout protocol of ABCB1 cell lines AuxB1 and $CHO^{R}C5$ [16] cells and their characterization is described elsewhere (Limniatis and Georges, ABCB1 CRISPR/Cas9 knockout in multidrug-resistant cells shows complete reversal of drug resistance and collateral sensitivity phenotypes 2020; manuscript in preparation). For cell proliferation, without and with increasing concentrations of drugs, AuxB1, $CHO^{R}C5$, and their respective ABCB1-knockout clones were seeded in 48-well plates at 200-700 cells/well. After 24h in culture, drugs were added to each well and cells were allowed to proliferate for 7 days. The effects of drugs on cell proliferation was determined using methylene blue dye (0.2% methylene blue in ethanol:water (1:1 v:v). Cells were fixed and stained for 20 minutes, washed with cold water and plates were left to dry overnight. To quantify the effects of drugs on the growth of cells, stained cell colonies were solubilized in 0.1% SDS/PBS for 1h

at room temperature. The released dye in the 48-well plates was read using the Synergy H4 plate reader at 660 nm.

ATPase activity assay - Measurements of ABCB1-ATPase activity in purified plasma membrane fractions were performed as previously described by Litman *et al.* [24] with some modifications. Briefly, plasma membrane fractions were isolated from AuxB1 and $CHO^{R}C5$ cells and diluted to 20 µg/ml in ice-cold ATPase assay buffer (3 mM ATP, 100 mM KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 100 mM Tris pH 8.0, 4 mM EGTA, 2 mM ouabain, and 10 mM NaN_3). In a 96-well plate, 1 µg of protein was loaded (corresponding to a total volume of 50 µl). Membranes were incubated with increasing concentrations of trifluoperazine (0-400 µM) for 1hr at 37°C, and the assay was terminated using 200 µL of ice-cold stopping medium (0.2% ammonium molybdate, 1.3% sulfuric acid, 0.9% SDS, 2.3% trichloroacetic acid, and 1% ascorbic acid) to each well and incubated for 75 minutes. The released phosphate was quantified calorimetrically in a microplate reader (Dynatech Laboratories, MR5000) at 630 nm. The experiment was repeated three separate times using triplicates.

ROS measurements - AuxB1 and $CHO^{R}C5$ cells were seeded at a density of 50,000 cells/well in a clear-bottomed black-well plate and allowed to settle for 24 hours, after which the medium was removed and the cells were allowed to incubate with 100 µM H_2DCFDA for 45 minutes at 37°C. Wells were washed with sterile, cold HBSS, and then 100 µl of fluorobrite αMEM + 8% FBS was added in each well. Dilutions of TFP or hydrogen peroxide (2.5 mM) were added and allowed to incubate for another 24 hrs, after which the plate was read in

the H4 Synergy plate ready (485 $_{ex}$, 527 $_{em}$). The data were analyzed and the fold increase in fluorescence compared to respective solvent control was plotted using GraphPad Prism (GraphPad Software, version 8.0.1).

Measurement of total in-situ reduced sulfhydro levels - AuxB1 and CHO^{R}C5 cells were seeded at a density of 100,000 cells/well in a 48-well plate and allowed to settle for 24hrs, after which the trifluoperazine dilutions were added. Following another 24-hour incubation, media was removed and 250 µl of RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, pH 8) was added. Plates were placed on a gentle shaker for 20 minutes and 50 µl of each sample was removed for use in a microBCA assay following the protocol provided by the manufacturer. In the remaining samples, 50 µl of 50 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) (DTNB) was added and allowed to incubate for 30 minutes. Plates were read at 412 nm on Synergy H4™ Hybrid Multi-Mode Microplate reader (Biotek Inc.). Analysis was done comparing the absorbance of each sample to the total amount of protein in the sample, yielding the relative amount of reduced sulfhydro compared to solvent control. The data was analyzed and the total amount of reduced sulfhydro groups compared to respective solvent control was plotted using GraphPad Prism (GraphPad Software, version 8.0.1).

Measurement of apoptotic events - AuxB1 and CHO^{R}C5 cells were seeded at a density of 10,000 cells/well in black, clear-bottomed 96-well plates and allowed to settle for 24 hours, after which the trifluoperazine dilutions were added to the media, containing propidium iodide at a final concentration of 1.5 µM. The IncuCyte was set up to track cell number, cell

confluency and red apoptotic events (red-stained cells) over time. A 30-hour time point was selected for analysis to compare total cell number, as well as the proportion of apoptotic cells (apoptotic cells/ total number of cells) with increasing concentrations of trifluoperazine. Relative apoptosis is indicative of the ratio of red-stained cells to the total number of cells.

SDS-PAGE and Immunoblotting - Total cell extracts (20 µg) from AuxB1, $CHO^{R}C5$ cells and their respective ABCB1-knockout clones were obtained using RIPA buffer and loaded onto an 8% SDS-PAGE gel. Proteins were then transferred onto a polyvinyldene difluoride (PVDF; Immobilon®-P) membrane for 2.5 hours at 400 mA. The PVDF membrane was then blocked in 5% milk/PBS-Tween20 (0.05%) at 4°C overnight. ABCB1 expression was detected using the C494 monoclonal antibody at a dilution of 1:1000 [25] and HRP-conjugated goat anti-mouse IgG was used as the secondary antibody, also at a 1:1000 dilution. PARP expression was detected using the C2-10 anti-PARP antibody from R&D Systems in a 1:1000 dilution. The chemiluminescent reaction was achieved using the Thermo Fisher Pierce™ ECL kit and the signal was captured using the Thermo Fisher MyECL digital imager. Alpha-tubulin was detected using supernatant from hybridoma cultures (1:200, mouse hybridoma 12G10, deposited to the DSHB by Frankel, J. / Nelsen, E.M. {DSHB Hybridoma Product 12G10 anti-alpha-tubulin}) in 5% milk/PBS-Tween20 as described above and the same HRP-conjugated goat anti-mouse IgG was used as the secondary antibody, at a 1:1000 dilution.

Data and statistical analyses - The data was analyzed and the changes are compared to respective solvent control and plotted using GraphPad Prism (GraphPad Software, version 8.0.1) and statistical analysis was done using a one-way ANOVA, where $P<0.05$ (*), $P<0.01$ (**), $P<0.005$ (***), or $P < 0.001$ (****).

RESULTS AND DISCUSSION

Collateral sensitivity to trifluoperazine - We have previously shown that drugs that stimulate the ABCB1 ATPase preferentially target multidrug resistant cells expressing high levels of ABCB1 protein [17, 18, 26]. Trifluoperazine (TFP), an antipsychotic drug [27], was shown to stimulate ABCB1 ATPase using plasma membranes from CHO^{R}C5 cells (**fig. 1A** and **1B**, with max ATPase activity at 3 μM) as well as purified ABCB1 from CHO^{R}C5 cells reconstituted into membrane vesicles [28-30]. Given TFP's capacity to stimulate the ABCB1 ATPase, it was of interest to study the potential selective targeting of CHO^{R}C5 cells by TFP relative to the parental AuxB1 cells, which show lower detectable levels of ABCB1 compared to the CHO^{R}C5 cells **(fig. 1A)**. Figure 1C shows the effects of increasing TFP on the proliferation of AuxB1 and CHO^{R}C5 cells in the absence and in the presence of the established ABCB1 ATPase inhibitor PSC-833 (2 μM, [31]), respectively. In response to increasing concentrations of TFP, CHO^{R}C5 cells show dose-dependent sensitivity (IC_{50} 0.232 ± 0.07 μM), or collateral sensitivity, to TFP, compared to the parental cell line AuxB1 (5.07 ± 0.99 μM). Moreover, the addition of PSC833 (Valspodar) completely reversed the collateral sensitivity of CHO^{R}C5 cells to TFP (**fig. 1C**; IC_{50} of 4.3 ± 0.78 μM versus 3.07 ± 0.25 μM for AuxB1). These results suggest that ABCB1 with functional ATPase activity plays an essential role in the collateral sensitivity effect of drug resistant cells to clinically relevant concentrations of TFP [35], and inhibition of the ABCB1 ATPase abolishes TFP-mediated collateral sensitivity. Similar effects of TFP were observed using doxorubicin-selected human triple negative breast cancer cells (MDA-DOXO^{400}), which express much lower levels of ABCB1 than CHO^{R}C5 cells **(fig. 2A)**. The results in figure 2B show MDA-DOXO^{400} cells to be more sensitive than the parental MDA-MB-231 cells to TFP. The IC_{50} for the

MDA-MB-231 cells is 3.59 ±0.49 μM TFP whereas the IC_{50} value for MDA $^{400\ nM\ doxo}$ cells is 0.99 ±0.11 μM. These results show a correlation between ABCB1 expression and the sensitivity of ABCB1 expressing cells to TFP.

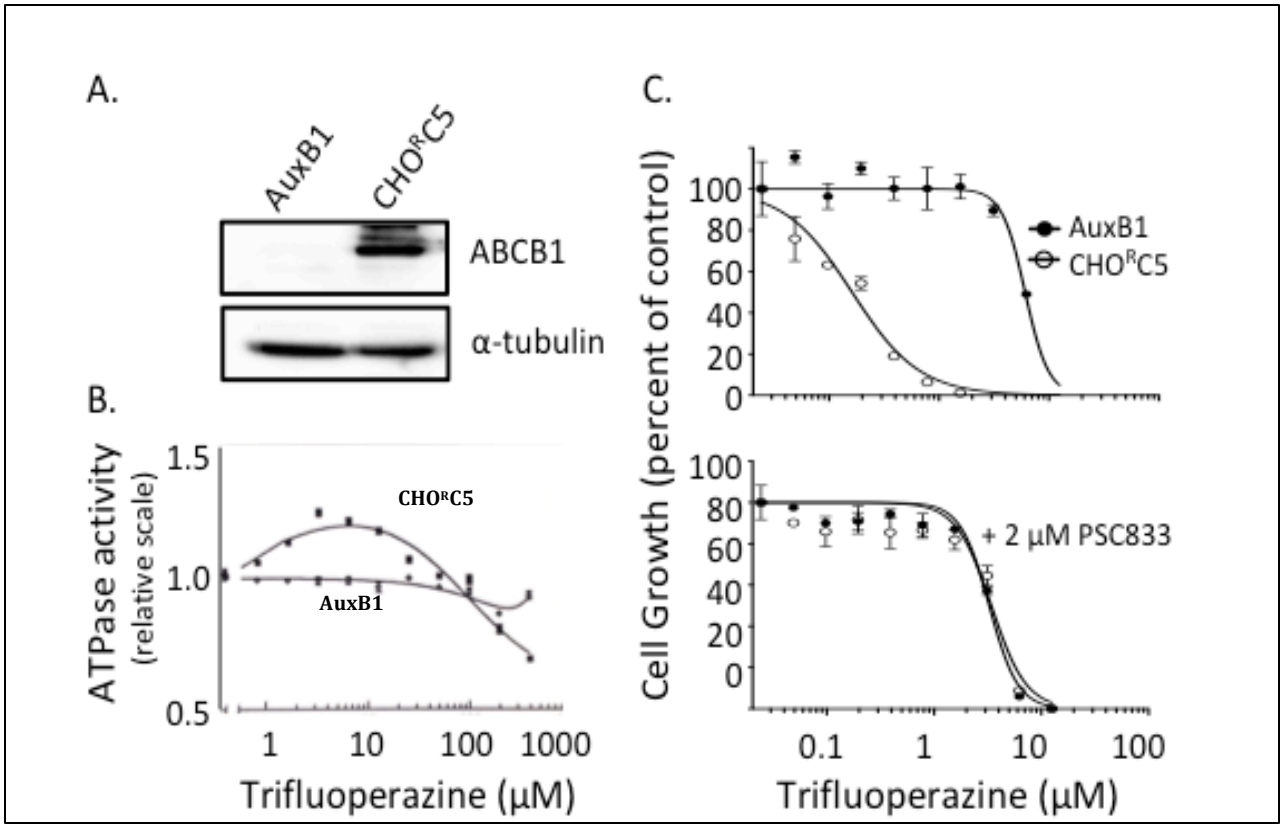

Figure 1. Multidrug-resistant cells are collaterally sensitive to trifluoperazine - Panel-1A shows Western blot analysis of drug-sensitive (AuxB1) and -resistant (CHO^RC5) cell extracts probed with anti-ABCB1-specific (C494) and anti-α-tubulin monoclonal antibodies, respectively. Panel 1B shows the effects of increasing concentrations of TFP on ABCB1-ATPase activity measured in purified membrane fractions from AuxB1 and CHO^RC5 cells, with peak ATPase activity measured at 3 μM TFP (see methods). Panel C shows cell proliferation assays measuring the cell growth of AuxB1 and CHO^RC5 cells cultured with increasing concentrations of TFP (1-100 μM) alone or in the presence of 2 μM PSC-833. Graphs represent the mean **±** SD of three independent experiments done in triplicate.

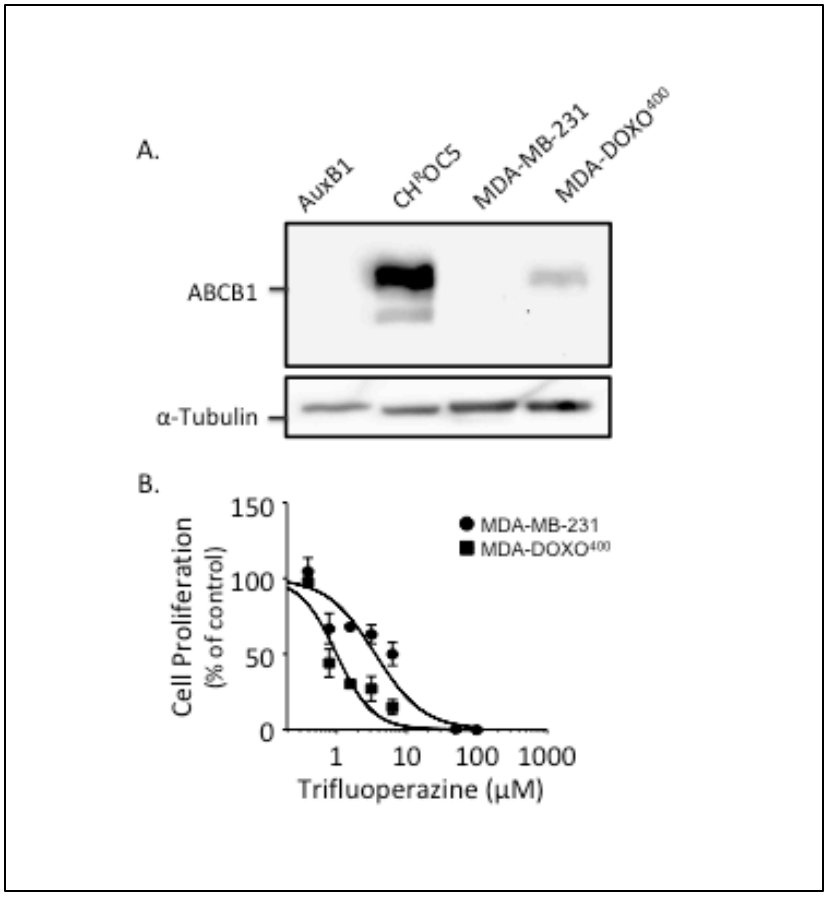

Figure 2. Trifluoperazine preferentially inhibits the growth of doxorubicin-resistant human triple negative breast cancer cells – Panel A shows the relative expression of ABCB1, by Western blot analysis, of drug-sensitive (AuxB1 and MDA-MB-231) and –resistant (CHO^{R}C5 and MDA-DOXO^{400}) total cell lysates. The Western blot was probed with anti-ABCB1 (C494) and anti- α-tubulin monoclonal antibodies, respectively. Panel B shows the proliferation of drug-sensitive (MDA-MB-231) and –doxorubicin-resistant (MDA-DOXO^{400}) cells cultured *in vitro* in the presence of increasing concentrations of TFP (1-100 μM). Graphs represent the mean **±** SD of three independent experiments done in triplicate.

ABCB1 expression is essential for TFP-induced collateral sensitivity - The above results suggest a strong correlation between ABCB1 expression in CHO^{R}C5 cells and TFP-induced collateral sensitivity. To confirm the direct role of ABCB1 expression in TFP collateral sensitivity, we made use of ABCB1-knockouts from both AuxB1 and CHO^{R}C5 cells using the CRISPR approach. AuxB1 and CHO^{R}C5 were transfected with eSpCas9(1.1)

plasmid or eSpCas9(1.1) containing ABCB1-specific guide RNA as previously described. Figure 3A shows complete knockout of ABCB1 expression in all three CHO^{R}C5 clones.

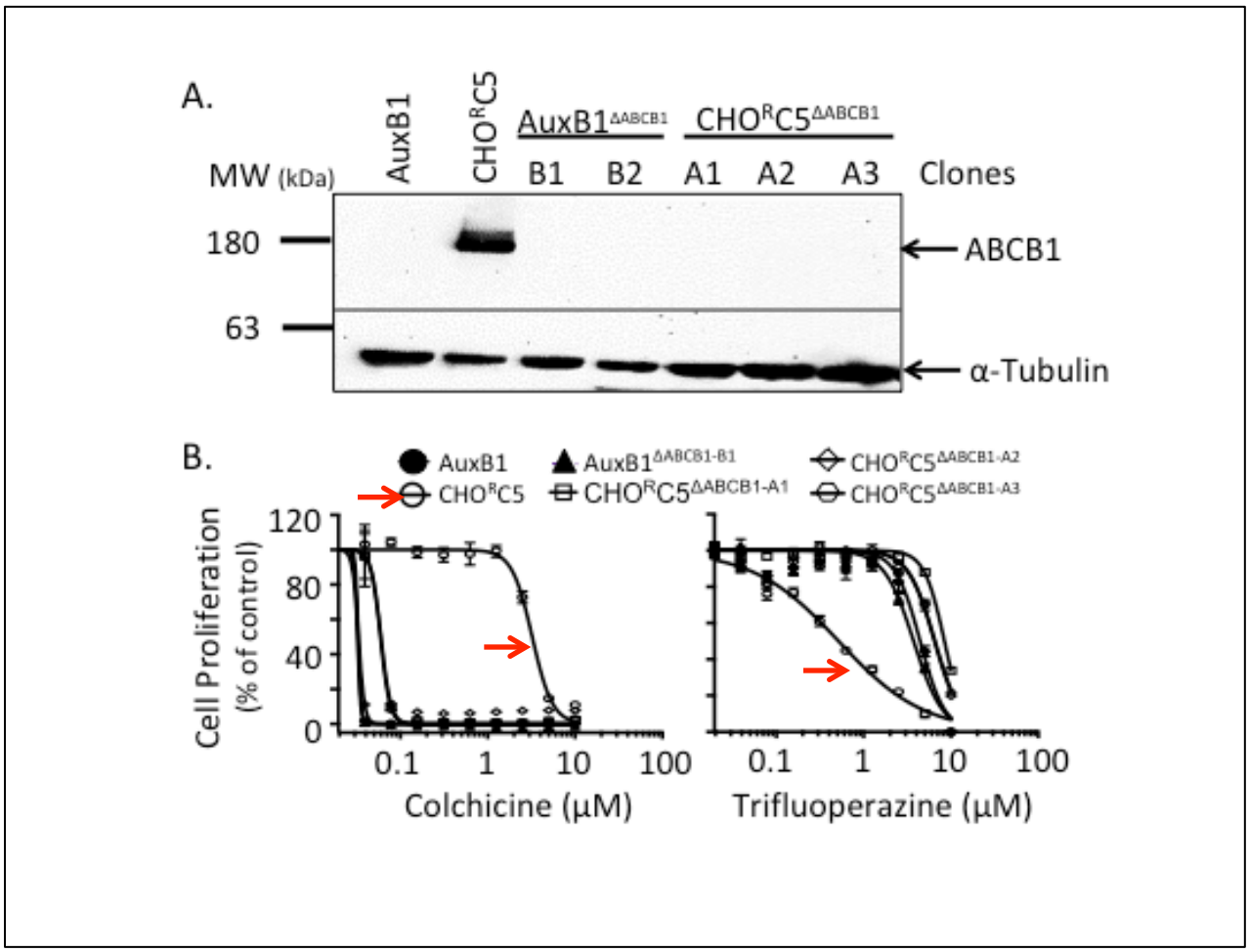

Figure 3. Knockout of ABCB1 expression abolishes collateral sensitivity to trifluoperazine - CRISPR/Cas9 with an ABCB1-specific guideRNA was used to knock out *ABCB1* in CHO cells. Panel A shows ABCB1 expression, by Western blotting analysis of total cell lysates from vector-transfected drug sensitive and resistant cells (e.g, AuxB1 and CHO^{R}C5, respectively), ABCB1-specific guide RNA drug sensitive (AuxB1$^{\Delta ABCB1}$ clones B1 and B2) and resistant (CHO^{R}C5$^{\Delta ABCB1}$ clones A1, A2, and A3) cells probed with anti-ABCB1-specific (C494) and anti-α-tubulin monoclonal antibodies. Panel B shows the proliferation of the above cell lines in the presence of increasing concentrations of colchicine and trifluoperazine (0-10 μM), respectively. Graphs represent the mean ± SD of three independent experiments done in triplicate.

Having established the successful knockout of ABCB1 from AuxB1 or CHO^{R}C5 cells, and to confirm the essential role of ABCB1 in drug resistance relative to the parental AuxB1 cells despite other cellular changes, figure 3B shows the effects of increasing concentrations of colchicine and TFP on proliferation of mock and ABCB1-knockout cells, respectively. The results of figure 3B show CHO^{R}C5 cells to be highly resistant to colchicine while all three ABCB1 CHO^{R}C5 knockouts (e.g. CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3) display increased sensitivity to colchicine compared to the parental CHO^{R}C5 cells. CHO^{R}C5$^{\Delta ABCB1}$ clones A1 and A3 show slight resistance to colchicine relative to parental AuxB1, AuxB1$^{\Delta ABCB1}$ and CHO^{R}C5$^{\Delta ABCB1}$ clone A2 **(fig. 3B)**. Although the reason for this discrepancy is not entirely clear, it is likely due to differences between the three CHO^{R}C5$^{\Delta ABCB1}$ clones A1-A3. By contrast, the results of figure 3B show that all three CHO^{R}C5$^{\Delta ABCB1}$ clones have lost their sensitivity to TFP and behave similarly to AuxB1 mock and AuxB1$^{\Delta ABCB1}$ clone B1. Taken together, these results reveal TFP as a collateral sensitivity drug capable of preferentially targeting ABCB1-expressing multidrug resistant cells, and this targeting capacity is dependent on ABCB1 expression and ATPase activity.

Trifluoperazine increases reactive oxygen species in CHO^{R}C5 cells - Earlier reports relating to the underlying mechanism of drug-induced collateral sensitivity in CHO^{R}C5 resistant cells demonstrated an ABCB1-dependent rise in reactive oxygen species (ROS) and oxidative cell death [16]. To investigate the mechanism of ABCB1-dependent collateral sensitivity to TFP, we measured the level of ROS in response to increasing molar concentrations of TFP for AuxB1 and CHO^{R}C5 cells. The results in figure 4A show significant increase in ROS levels in CHO^{R}C5 cells, relative to AuxB1, when cells were

exposed to 2 - 8 μM TFP. It is important to point out that the rise in ROS following the exposure of CHO^{R}C5 cells to TFP correlates with the effect of TFP on ABCB1-ATPase activity, whereby lower concentrations of TFP led to higher levels of ROS **(fig. 4A and fig. 1B)**. Moreover, the results in figure 4A show that the addition of PSC-833 (2 μM) reversed the increase of ROS levels in CHO^{R}C5 cells consistent with the effect of TFP on ABCB1 ATPase and in turn TFP-induced collateral sensitivity. In support of the TFP-induced ROS results in the CHO^{R}C5 versus AuxB1 cells, figure 4B shows the relative depletion in total levels of reduced thiols in cells exposed to 2 - 8 μM TFP for 24hr without or with the addition of 1 mM of the antioxidant N-acetyl-L-Cysteine. Together, these results demonstrate that cells expressing ABCB1 exposed to low concentrations of TFP show an increase in ROS and decrease in total reduced sulfhydro levels.

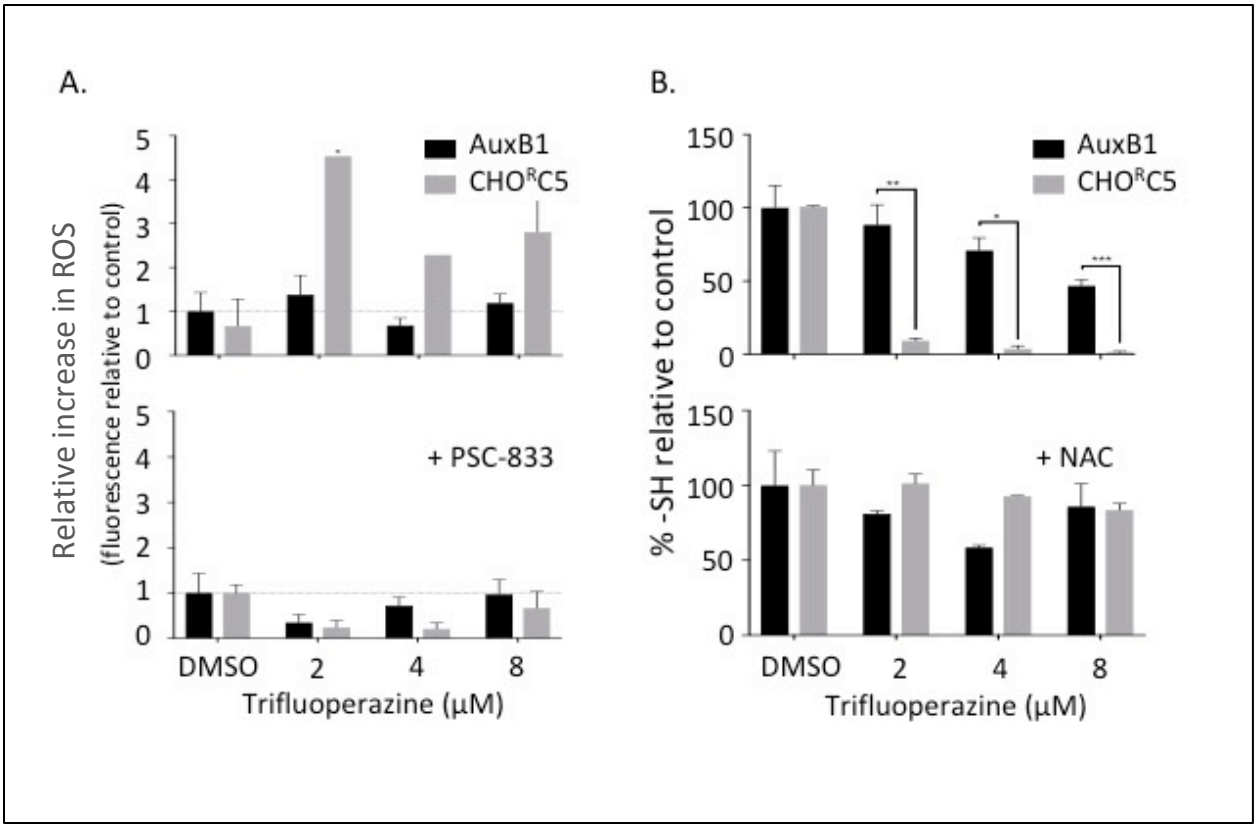

Figure 4. Trifluoperazine modulates the oxidative state of MDR cells – Panel A shows the effect of TFP (2, 4, and 8 μM) alone or in the presence of an ABCB1-ATPase inhibitor (PSC-833 at 2 μM) on the relative production of ROS in drug sensitive (AuxB1) and –resistant (CHO^RC5) cells, as measured using a ROS-dependent fluorescent probe, H_2DCFDA. Panel B shows the effect of the same TFP concentrations (2, 4, and 8 μM) alone or in the presence of N-acetyl cysteine (NAC at 1 mM) on the relative levels of reduced thiols (see methods). Graphs represent the mean **±** SD of three independent experiments done in triplicate. One asterisk indicates $P < 0.05$.

Trifluoperazine induces ROS-mediated apoptosis in CHO^RC5 cells - To determine if the collateral sensitivity effect of low concentrations of TFP on CHO^RC5 cells, relative to AuxB1, is due to inhibition of cell proliferation or increased cell death, cells were exposed to TFP

for 24 hrs while simultaneously monitoring both cell confluency and cell death by propidium iodide staining [32]. AuxB1 and CHO^{R}C5 cells were exposed to increasing concentrations of TFP (0.4 – 12.5 μM) and cell confluency was compared to the number of PI-positive events calculated proportionally to cell number **(fig. 5A)**. The results in figure 5A show a steady decrease in cell confluency and an increase in PI staining (or relative apoptosis) for CHO^{R}C5 at 1- 12.5 μM TFP. By contrast, TFP did not significantly affect the confluency or PI staining of AuxB1 cells at these same concentrations **(fig. 5A)**. Similarly, to determine the apoptotic effect of TFP, AuxB1 and CHO^{R}C5 cells were treated with 2 μM TFP for 36hr and 48hrs followed by analysis of poly (ADP-ribose) polymerase (PARP) cleavage in cell lysate by Western blotting. Figure 5B shows the characteristic 86 kDa cleavage fragment of PARP-1, indicative of apoptosis, relative to the native protein migrating as 110 kDa polypeptide following a 48 hr treatment of CHO^{R}C5 cells with TFP. A much fainter 86 kDa fragment was also seen in AuxB1 cells treated for 48hrs with TFP **(fig. 5B)**. As expected, treatment of AuxB1 and CHO^{R}C5 cells with the cytotoxic alkylating agent, Cis-platinum, for 36 hrs also caused a significant degradation of PARP-1 polypeptide in CHO^{R}C5 cells and to a lesser degree in AuxB1 cells **(fig. 5B)**. Taken together, the results in figure 5 are consistent with TFP preferentially targeting CHO^{R}C5 (or ABCB1-expressing) cells.

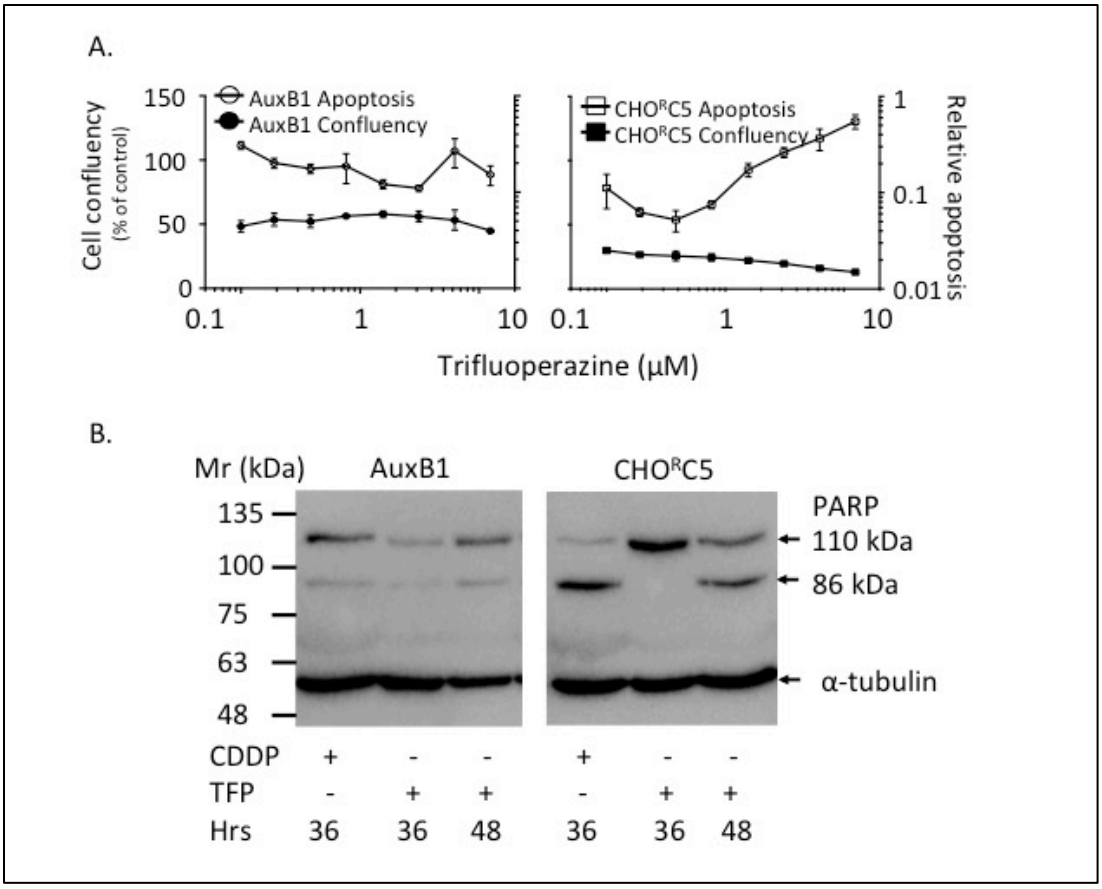

Figure 5. Trifluoperazine preferentially induces apoptosis of MDR cells – Panel 5A shows propidium-iodide staining of AuxB1 and CHO^{R}C5 cells (normalized to cell confluency) with increasing concentrations of TFP (see methods). Panel 5B shows the effects of TFP treatment on AuxB1 and CHO^{R}C5 cells PARP status. Cells were treated (Cis-platinum [or CDDP, 50 μM] for 36hrs or TFP [2 μM] for 36 - 48hrs) and their protein extracts analyzed by Western blotting using anti-PARP and anti-α-tubulin monoclonal antibodies, respectively. Intact PARP migrates at 116 kDa, while its cleavage product (a marker for apoptosis) migrates as an 86 kDa polypeptide. Significant levels of the 86 kDa PARP cleaved product was observed in CHO^{R}C5 cells following the treatment of cells with CDDP and TFP for 36hrs and 48hrs, respectively. The positions of PARP native and cleaved fragment are indicated with arrows on the right of Panel 5B, while the migration of the molecular weight markers are indicated to the left of the figure.

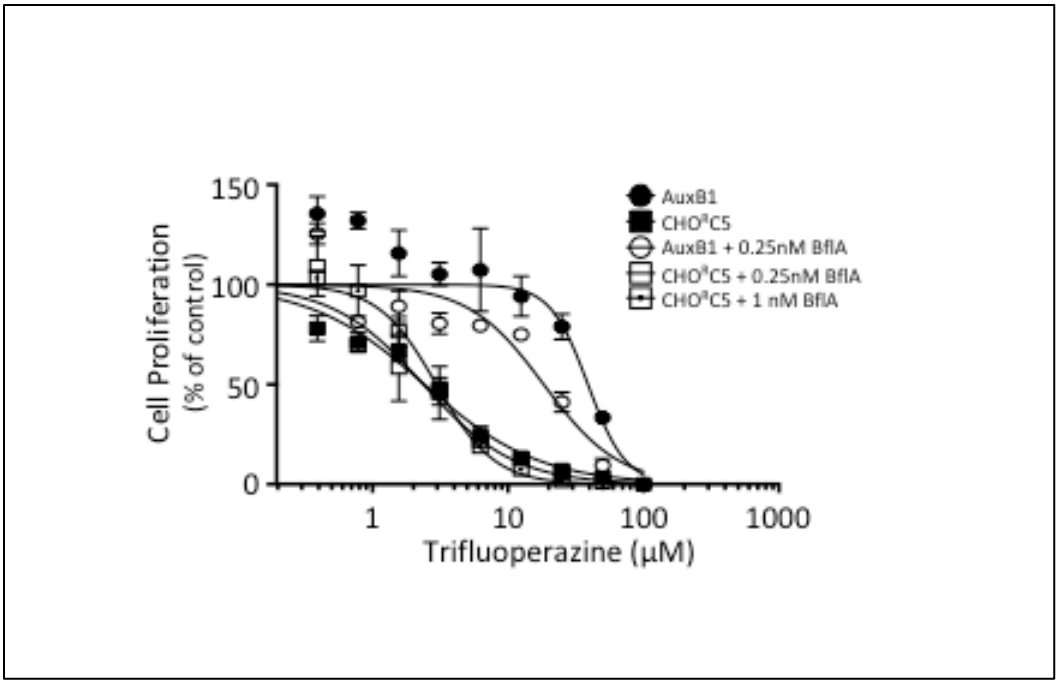

Figure 6. Effect of Bafilomycin A1 on TFP-induced collateral sensitivity of MDR cells – Drug-sensitive (AuxB1) and –resistant (CHO^RC5) cells were cultured in the presence of increasing concentrations of TFP, in the absence or presence of Bafilomycin A1 (BflA, 0.25 nM and 1 nM). Graphs represent the mean **±** SD of three independent experiments done in triplicate.

To determine whether TFP-selective targeting and cell death of CHO^RC5 cells was mediated by autophagy as previously suggested [20], AuxB1 and CHO^RC5 cells were allowed to proliferate in the presence of increasing concentrations of TFP, in the absence and presence of bafilomycin A, an inhibitor of autophagy [36]. The results in figure 6 shows similar growth curves for CHO^RC5 without and with bafilomycin A, suggesting that unlike thiosemicarbazone, TFP does not induce preferential autophagy in ABCB1-expressing cells [20]. We have demonstrated the potential use of TFP as a collateral sensitivity drug that

should be effective at or below clinically achievable plasma concentrations of up to 10 μM [33]. Moreover, given our earlier work with tamoxifen (Bakadlag *et. al.* 2020, Submitted), another clinically used drug that acts as a collateral sensitivity agent, it was of interest to determine if the two drugs can act synergistically as combination collateral sensitivity drugs. Figure 7A shows the effects of tamoxifen on the proliferation of drug sensitive (AuxB1) and resistant CHO^{R}C5 cells, alone and in the presence of 50 nM of TFP. The presence of 50 nM TFP caused a shift in the IC_{50} of CHO^{R}C5 cells for tamoxifen from 0.6 μM to 0.3 μM **(fig. 7A)**. In contrast, the addition of 50 nM TFP, together with tamoxifen, did not have a significant effect on the proliferation of AuxB1 cells **(fig. 7A)**. A graph representation for the combined effect of TFP with tamoxifen based on figure 7A shows a strong synergism between the two drugs, using the method previously described by Ferguson *et al*. [37] **(fig. 7B)**. The method is based on the comparison of TAM IC_{50} with and without the addition of TFP, whereby the change in IC_{50} value is described as a % decrease (synergy) or increase (antagonism) **(Supplemental Table 1)**. Moreover, the same drug combination was shown to be effective in inhibiting malignant peripheral nerve sheath tumor growth in pre-clinical tumor models [34].

Multidrug-resistant, ABCB1-expressing CHO^{R}C5 cells are collaterally sensitive to trifluoperazine compared to the drug sensitive parental cell line, AuxB1. The mechanism of collateral sensitivity is similar to that of verapamil and progesterone, and is mediated by an increase in the production of ROS and a decrease of cellular thiol levels in the MDR cells. This collateral sensitivity is dependant on the expression of ABCB1, since it is absent in cells where ABCB1 has been knocked out, underlining the importance of ABCB1 in the mechanism of collateral sensitivity of MDR cells. Additionally, TFP shows synergism with

another collateral sensitivitiy drug, tamoxifen. Consequently, the latter findings are encouraging since both drugs (i.e., tamoxifen and TFP) are safe at these concentrations and are currently in clinical use. Thus, collateral sensitivity of ABCB1-expressing MDR cells with drugs such as trifluoperazine has potential as a treatment against the development of MDR tumors.

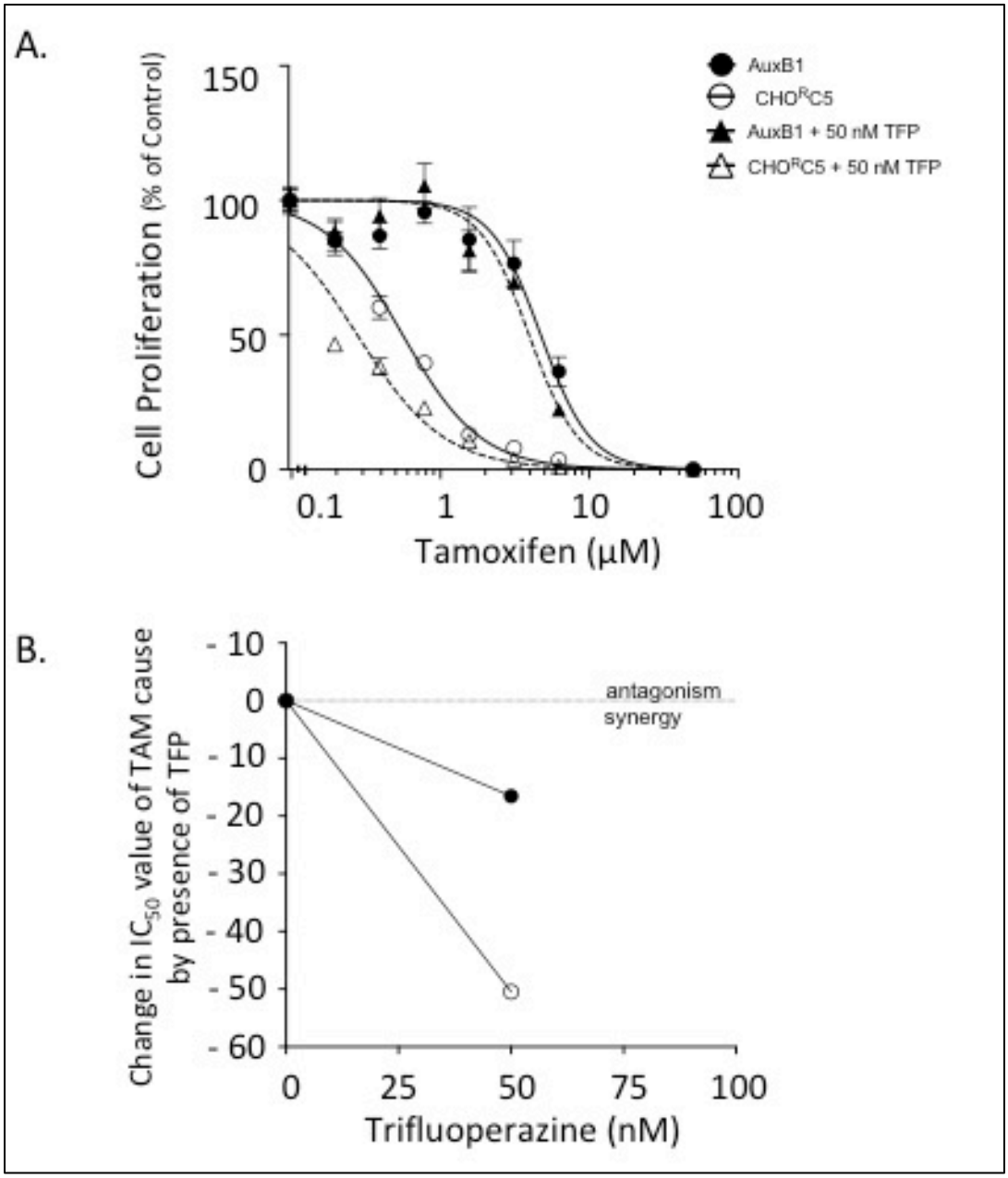

Figure 7. Trifluoperazine-induced collateral sensitivity synergizes with tamoxifen – Panel 7A shows the proliferation of AuxB1 and CHO^{R}C5 cultured with increasing concentrations of tamoxifen (0- 500 μM) in the absence and presence of 50 nM TFP (tfpz). Panel 7B shows synergy analysis of interactions between tamoxifen and TFP to induce collateral sensitivity in drug resistant cells (CHO^{R}C5). TFP (50 nM) shows strong synergy with approximately 0.28 μM tamoxifen, as shown by a reduction in IC_{50} value caused by the presence of TFP as a percentage of the IC_{50} value TAM in the absence of TFP. Graphs represent the mean **±** SD of three independent experiments done in triplicate.

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Conflicts of Interest – There are no conflicts of interest to report from either of the authors.

	IC_{50} TAM (µM)			
	AuxB1		**CHO^RC5**	
	0 nM TFP	**50 nM TFP**	**0 nM TFP**	**50 nM TFP**
	4.72	3.94	0.58	0.28
% change in IC_{50}	0	-16.52	0	-51.72

Supplemental Table 1. Synergy calculations (as described by Ferguson et al., 2018) [37] – This table summarizes the calculations used to create the synergy graph in Figure 7B. Cell growth assays were carried out as described, with tamoxifen alone or in combination with 50 nM trifluoperazine, a concentration that does not affect the growth of AuxB1 or CHO^RC5 cells. IC_{50} values changes were obtained by dividing the combination value (TAM + 50 nM TFP) by the value obtained by treating cells with TAM only.

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GENERAL CONCLUSIONS

Drug resistance to chemotherapy is an obstacle in the clinical treatment of many different diseases and disorders, including cancer [1]. Tumors often develop drug resistance by overexpressing ATP binding cassette transporters such as ABCC1, ABCG2 and ABCB1 [2]. The latter, also known as P-glycoprotein, is the most well studied and characterized of all, yet the native structure and function of this protein remain elusive. P-glycoprotein is a drug efflux transporter that removes compounds from cell membranes, in a mechanism that is dependent on the energy derived from ATP hydrolysis, allowing tumor cells to evade chemotherapy [3, 4]. Efforts to inhibit ABCB1 function as a method to overcome clinical multidrug resistance were unsuccessful, largely due to the fact that ABCB1 exhibits an undetermined protective native function in cells and tissues, and using ABCB1 inhibitors in addition to chemotherapy caused high levels of toxicity in patients [5-7]. However, certain compounds were shown to target ABCB1 expressing multidrug resistant (MDR) cells in a phenomenon called “collateral sensitivity” (CS) [8]. Collateral sensitivity is defined as the process the higher sensitivity of ABCB1-expressing MDR cells to some compounds, compared to the parental cell lines from which they are derived. This process has been shown to involve three different types of compounds: a) compounds that are transported and stimulate ATPase activity, such as verapamil [9]; b) compounds that interact with and stimulate the ATPase but are not transported, such as tamoxifen (Bakadlag and Georges, in preparation) or progesterone [10]; and c) compounds that do not interact with the ATPase but increase the reactive oxygen stress in MDR cells, such as NSC73306 [11].

While the collateral sensitivity drugs were shown to target ABCB1-expressing MDR cells, some of the cells do survive treatments. Additionally, few clinically approved compounds at relevant concentrations have been identified as collateral sensitivity agents. Moreover, until now, very few tools were available to study P-glycoprotein, for several reasons: it has over 300 different substrates, ABCB1 inhibitors can also inhibit other transporters and siRNA silencing is transient. For these reasons, the major objective of this project was to further study the mechanisms of collateral sensitivity and drug resistance in ABCB1-expressing MDR cells, by creating tools and identifying new compounds for this purpose.

The first objective of this project (Chapter 2) was to determine the effects of collateral sensitivity drugs (tamoxifen and verapamil) at IC_{50} and IC_{90} concentrations on the MDR clones, in order to better understand the feasibility of collateral sensitivity as a clinical treatment option. Most of the clones did not survive the treatments. However, surviving clones were further characterized and were found to express lower levels of ABCB1 compared to untreated clones, which related to increased sensitivity to conventional chemotherapy. Additionally, these clones had increased expression of α-enolase compared to untreated cells, suggesting that a metabolic change may be associated with ABCB1 expression. As the possibility of using CS as a treatment became more prominent, more information was needed on the role of ABCB1 in the collateral sensitivity of MDR cells in order to better understand the protein.

The second objective of this project (Chapter 3) was to use the CRISPR/Cas9 gene editing system to specifically knock out *ABCB1* and create stable clonal cell lines to study CS and MDR. Additionally, characterization of these clones shed some light on the role of

ABCB1 and other transporters. In this manuscript, we showed that ABCB1 expression was absolutely necessary for both multidrug resistance and collateral sensitivity. The emergence of multidrug resistance as a result of drug selection not only increases ABCB1 expression. Other cellular changes are often defined, which can affect collateral sensitivity. We show, for the first time, that despite the possibility of other cellular changes, ABCB1 is required for both the MDR and CS phenotypes to occur. The ABCB1 knockout clones we developed show a complete reversal of both multidrug resistance and collateral sensitivity. Increased ROS and decreased thiol levels, both involved in the mechanism of collateral sensitivity, in cells exposed to the CS agents verapamil and progesterone are completely absent in the knockouts. Furthermore, we studied the efflux capacities of the cell lines by incubating them with fluorescent dyes that are substrates for ABCB1 such as Rh123 and Hoechst 33342. In these experiments, we showed that the efflux capacities are absent in the ABCB1 knockouts, but also that these knockouts accumulated even more dye than ABCB1-negative cell lines, which requires further research.

The final objective of this project (Chapter 4) used the aforementioned tools to identify and characterize a novel CS agent, trifluoperazine, which shows collateral sensitivity at clinically relevant concentrations. Trifluoperazine (TFP) stimulates the ABCB1 ATPase, increases cellular ROS levels and leads to apoptosis. We showed that ABCB1 knockouts did not show collateral sensitivity towards TFP. Additionally, TFP synergizes with another collateral sensitivity drug, tamoxifen, at concentrations well within the clinically achievable levels. Thus, we open up the possibility of using CS drugs in the clinic, to prevent or treat ABCB1-mediated multidrug resistance without the total inhibition of ABCB1 function, which may affect pharmacokinetics of other treatments.

In conclusion from a clinical perspective, the importance of ABCB1-mediated multidrug resistance cannot be underestimated. The circumvention of this process by inhibiting the function of ABCB1 is problematic. Therefore, we propose to consider using the above-mentioned novel findings in this work to open up the possibility of collateral sensitivity cycling as a treatment option. Animal study models are needed to validate the findings *in vivo*.

From a fundamental scientific perspective, our $CHO^{R}C5$ ΔABCB1 clones may be used to finally reveal the native substrate and function of the elusive transporter. We also used these clones to solidify our working mechanism of collateral sensitivity, and opened up the possibility to study other resistance-associated proteins and transporters in the process. As scientists, we believe that the findings in this work contributed greatly to the knowledge of ABCB1, and provided useful and practical tools to continue these efforts to better understand ABCB1 and contribute to the fight against multidrug resistant cancer.

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