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IDENTIFICATION AND CHARACTERIZATION OF MODULATORS OF HUMAN
MRP1 (ABCC1) AND HUMAN MRP2 (ABCC2) EXPRESSION

BY

VIVIAN OSEI POKU

A dissertation submitted in partial fulfillment of the requirements for the Doctor of
Philosophy

Major in Biochemistry

South Dakota State University

2021

DISSERTATION ACCEPTANCE PAGE

Vivian Osei Poku

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this dissertation to my father; Mr. Emmanuel Osei Poku, my mother; Madam Agatha Owusu, my Siblings (Jennifer, Sandra, and Caren), and to Mr. Kwasi Yiadom Konadu for their unswerving support, and prayers. I have achieved this milestone because you always believed in me.

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ABBREVIATIONS

ABC	ATP-binding cassette
ADMET	absorption, distribution, metabolism excretion and toxicity
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCA	bicinchoninic acid assay
BCRP	breast cancer resistance protein
CFTR	cystic fibrosis transmembrane conductance regulator
cMOAT	canalicular multi-specific organic anion transporter 1
DJS	Dubin-Johnson syndrome
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FACE	Fast Activated cell-based ELISA
FDA	Food and Drug Administration
GSH	reduced glutathione
GSSG	oxidized glutathione
HEK293	human embryonic kidney 293
HCl	Hydrochloride
HIF	hypoxia-inducible factor

HIV	human immunodeficiency virus
HKP	housekeeping proteins
ICE	In-Cell ELISA
MDR	multidrug resistance
MRP1	multidrug resistance protein 1
MRP2	multidrug resistance protein 2
MSD	membrane spanning domain
MTX	Methotrexate
NBD	nucleotide binding domain
P-gp	permeability glycoprotein
PI3K	phosphoinositide 3-kinases
PKB	protein kinase B
PTM	post-transcriptional modification
SUR	sulfonylurea receptor
TBHQ	tert-Butylhydroquinone
TF	transcription factor
TMD	transmembrane domain

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF MODULATORS OF HUMAN MRP1 (ABCC1) AND HUMAN MRP2 (ABCC2) EXPRESSION

VIVIAN OSEI POKU

2021

ATP-binding cassette (ABC) transporters are known to play a critical role in conferring multidrug resistance (MDR) in various cancers. Several retrospective analyses of chemotherapy results have reported high expression of Multidrug Resistance Protein 1 (MRP1) and Multidrug Resistance Protein 2 (MRP2) in tumor cells exhibiting the MDR phenotype. High MRP1 and MRP2 expression in cancer patients predict a higher risk of treatment failure, resulting in relapse and disease recurrence as well as shortened survival rates. The key role of MRP1 and MRP2 play in the development of MDR makes them important therapeutic targets that hold a great promise for addressing multidrug resistance in cancer cells. Since MRP1 and MRP2 play critical roles in the regulation of various cellular pathways by altering the levels of several key signaling molecules, finding ways of modulating the activities and expression of these transporters in cancer cells is of great clinical interest in oncology research. We identified four novel modulators of MRP1 from our initial screening of 30 therapeutic compounds using an In-Cell ELISA assay. Three of these compounds; Amuvatinib, SB743921 HCl, TG101348 (SAR302503), which were identified to be ATP competitive inhibitors based on their mode of action, decreased MRP1 expression whereas Felbamate (a recently approved FDA drug) increased MRP1 protein expression. Our findings revealed that these ATP competitive inhibitors decreased MRP1-mediated calcein accumulation. These compounds inhibited the growth of HEK293 MRP1-

overexpressing cells at clinically achievable concentrations, and also reversed MRP1-mediated resistance in these cells. Since regulation of the activity of activators and effectors of specific biochemical pathways provide a means of regulating downstream signaling, we investigated the effect of a novel Tie2 kinase inhibitor and mTOR inhibitor, Everolimus, on MRP1 activity and expression. Tie2 is an activator of the PI3K/Akt pathway (a pathway known to modulate MRP1 activity and expression) whereas mTOR is a downstream effector of this pathway. We demonstrated using a flow cytometry-based calcein accumulation assay, and MTT based reversal resistance studies that Tie2 kinase inhibitor and Everolimus can decrease MRP1 mediated calcein efflux and reverse MRP1 mediated resistance towards vincristine in HEK293 MRP1-overexpressing cells. Lastly, we identified 49 modulators of MRP2 from our initial screening of 372 FDA-approved drugs from a recently approved FDA drug library representing 13.17% of total compounds screened. Thirty-nine (39) drugs increased MRP2 expression whereas 10 drugs lowered expression of MRP2 after drug treatment. Results from this screening reaffirm the promiscuous nature of the MRP2 transporter, and how important it is to investigate the interaction between both old and newly developed drugs with MRP2. The modulators identified from this study would be further characterized in future projects. Overall, our findings signify the importance of profiling drug interactions with these transporters, and the data obtained would provide essential information to improve combinatorial drug therapy and precision medicine as well as reduce drug toxicity of various cancer chemotherapies.

Chapter 1.0

1.0 Scope of the Study

The main objective and significance of this study is to identify and characterize drugs from various drug libraries as modulators of Multidrug Resistance Protein 1 (MRP1) and Multidrug Resistance Protein 2 (MRP2) protein expression in cancer cells. This section reviews important literature on ATP Binding Cassette (ABC) transporters with a focus on the ABCC subfamily, their structure, function, and mode of transport. It also details the role of ABC transporters in the development of Multidrug Resistance (MDR), the role of modulators in chemotherapy, and some current modulators of ABC transporters as well as some mechanisms through which protein expression of ABC transporters can be modulated. This chapter also captures relevant literature on common assays employed in screening for modulators of ABC transporters such as MRP1 and MRP2. In-cell ELISA assay, which was the main high-throughput screening tool used in this present study is carefully examined. Finally, this section concludes by detailing the rationale of this present study and the essence of identifying modulators of ABC transporters protein expression. It also describes how findings from this project can be utilized in curbing multidrug resistance and improving the effectiveness of combinatorial chemotherapy.

1.1 Introduction to ABC transporters

One of the largest superfamilies of transporters reported to be present in almost every kingdom of life is the ATP-binding cassette superfamily of transporters (ABC transporters). They are described as a diverse and ubiquitous superfamily of transporters encoded by the ABC genes [1]. ABC transporters function basically as primary-active transporters, as such they require ATP hydrolysis for their transport activity [2]. These transporters were previously known as the traffic ATPases [2]. ABC transporters are reported to facilitate the transport of a broad spectrum of molecules ranging from small molecules to highly charged and highly hydrophobic molecules such as peptides, lipids, vitamins [3, 4]. Based on the direction of transport relative to the cytoplasm, ABC transporters can be classified as importers or exporters [5]. This superfamily of transporters function as both influx and efflux transporters in prokaryotes, but function mainly as efflux transporters in eukaryotes. As influx transporters, they are responsible for the influx or transport of nutrients into the cells. Moreover, as efflux transporters, they are responsible for the efflux of toxins and drugs across biological membranes [6]. In microorganisms, ABC transporters have been associated with the development of antibiotic and antifungal resistance [3].

In humans, 49 ABC transporter proteins have been discovered. These transporters have been categorized into seven subfamilies based on their amino acid sequence and protein domain (Table 1.0) [7, 8]. ABC transporters have been reported to play key roles in the transport of drugs and their metabolites, toxins, steroids, heavy metals as well as aid in maintaining physiological homeostasis [9]. ABC transporters are expressed in the lungs, kidney, intestines, and at sacred pharmacological regions like the blood-placenta barrier,

blood-brain barrier, blood-testis barrier, and the blood-cerebrospinal fluid barrier [10]. Mutation in some ABC transporters have been associated with several human genetic diseases, and immune deficiencies. Examples include Cystic fibrosis (ABCC7/CFTR), Stargardt disease and age-related macular degeneration (ABCA4/ABCR), Tangier disease and familial HDL deficiency (ABCA1), Progressive familial intrahepatic cholestasis (ABCB11/SPGP), Dubin-Johnson syndrome (ABCC2/MRP2), Pseudoxanthoma Elasticum (ABCC6/MRP6), Persistent hypoglycemia of infancy (ABCC8/SUR1), Sideroblastic anemia (ABCB7), Adrenoleukodystrophy (ABCD1), Sitosterolemia (ABCG5/ABCG8), Immune deficiency (ABCB2 /Tap1, ABCB3/Tap2) [11].

Table 1.1: List of subfamilies and members of ABC superfamily

Superfamily	Subfamily	Members
ABC Superfamily	ABCA	A1
		A2
		A3
		A4
		A5
		A6
		A7
		A8
		A9
		A10
		A12
		A13
		ABCB
	B4	
	B5	
	B6	
	B7	
	B8	
	B9	
	B10	
	B11	
	TAP2	
	ABCC	TAP1
		C1
		C2
		C3
		C4
		C5
		C6
		C8
		CFTR
		C9
	C10	
C11		
C12		
C13		

Table 1.1 (Continued): List of subfamilies and members of ABC superfamily.

Superfamily	Subfamily	Members
ABC Superfamily	ABCD	D1
		D2
		D3
		D4
	ABCE	E1
	ABCF	F1
		F2
		F3
	ABCG	G1
		G4
		G2
		G5
G8		

1.2 Structural organization and function of ABC transporters

Structural analysis reveals that most ABC transporters consist of two sets of membrane-spanning domains (MSD1 and MSD2) also referred to as the transmembrane domains (TMDs) and two nucleotide-binding domains (NBD1 and NBD2) [12] (Figure 1.1). The NBDs consist of two conserved Walker motifs (A and B) regions and a dodecapeptide region or linker that lies between the Walker motifs known as the C region. The Walker A and Walker B motifs are reported to be pivotal for ATP binding and hydrolysis [13]. The Walker A motif specifically binds to β - γ phosphate of ATP to the Glycine loop of γ -phosphate linker, whereas the Walker B motif interacts with the magnesium ions. Region C or the LSGGQ motif serves as the ABC signature motif [14]. A typical MSD consists of six transmembrane α -helices. The MSDs are responsible for substrate recognition and translocation across biological membranes [9]. In the ABC superfamily, full transporters

refer to transporter proteins with at least two MSDs and two NBDs, whereas transporter proteins with one of each domain are referred to as half transporters. Recent studies have revealed that some ABC transporters like ABCC1/MRP1 and ABCC2/MRP2 possess an extra NH₂ – proximal membrane-spanning domain known as the MSD0 [15]. The MSD0 contains five transmembrane helices and is reported to help with the retention and recycling of the transporter to the plasma membrane [16] (Figure 1.1).

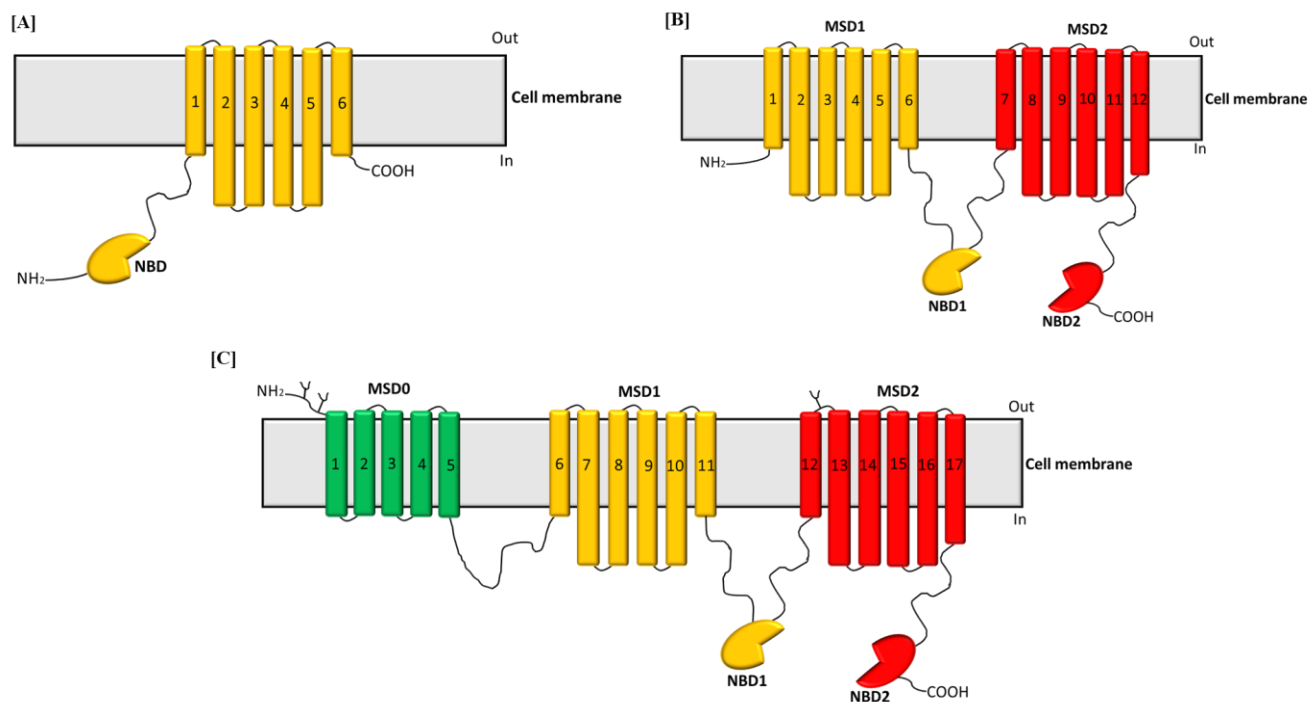


Figure 1.1 Structure of ABC transporters.

The membrane-spanning domains; MSD0 (green), MSD1(yellow), MSD2 (red), and nucleotide-binding domains (NBDs). [A] The predicted topology of half ABC transporters like BRCP/ABCG2. [B] The predicted topology of ABC transporters like ABCB1/P-gp, and short MRPs (MRP 4,5,6,8). [C] The predicted topology of ABC transporters having an extra MSD (MSD0) like long MRPs (MRP1, 2, 3, 6, and 7).

1.3 The ABCC subfamily

A total of thirteen transporters can be found in this subfamily. These transporters are referred to as full transporters and are grouped into the multidrug resistance protein subgroup (9 members), and the sulfonylurea receptor subgroup (SURs, 3 members). The cystic fibrosis transmembrane conductance regulator (CFTR) can also be found in this subfamily [17]. The summary of members of this subfamily is listed in table 1.2. Members of the MRP subgroup can further be categorized into long and short MRPs based on their predicted topology [18]. The long MRPs are described as transporter proteins that have an additional NH₂-proximal MSD0 to their set of membrane-spanning domains (MSD1 and MSD2), and two nucleotide-binding domains (NBD1 and NBD2) (Figure 1.1). Examples of MRPs with this predicted topology include ABCC1/MRP1, ABCC2/MRP2, ABCC3/MRP3, ABCC6/MRP6, and ABCC10/MRP7. The short MRPs on the other hand consist of two membrane-spanning domains and two nucleotide-binding domains. Members with this predicted topology include ABCC4/MRP4, ABCC5/MRP5, ABCC11/MRP8, and ABCC12/MRP9 [19]. ABCC6 is reported to be associated with the genetic disease, Pseudoxanthoma Elasticum (PXE) [20]. This disease is characterized by abnormal accumulation of calcium and other minerals in the elastic fibers of connective tissues. Mutations in CFTR that cause protein misfolding and abnormal processing provide the molecular basis of genetic disease, Cystic fibrosis [21]. CFTR plays a key role in chloride transport. Thus mutation in this gene affects the chloride ion channel function resulting in dysregulation of epithelial fluid transport in the lungs, pancreas and in other organs leading to Cystic fibrosis. Loss of ABCC2 activity is also reported to be associated with the Dubin-Johnson syndrome which is characterized by hyperbilirubinemia.

Moreover, members of the ABCC subfamily like ABCC1 (MRP1) and ABCC2 (MRP2) have been associated with the development of multidrug resistance in several carcinomas.

Table 1.2: Summary of members of the ABCC subfamily

ABCC Subgroup	Symbol	Alternative Name	Tissue Localization	References
MRPs	ABCC1	MRP1	Ubiquitous (lungs, kidney, placenta, blood-brain barrier)	[22, 23]
	ABCC2	MRP2	Canicular membrane of hepatocytes. Apical membrane of proximal renal tubule endothelial cells	[24, 25]
	ABCC3	MRP3	Liver, colon, intestine, adrenal gland	[24]
	ABCC4	MRP4	Prostate, testis, ovary, intestine, pancreas, lung	[11, 26]
	ABCC5	MRP5	Skeletal muscle, brain, heart	[27]
	ABCC6	MRP6	Liver, kidney	[28]
	ABCC10	MRP7	Liver, peripheral blood cells, intestines	[29]
	ABCC11	MRP8	Breast, lung, colon, prostate, ovary	[30]
	ABCC12	MRP9	Testicular germ cells, sperms	[31]

Table 1.2 (Continued): Summary of members of the ABCC subfamily

ABCC Subgroup	Symbol	Alternative Name	Tissue Localization	References
	ABCC8	SUR1	Neuronal cells, pancreatic B-cells	[32]
SURs	ABCC9	SUR2	SUR 2A - cardiac and skeletal muscle SUR 2B - vascular smooth muscle	[33, 34]
	CFTR	ABCC7	Apical membrane of epithelial cells in exocrine glands	[35]
	ABCC13	MRP10	Liver, fetal spleen, colon, placenta, brain, ovary, liver	[36]

Multidrug Resistance Protein 1 (MRP1) is a 1531 amino acid integral-membrane protein with a molecular weight of 190-kDa, it is encoded by the gene ABCC1 [37]. MRP1 is expressed at normal levels in the lungs, kidney, placenta, heart [22, 23], with lower expression levels observed in the colon, brain, small intestine, and peripheral blood mononuclear cells. High expression levels of the transporter are observed in cells at various pharmacological sanctuary sites like the blood-brain barrier, blood-testis barrier, and in the basolateral membrane of polarized cells [38] as well as in cells with high proliferative status such as the reactive type II pneumocytes in the alveoli of the lungs [39]. MRP1 as an ATP-dependent efflux transporter plays a major role in transporting broad spectrum substrates. These substrates include organic anions, metalloids (sodium arsenite, potassium antimonite), toxicants (aflatoxin B1, methoxychlor) folic acids, bilirubin, vitamins,

glutathione and glucuronide-conjugates of steroids, leukotrienes, and prostaglandins B12 [18, 40, 41]. Endobiotics transported by MRP1 include doxorubicin, vincristine, paclitaxel, ritonavir, irinotecan, methotrexate, saquinavir [41]. Due to the ability of MRP1 to transport drugs from different multiple families irrespective of their molecular target, structure and mode of action, MRP1 has been reported to regulate the absorption and disposition of drugs as well as their metabolites across cells [42]. MRP1 is also reported to be a major player in the regulation of several physiological processes like redox homeostasis, steroid metabolism, tissue defense and in the etiology of neurodegenerative and cardiovascular diseases [43].

MRP2 (ABCC2) on the other hand is also known as canalicular multi-specific organic anion transporter 1 (cMOAT) [44]. It functions as an ATP-dependent unidirectional efflux pump and is highly expressed in the liver where it governs the elimination of bilirubin glucuronides and drug conjugates into the bile. MRP2 is also involved in renal elimination in the kidneys, and distribution of its substrates in the placenta and the gastrointestinal tract [45]. It is involved in the transport of numerous clinically important compounds across multiple drug classes such as antibiotics, HIV drugs, antihypertensives, and anticancer agents as well as conjugates of lipophilic substances with glutathione, glucuronate, and sulfate [46]. MRP2 plays a critical role in conferring resistance to various chemotherapeutics as such it has been implicated in multidrug resistance (MDR) of several cancers like ovarian, colorectal, lung carcinomas. Moreover, the absence of functional MRP2 protein leads to Dubin-Johnson syndrome (DJS) associated with conjugated hyperbilirubinemia [47].

As members of the long MRPs subgroup, MRP1 and MRP2 possess two nucleotide-binding domains (NBDs) and two membrane-spanning domains (MSDs), and an additional third N-terminal membrane-spanning domain (MSD0) which comprises of 5 transmembrane spanning helices [26, 48] (Figure 1.1[C]). Studies have shown that MSD0 facilitates interactions between the transporter and other protein partners [49]. Structural analysis has shown that when these transporters are not bound to any substrate or ATP, they assume an inward-facing conformation, while the NBDs are widely separated and the translocation pathway remains continuous with the cytoplasm [50]. On the other hand, the MSDs get closer to form a high-affinity substrate binding pocket to which the substrate binds. The NBDs move closer to each other and align themselves for dimerization. ATP binds to the NBDs, leading to dimerization of the NBDs which causes a conformational change that results in rearrangement of the MSDs to the outward-facing conformation of the transporter (rotates and opens towards the extracellular space). Sequentially, the residues forming the substrate-binding site tend to be pulled apart as the extracellular ends of the helices of the MSDs peel outward leading to a significant reduction in the binding affinity of the substrate to the transporter. As a result, the substrate is released into the extracellular space [50]. ATP hydrolysis begins which causes the dissolution of the closed NBD dimer conformation. The MSDs move into the open conformation as ADP (adenosine diphosphate) and phosphate is released [2]. A pictorial diagram of the transport mechanism of ABC transporters like MRP1 based on the ATP-switch model is shown in Figure 1.2.

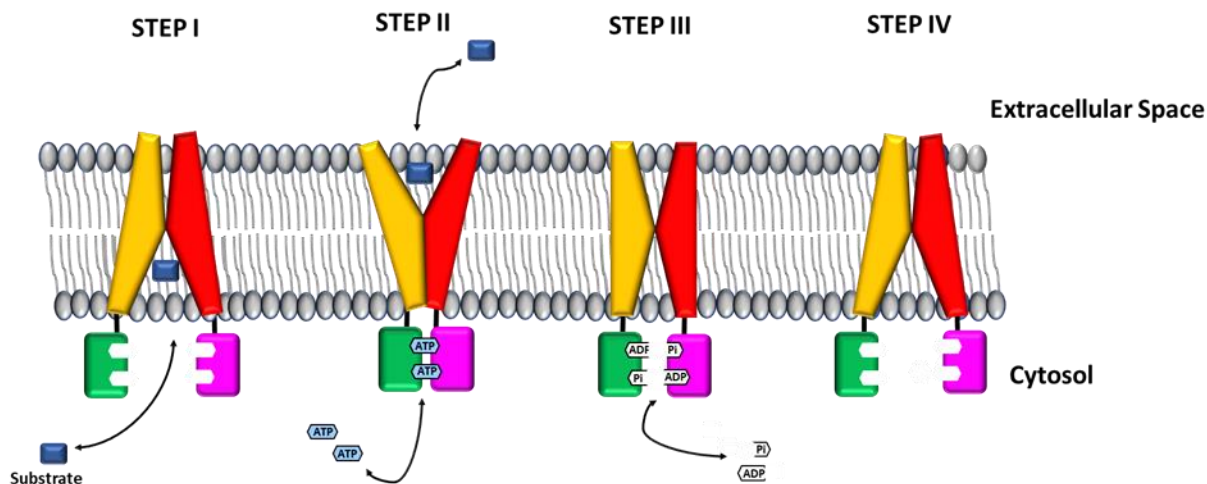


Figure 1.2 Mechanism of ATP mediated transport of ABC exporters based on the ATP switch model.

The substrate (blue rectangle) binds to the high-affinity binding pocket formed by the MSDs (yellow and red). This leads to a conformational change in the NBDs (green and pink), ATP (turquoise hexagon) binds and subsequent NBDs dimer closure occurs. A conformational change in the MSDs transpires upon the NBDs dimer closure, resulting in the rotation and opening of the TMDs to the extracellular space, and subsequent substrate translocation (Step II). The closed NBD dimer conformation is annulled as ATP hydrolysis commences, leading to conformational changes in the MSDs (Step III). The MSDs move into the open NBDs dimer conformation as ADP (adenosine diphosphate) and phosphate is released (Step IV).

1.4 Multidrug resistance and Cancer

Cancer is described as the abnormal and uncontrolled growth of cells. The United States recorded approximately 599,274 deaths due to cancer in 2018 [51]. Moreover, 18.1 million new cancer cases were recorded globally in the same year [52]. Although several cancer treatment options exist, one of the most effective treatment modalities for metabolic tumors is chemotherapy. Chemotherapy is a type of systemic treatment that involves the use of

drug formulations to target, control, and kill tumor cells [53]. Although oncology drug development has seen a paradigm shift from the low-budget, government-supported research effort to a high-stakes, multi-billion dollar industry [53], the challenge and limitations of chemotherapy experienced by early research still exist. One of the major challenges posed to the effectiveness and success of the chemotherapeutic regime is Multidrug Resistance (MDR). Multidrug resistance is described as a phenomenon in which tumor cells develop resistance to several drugs that may vary in both structure and mode of action [54]. Research has revealed that there are several mechanisms involved in the development of MDR. These include; cellular changes in cells that reduce the ability of the cytotoxic drug to kill cells such as changes in the cell cycle, elevated repair of DNA damage, decreased apoptosis occurrence, and altered drug metabolism, decreased uptake of water-soluble drugs including cisplatin and folate antagonists that need the service of transporters for cell entry, and increased energy-dependent efflux of hydrophobic drugs that can diffuse through the plasma membrane into the cell [55]. Studies have shown that the efflux of hydrophobic cytotoxic drugs by energy-dependent transporters like the ABC transporters is most common among the mentioned mechanisms. ABC transporters are major players in the absorption, excretion, metabolism, and elimination of drugs and their metabolites. For instance, ABC transporters like P-gp, MRP1, MRP2, and BCRP among others play pivotal roles in phase O and phase III of drug metabolism [5]. In phase O, these transporters are known to regulate the entry and extrusion of drugs before they reach their pharmacological target [5]. Moreover, ABC transporters are responsible for the complete elimination of metabolized molecules in phase III [5]. Transporters like P-gp have been associated with the transport of cationic drugs and their metabolites, whereas MRP2 and

BCRP are implicated in the transport of conjugate anionic drugs like conjugated glutathione across the plasma membrane [56]. The pharmacological aim of administering chemotherapeutic agents is to ensure the delivery of active compounds as much as possible to the molecular target in cancer cells to institute sufficient cellular damage to cause cell death. However, reduction in the intracellular drug accumulation has been reported to be one of the key factors that decrease the amount of active drug component that reaches these tumor cells [57]. Due to the essential role played by ABC transporters in drug metabolism, cancer cells in their intelligence overexpress these transporters as a means of protection against chemotherapeutic drugs and to ensure their survival. This is accomplished as the overexpressed transporters cause substantial reductions in the intracellular concentration of the anticancer drugs resulting in reduced bioavailability and decreased pharmacological toxicity and potency in cancer patients. As such, MDR remains one of the major barriers to the effectiveness of chemotherapy and is reported to be responsible for a larger percentage (about 90%) of cancer related deaths. Thus there is the need for more critical and careful research to be conducted into the role of ABC transporters in MDR.

Although recent advancement in cancer research has explored the role of transporters like P-gp and BCRP in MDR to a greater extent, the role of transporters like MRP1 and MRP2 are severely understudied. Recently, several retrospective analyses of chemotherapy results have reported high expression profiles of MRP1 and MRP2 [58, 59]. In addition, overexpression of MRP1 and MRP2 has been associated with higher incidence of treatment failure, resulting in cancer relapse and poor survival rates in some cancer patients [60, 61]. MRP1 is reported to confer resistance to anticancer drugs like methotrexate (MTX), anthracyclines (doxorubicin), etoposide, vincristine, paclitaxel, vinblastine among others

[41]. MRP2 also confers resistance to anticancer drugs like cisplatin, irinotecan, methotrexate, teniposide, mitoxantrone [46]. Aside from anticancer drugs, MRP1 and MRP2 also affect the bioavailability and efficacy of various antivirals, antimalarials, and antibiotics [19, 62]. The US food and drug administration recommendation in 2017 greatly encouraged the need to profile drug-transporter interactions for drugs in clinical trials with MDR transporter proteins like BRCP and P-gp [63]. But this recommendation excluded MRP1 and MRP2 although several studies have shown the effect of overexpression of MRP1 and MRP2 in MDR. Nonetheless, there is no specific recommendation for these transporters (MRP1 and MRP2) in the current FDA or EMA guidelines. Considering the essential role of MRP1 and MRP2 as well as their contribution to MDR, it is of great importance to explore the pharmacological essence and impact of these transporters by investigating their biochemical interactions with both new and promising drug targets.

1.5 Role of modulators in chemotherapy

The key role of ABC transporters in MDR cannot be overemphasized, as such finding ways of curbing the menace of ABC transporters in chemotherapy is of great importance to oncology research. Since ABC transporters are also essential for regulating cellular function and cellular balance, great consideration must be taken in considering the possible solutions in managing their role in MDR development. One approach that has been proposed by researchers is to completely shut down the efflux activity of these pumps. Although the aforementioned approach seems laudable since overexpression of the ABC transporter greatly hinders the bioavailability of the therapeutic drugs, this approach would be suicidal to the cells. This is because ABC transporters also play vital roles in tissue

defense and maintaining the physiological balance of cells. Thus, a complete shutdown of these transporters would mean a collapse in physiological stability and equilibrium. An alternate approach that provides a superior advantage is the modulation of the activity of ABC transporters in cancer patients through biochemical modulation. Biochemical modulation involves the modification of pathways and molecular targets by therapeutic agents to enhance the selective cytotoxic effect of anticancer drugs on tumor cells as well as to decrease their toxic side effects on normal cells [64, 65]. In clinical oncology research, biochemical modulation can also be described as a phenomenon in which the cytotoxicity of an active chemotherapeutic agent is modulated by one or several agents that may not have inherent cytotoxicity against a given normal or tumor cell population [66]. Modulation via this approach can result in the reduction of the cytotoxicity impact of the anticancer drug on normal tissues, and an elevation in the cytotoxicity effects of the anticancer agent on tumor cells. Furthermore, biochemical modulation can also be employed by using exogenously supplied metabolites to ensure selective manipulation of tumor cell metabolism to ensure the more selective response to the action of anticancer agents [67]. Several forms of biochemical modulation have been explored in oncology research and cancer therapy. This includes modulation to overcome drug resistance in drug-resistant cells, modulation of the transport of anticancer agents, modulation of intracellular thiol levels to affect the extent of damage caused by radiation or chemical DNA damaging agents, increasing the sensitivity and exposure of tumor cells to chemotherapeutic agents by modulating the cytokine profile of tumor cells and normal cells [66]. With regards to overcoming MDR in refractory cancer cells, ligands that interact with ABC transporters can be grouped into substrates, inhibitors, activators, and inducers. Substrates are small

molecules or drugs that are effluxed by the transporters. Inhibitors are described as molecules that decrease or impair the activity of the transporter either by binding directly or indirectly to it. Inducers are molecules that enhance the expression levels of the transporter either by altering protein or gene expression levels. Activators are described as molecules that are reported to elevate the activity of the transporter. Activators exert their effect by binding to the transporter protein and cause a conformational change which provokes the transport of a substrate [68]. Via biochemical modulation, the activity of a specific transporter can be modulated without impacting the physiological steadiness of normal cells. Moreover, the biochemical modulation approach makes it possible to combine two or more pharmacological agents that may work by different molecular mechanisms at their respective effective doses without unacceptable side effects. As such, an anticancer drug that inhibit the activity of a specific transporter can be used together with a chemotherapeutic agent that is known to be a substrate of the transporter, yet has superior therapeutic potency against tumor cells. In this instance, the inhibitor can be used to decrease the efflux activity of the transporters, allowing the more potent anticancer drug to accumulate at the appropriate intracellular concentration and be bioavailable to elicit its effect. Thereby enhancing the effectiveness of combinatorial drug therapy. Combinatorial therapy is described as the use of two or more pharmacologic agents administered separately or in a fixed- dose as a single formulation.

1.6 Current modulators of MRP1 and MRP2

MRP1 and MRP2 were discovered several years after the initial characterization of P-gp, yet there is little scientific information on modulators of MRP1 and MRP2 compared to

the diverse range of modulators for P-gp that have been well explored and characterized.

Some current modulators of MRP1 and MRP2 are shown in table 1.3

Table 1.3: Summary of the current modulators of MRP1/ABCC1 and MRP2/ABCC2

Protein/ Gene name	Substrates	Inhibitors	Inducers	Activators	Reference
MRP1 (ABCC)	Vinca alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), taxanes (paclitaxel), epipodophyllotoxins (etoposide, teniposide), camptothecins (topotecan, methotrexate, irinotecan) glucuronosylbilirubin, estradiol-17- β -D-glucuronide, etoposide-glucuronide, SN-38-glucuronide, leukotrienes C4, D4 and E4, glutathione disulfide, prostaglandin A2-SG, hydroxynonenal-SG, aflatoxin B1-epoxide-SG, cyclophosphamide-SG, doxorubicin-SG, estrone-3-sulfate, dehydroepiandrosterone-3-sulfate, sulfatolithocholyl taurine), difloxacin, grepafloxacin, folic acid, L-leucovorin, mitoxantrone, curcuminoids, sodium arsenate, sodium arsenite, potassium antimonite, fenitrothion, methoxychlor, aflatoxin B1, calcein, fluorescein, Fluo-3, BCECF, indinavir, adefovir	Sulfinpyrazone, biricodar, probenecid, MK571, LTC4, cyclosporin A, verapamil, PSC 833, benzbromarone, indomethacin, probenecid, agosterol A and analogs, verapamil derivatives, flavonoids derivatives (genistein and flavopiridol), raloxifene-based inhibitors (LY117018, LY329146, and indomethacin), piperazine and piperidine-based compounds as dual MRP1/P-gp inhibitors (N, N-disubstituted piperazines), isoxazole-based compounds (LY402913), quinazoline- and quinaxoline-derived molecules	Dexamethasone, Rifampicin, Sulindac, Tert-Butylhydroquinone (TBHQ), quercetin, Vinblastine	Verapamil and derivatives	[69], [70] [71],[72] [73], [74] [75], [76] [77], [78] [79], [80] [81]

Table adapted from [75] and [82]

Table 1.3 (Continued): Summary of the current modulators of MRP1/ABCC1 and MRP2/ABCC2

Protein/Gene name	Substrates	Inhibitors	Inducers	Activators	Reference
MRP2 (ABCC2)	Bilirubin-glucuronides, GSSH, GSH, including cotransport, estradiol-17- β -d-glucuronide, acidic bile salts. Anionic drug conjugates, cisplatin, doxorubicin, epirubicin, etoposide, indinavir, phenytoin, ritonavir, saquinavir, sulfipyrazone, vinblastine	Probenecid, Cyclosporine, PSC833, MK571, delavirdine, efavirenz, emtricitabine, benzbromarone, probenecid,			[82], [83]

Table adapted from [75] and [82]

Several decades of research have focused on overcoming MDR via pharmacological inhibition of ABC transporters like MRP1 and MRP2. However, there has been limited success due to high non-specific toxicity, low multidrug reversal effects, low potency, and undesirable off-target effects. Hence, contemporary clinical research strategies aim at identifying new selective modulators of ABC transporters that are more potent, well-tolerated, and have limited non-specific toxicity. Moreover, taking a critical look at the modulators identified for P-gp, MRP1, and MRP2 in literature, it can be ascertained that the current knowledge base on MRP1 and MRP2 is still narrow. Thus, it is expedient for more research to be conducted to identify more potent modulators of MRP1 and MRP2 as

these transporters also play an essential role in MDR development. Interestingly, most research studies that aimed at identifying such modulators investigated the impact of these therapeutic agents on MRP1 and MRP2 activity but hardly examined their effect on protein or gene expression levels of MRP1 and MRP2. Thus it is needful to explore how such therapeutic agents may affect protein or gene expression levels since this may also reveal other molecular targets that may be of great therapeutic importance in our fight against MDR and cancer.

1.7 Mechanisms for protein expression regulation

Proteins represent one of the abundant macromolecules in living systems. This group of macromolecules has the most diverse range of functions across the various classes of macromolecules [84]. Proteins synthesis involves the conversion of information on the DNA (deoxyribonucleic acid) into messenger RNA (ribonucleic acid, mRNA) through a process called transcription. After which the information on the mRNA is then converted into a protein sequence via translation (Figure 1.3). The protein sequence then determines the protein folding, its conformation, biochemical role, stability, and half-life [84]. The protein expression levels for a specific protein of interest in the eukaryotic cell can be regulated at the gene expression level through transcriptional control or by regulating the translation process.

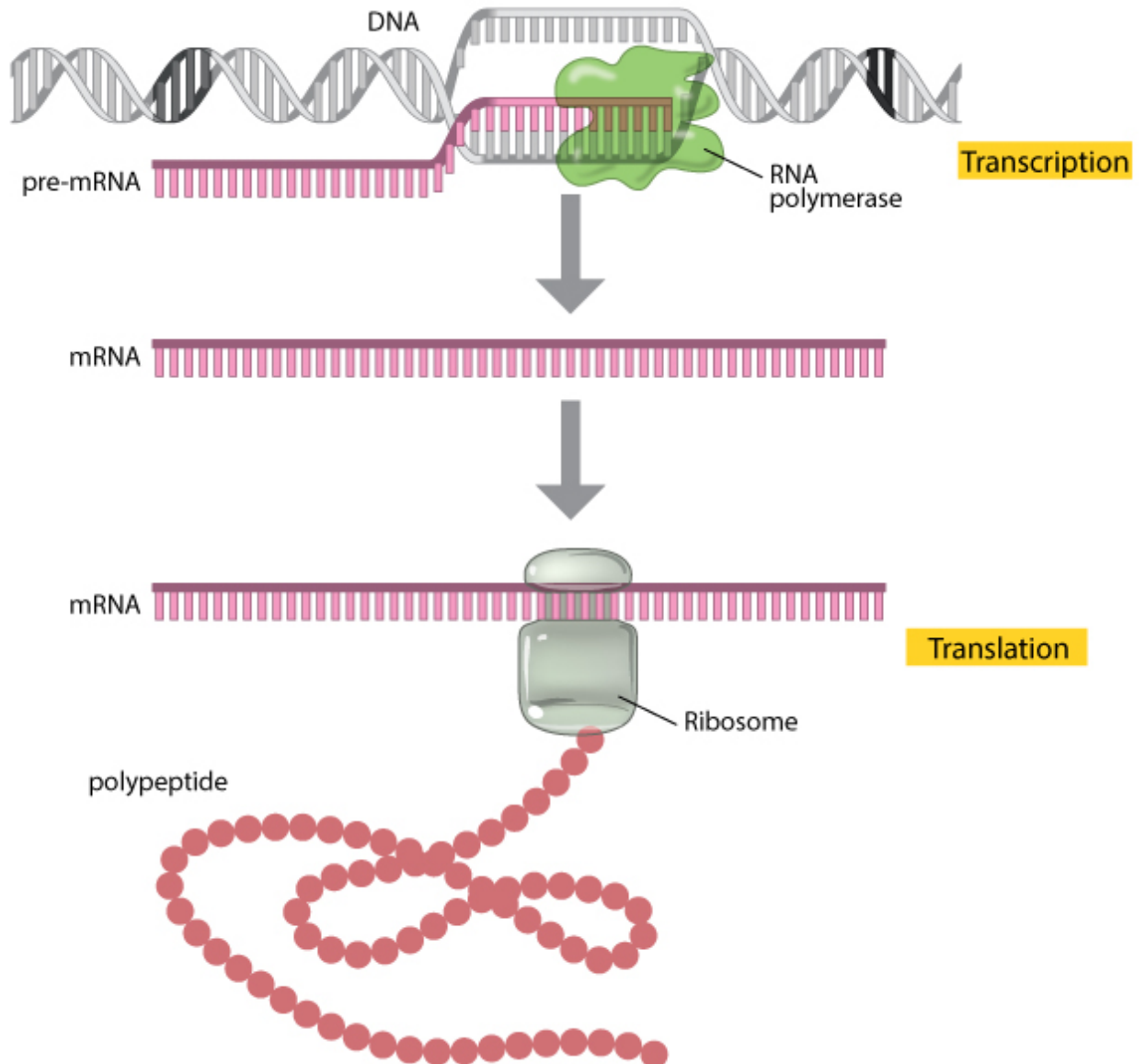


Figure 1.3 Protein synthesis illustrating transcription and translation steps

[85]

1.8 Transcriptional regulation

Transcriptional control in the eukaryotic cell can be achieved by transcription factors (TFs) activation, TFs binding with specific DNA recognition sequences, and chromatin

remodeling [84]. TFs bind to their target site at once to form the transcription complex, thus they can regulate several genes disseminated in the genome [86]. TFs can be activated through small molecules that physically bind or allosterically alter the protein structure [84]. These small molecules act as modulators of protein expression and function by depending on specific transcription factors to exert their effect on their target [86]. TFs can also be activated through cellular signaling pathways that create post-transcriptional protein modifications (PTMs). For instance, translation of the transcription factor, hypoxia-inducible factor (HIF-1 α and HIF-2 α), can be elevated by signaling through the phosphatidylinositol 3 kinase (PI3K)/Protein kinase B (PKB/Akt) or the PI3K-Akt pathway [87]. The PI3-Akt signaling pathway also regulates a lot of downstream transcription factors like NF-kappa B (NF-kB) and activator protein 1 (AP-1) that play key roles in cell proliferation, cell survival among others in carcinogenesis [88]. TFs activation is greatly pleiotropic and has many cellular effects, as such several downstream target genes can be inactivated or activated based on the cell type and environmental conditions. TFs possess the capacity to rapidly and selectively find their target site. Thus they can bind to the target DNA site to either institute recruitment of the transcription machinery onto the promoter region of the gene of interest or hinder the recruitment of the transcriptional machinery [84]. This can either upregulate or suppress gene expression which in turn affects translation and protein synthesis.

One way in which transcription can be regulated is via rearrangement of the chromatin structure. Chromatin consists of nucleosomes. Nucleosomes are described as DNA wrapped around a histone octamer. Modification to chromatin structure during transcription can be achieved by histone modifications, eviction or repositioning of

histones by histone chaperones, chromatin remodeling, and histone variant exchange [84]. Thus, Post-transcriptional modifications (PTMs) in the form of covalent modifications can be made on the histone tails by histone modifiers. The modifications alter the interaction and contact between histones and the DNA. Major modifications include acetylation and methylation of lysine residues. ATP-dependent chromatin remodelers can also use the energy from ATP hydrolysis to facilitate chromatin remodeling, which can be achieved through nucleosome sliding, nucleosome displacement, or incorporation and exchange of histone variants [89].

1.9 Translational regulation

Translation describes the process of converting the information on the messenger RNA (mRNA) into a protein sequence. The integrity of protein synthesis must be greatly upheld to ensure minimal error during the process to warrant the synthesis of a functional protein. Ribosomes are the machinery that ensures the integrity of protein synthesis is upheld with great care. It achieves this by matching the code from the template mRNA strand to the right amino acid. Due to the critical role the ribosomes play, the ribosome filter hypothesis by Mauro and Edelman in 2002 proposes that ribosomes function as translation determination factors [90]. As such, based on the specific ribosomal proteins and rRNA sequence in the ribosome complex, they filter and select for specific mRNA that should be translated. Thus, regulating the translation of these genes into proteins. Considering how the transcription and translation programs regulate protein expression, it can be seen that small-molecules if well-investigated and employed can offer the opportunity to modulate

protein expression levels through either of the aforementioned mechanisms. Hence this project seeks to provide a methodological alternative to the identification and characterization of modulators of MRP1 and MRP2 protein expression levels by screening various therapeutic agents from different drug libraries.

1.10 In vitro assays to screen for modulators of ABC transporters

Identification of interactions between new and promising drugs with ABC transporters is very essential for drug development. Studying drug-transporter interactions would provide essential information on how these transporters can impact drug disposition, efficacy, and toxicity. It would also create a pivotal platform to assist in the selection and optimization of new drug candidates. In vitro studies of ABC transporters and their interaction can be carried out via membrane-based assays or cell-based assays. Membrane-based assays involve the use of membranes made from cells expressing ABC transporters to study the efflux function of these transporters [75]. Membrane-based assays include membrane vesicular transport assays, ATPase assays, and photoaffinity labeling assays [69]. Cell-based assays, on the other hand, are performed using intact cells, such assays include; protein determination assays (In-cell ELISA, western blot), flow cytometry assays, cytotoxicity assays among others.

1.10.1 In-Cell ELISA assay (ICE)

One of the valuable assays for rapidly characterizing a wide range of cell signaling parameters in the development of targeted therapeutics is the In-Cell ELISA assay (ICE). ICE is also known as In-cell western assay, Cell-based ELISA, Cytoblot, or FACE (Fast

Activated cell-based ELISA). It is cell-based immunocytochemistry that allows the quantification of target proteins or post-translational modifications of target proteins in cultured cells (adherent and non-adherent cells). This assay is based on the principle that using target-specific antibodies, proteins can be detected in fixed and permeabilized cultured cells. Moreover, the ICE assay bypasses the protein harvesting, lysate preparation, electrophoretic separation, and electrophoretic transfer steps of western blot. Thus, it provides a simple and rapid platform for immunodetection of target proteins using antibodies. Since this assay requires no protein separation, the primary antibodies used must be highly specific for the protein of interest. This technique also demands the segregation of signals due to the protein of interest from the normalization signal due to a reference protein (Actin, Tubulin, Glyceraldehyde -3 - phosphate - dehydrogenase (GAPDH) into two detection channels [91]. To achieve greater precision, normalization can be done to correct for well-to-well variation in cell numbers. This can be achieved by normalizing signals from the protein of interest to an internal control protein/ reference protein or cell using a cell tag staining (example Cell tag 700) or cell labeling with a reactive dye. In addition to being simple and rapid, ICE has the added advantage of amalgamating the specificity of western blot and the replicability and high throughput of ELISA. As such it can be performed in either 96 or 384 well-formatted plates. Studies have also shown that ICE yields similar results to western blot whilst providing superior replicability and precision [91, 92]. Due to its flexibility and high throughput nature, it can be used for various screening purposes. In this project, ICE was adapted as the major high-throughput tool for screening the various drug libraries that were explored.

1.10.2 Western blot assay

Changes in the protein expression levels of cells after stimulation can be determined using traditional western blot. It is one of the common assays used in the detection and quantification of proteins in biomedical research [93]. In this immunodetection technique, proteins are first separated and then identified using target-specific antibodies [94]. The procedure involves the preparation of whole cell lysate from harvested cells and the separation of proteins based on their molecular weight. The separation of proteins in the whole cell lysate can be achieved by conducting electrophoresis. The proteins are then transferred onto a membrane (normally a nitrocellulose or polyvinylidene difluoride (PDVF)) and exposed to labeled target-specific antibodies [94]. The membrane is then washed to get rid of unbound antibodies. Antibodies that bind to the protein of interest give rise to a signal band that is specific for the protein of interest. This band can be detected using a chemiluminescent or fluorescent substrate. The signal can then be developed as a film or detected using a camera-based detection. Although the film detection technique is reported to be robust and sensitive, its dynamic range of quantification is poor compared to the camera-based detection which offers superior sensitivity and excellent quantification range [95]. In the western blot assay, the amount of protein present in the sample is representative of the intensity and thickness of the signal band that is visualized. To cater for differences in the electrophoresis loading, detection of housekeeping protein like; tubulin, actin, and GAPDH is performed [96]. Normalization of target signals can be done using reference signals obtained either from housekeeping proteins (HKPs) after immunochemical staining or using the total protein (TP) intensity on blotting membranes after total protein staining [93]. Advantages of this assay include the fact that, it is sensitive

and the same protein transfer blot can be used for a different analysis of multiple proteins [75]. However, due to the several processing steps in the western blot assay, it is known to be a low-throughput and labor-intensive technique [91].

1.10.3 Fluorescence accumulation assays using flow cytometry analysis

The impact of a therapeutic agent on the activity of an ABC transporter can be assessed by measuring the intracellular accumulation of a fluorescent substrate in cells that overexpress the ABC transporter of interest, in the presence or absence of a test compound. As such, fluorescence accumulation assays are one of the tools that have gained popularity in understanding how various test compounds/drugs can impact the functional activity of ABC transporters. It is based on the analogy that, there would be a low accumulation of fluorescent substrate in non-treated cells that overexpress ABC transporters like MRP1, P-gp, and MRP2 among others. This is because the fluorescent substrate is effluxed out of the intracellular space by the transporter resulting in low fluorescence. However, in the presence of an inhibitor of the transporter, there would be a high accumulation of the fluorescent substrate. This is because the inhibitor dampens/decreases the efflux activity of the transporter, thus leading to an increase in the intracellular fluorescence accumulation. Low intracellular fluorescence accumulation can also be observed in the presence of inducers or activators. Since activators only induce conformational changes, they require less incubation time in eliciting their effect, inducers on the other hand would require de novo synthesis of the transporter thus they need extended incubation time to elicit their effect [97]. In most studies, detection using fluorescent substrate is preferred over radioactive and analytical tools like mass spectrometry because it provides superior

sensitivity, greater convenience, and lower cost [98]. Visualization of a fluorescent substrate can be achieved using a fluorescence microscope, however, this method does not give a quantitative measurement of parameters. A spectrophotometer with fluorescence abilities can also be used but this approach is also limited by its low sensitivity [98]. One method that is reported to offer greater sensitivity whilst allowing quantitative measurement of intracellular accumulation of fluorescent substrates is flow cytometry [99-101]. Flow cytometry involves the use of fluorescent dyes and fluorescent antibodies that can bind to specific cellular components such as proteins on cell membranes or cell surface molecules among others. It is based on the principle that fluorescently labelled cells when passed through a light source get excited to a higher energy state. On returning to the ground/rest state, the fluorochromes emit light energy at higher wavelengths. The fluorescence emitted is collected by the flow cytometer, spectrally filtered and detected using a photomultiplier tube [102]. Thus, this technique allows quantitative measurement of single cells/particles or cellular constituents at high-speed rates [82]. Since test compounds that emit inherent fluorescence at emission wavelengths similar or close to the fluorescent substrate can interfere with quantification, it is needful to consider the background fluorescence of the compounds of interest during quantification. Flow cytometry can be used to measure the fluorescence and optical characteristics relevant for the studying of mammalian cells, as such it has become an essential tool for studying the regulation and interaction of cell systems [75]. Recent advancement in flow cytometry assays has also paved the way for the use of multiple fluorochromes that emit light at specific and varying wavelengths but share similar excitation wavelengths. This has created a platform that enables the measurement of different cell properties concurrently

[102]. Hence, in the study of ABC transporters, flow cytometry has been commonly employed in understanding and characterization of the interaction between therapeutic agents and ABC transporters. Commonly used fluorescent substrates include calcein acetoxymethyl ester (Calcein-AM) and rhodamine 123, with Calcein-AM being the ideal reference fluorescent substrate for P-gp and MRP1 studies [103].

1.11 The rationale of the study

There is no doubt that the discovery of modulators of ABC transporters has had several potential therapeutic benefits especially for patients with drug-resistant tumors. Even though most identified modulators of ABC transporters had significant effects on regulating its transport activity, one of the key challenges encountered in clinical trials has been the efficacy and safety of these modulators. Some dreadful side effects and elevated levels of patient toxicities have been reported due to adverse pharmacokinetic interactions with administered anticancer drugs. For instance, the coadministration of cyclosporin A and etoposide to a patient with acute T-lymphocytic leukemia in relapse resulted in progressive hyperbilirubinemia and mental confusion [104]. Therefore, there is a need for more potent, low toxic, and well-tolerated drugs. Moreover, a critical review of literature on modulators of ABC transporters like MRP1 reveals that although most MRP1 modulators could influence transporter activity, little is known about their impact on the gene and protein expression levels of these transporters. Thus, further research must be conducted to investigate how current and future therapeutic agents that interact with ABC transporters may affect their protein and gene expression levels. This would provide

essential data on drug-transporter interactions, which is important for clinical trials. Moreover, therapeutic agents that show the ability to decrease protein expression could be used together with drugs that are known to be efficacious in treating cancer but are unfortunately substrates of these transporters. Thus in the presence of the drug that decreases protein expression of the transporter, the more potent drug would have higher bioavailability to exert its effect on the cancer cells, thereby enhancing combinatorial drug therapy. Identification of modulators of ABC transporters like MRP1 and MRP2 would also enable the scientific community and the pharmaceutical industry to gain greater insight into the causes of treatment failure and relapses experienced by cancer patients, as well as provide a possible therapeutic approach to enhance effectiveness of chemotherapy. Aside from cancer treatment, findings from such studies would provide great enlightenment for the treatment of other diseases in which these transporters are implicated whilst deepening our understanding of the pharmacological and physiological nature of these transporters.

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Chapter 2.0

Novel ATP competitive inhibitors downregulate Multidrug resistance protein 1 (MRP1) expression in MRP1- overexpressing cells

Abstract

Multidrug resistance protein 1 (MRP1) is an integral membrane protein that serves as an ATP-dependent drug efflux pump. It plays a pivotal role in the efflux of a wide variety of endogenous and exogenous substrates such as toxic chemicals, drugs, and their metabolites out of cells. Overexpression of MRP1 confers resistance against commonly used chemotherapeutic agents in tumor cells. In chemotherapy, the emergence of the combinatorial therapeutic approach led to a major improvement in survival rates of several cancers, however multidrug resistance (MDR) has been a major challenge to its effectiveness. Thus it is of great clinical interest to identify compounds which can modulate MRP1 expression and activity without perturbing physiological homeostasis. Using an In-Cell ELISA assay we screened 30 drugs which consisted of both clinically tested anticancer drugs and recently approved FDA drugs to investigate their effect on MRP1 expression. We identified a total of 7 modulators, of which 4 test compounds increased the protein expression levels of MRP1 whereas 3 test compounds decreased the protein expression of MRP1. Four of the modulators identified (Amuvatinib, SB743921 HCl, TG101348 (SAR302503), Felbamate) have never been reported as modulators of MRP1, thus these compounds were selected for further characterization in this study. Three of the novel modulators of MRP1 discovered (Amuvatinib, SB743921 HCl, and TG101348 (SAR302503)) decreased MRP1 protein expression and were identified to be ATP

competitive inhibitors based on their mode of action. Felbamate (recently approved FDA drug) increased MRP1 protein expression. Further characterization of our novel modulators using In-Cell ELISA assay showed that SB743921 HCl and Amuvatinib decreased MRP1 protein expression in a concentration and time-dependent manner. Calcein AM accumulation assay performed to ascertain the impact of the selected hit compounds on MRP1 efflux activity revealed that TG101348 (SAR302503), Amuvatinib, and SB743921 HCl decreased MRP1 efflux activity. Cell viability and reversal of MRP1-mediated resistance to vincristine studies carried out using MTT assay also showed that TG101348(SAR302503) and Amuvatinib were more potent at reversing MRP1-mediated resistance. The discovery of key and novel modulators of MRP1 is a step in the right direction to aid revert MDR in cancer patients. Findings from this project would provide essential information to improve combinatorial drug therapy and precision medicine as well as reduce drug toxicity of various cancer chemotherapies.

Keywords: ABC transporters; multidrug resistance; MRP1; ABCC1; MRP1 modulators; In-Cell ELISA; protein expression; anticancer drug; FDA approved drug; drug profiling; drug-transporter interactions

1) SB743921 HCl (PubChem CID: 49867937); 2) Amuvatinib (PubChem CID: 11282283); 3) TG101348 (SAR302503) (PubChem CID: 16722836); 4) Felbamate (PubChem CID: 3331)

1.0 Introduction

Cancer is the second leading cause of mortality and morbidity globally. In 2018, approximately 18.1 million new cancer cases, and an estimated 9.6 million cancer-related deaths were recorded worldwide [1]. Although several treatment modalities exist for cancer therapy, chemotherapy remains the standard treatment method for various types of cancers. Chemotherapy is described as the use of drug formulations to target, control, and kill tumor cells in a systemic treatment module [2]. Even though chemotherapy has been successful and beneficial in cancer therapy, the challenge of multidrug resistance (MDR) limits its effectiveness. MDR is a phenomenon in which cells develop resistance to several drugs that may differ in structure, molecular target, and mode of action [3]. Studies into the development of MDR revealed several mechanisms that are implicated in this phenomenon. These mechanisms include; cellular changes in cells that minimize the ability of cytotoxic drugs to kill cells such as elevated repair of DNA damage and evasion of apoptosis among others. The other mechanisms reported include decreased uptake of water-soluble drugs (folate antagonists and cisplatin) that utilize transporter proteins for cell entry and increased energy-dependent efflux of hydrophobic drugs through the plasma membrane of cells [3]. One superfamily of transporters whose overexpression has been implicated in MDR is the ATP-Binding Cassette superfamily of transporters (ABC transporters). ABC transporters represent a diverse and ubiquitous superfamily of transporters that utilize ATP hydrolysis for their transport activities [4]. This group of transporters is known to facilitate the transport of a variety of molecules ranging from small molecules to highly charged and hydrophobic molecules including peptides, vitamins, toxins, drugs, and their metabolites across biological membranes [5].

ABC transporters are present in every phylum of life. Based on their direction of transport relative to the cytoplasm, they can be categorized into importers and exporters [6]. In prokaryotes, this group of transporters can function as importers or exporters, however, they function solely as exporters in eukaryotes. A total of 49 ABC transporters have been identified in the human genome. These transporters have been classified into seven subfamilies (A-G) based on their protein domain and amino acid sequence [7, 8]. ABC transporters like P-glycoprotein (P-gp), Multidrug resistance protein 1 (MRP1), and Breast cancer resistance protein (BCRP) have been reported to play pivotal roles in the absorption, excretion, metabolism, and elimination of drug and their metabolites [6]. In phase O and phase III of drug metabolism, these transporters regulate the entry and extrusion of drugs before reaching their pharmacological target as well as ensure the complete elimination of metabolized molecules [6]. As major players in drug metabolism, the overexpression of some ABC transporters have been implicated in reduced intracellular accumulation and therapeutic potency in anticancer drugs in cancer patients [9]. The prototypical ABC transporter is characterized by a transport core consisting of four main domains; two sets of membrane-spanning domains (MSDs) and two sets of cytosolic nucleotide-binding domains (NBDs) [10]. The MSD comprises six transmembrane α -helices that form the substrate-binding site/s and facilitate substrate translocation across the plasma membrane [11]. The NBDs on the other hand consists of the Walker motifs (Walker A and Walker B) that bind to ATP for ATP hydrolysis [12]. Upon ATP binding and hydrolysis, the NBDs dimerize to cause conformational changes that result in the rearrangement of the MSDs to an outward-facing conformational and subsequent efflux of the substrate. MRPs in the ABCC subfamilies possess an extra NH₂-proximal membrane-spanning domain known as

the MSD0 [13]. The MSD0 is reported to aid the trafficking, retention and recycling of the transporter to the plasma membrane [14].

MRP1 is a 190-kDa ATP-dependent efflux transporter [15]. It is expressed in the lungs, kidney, small intestines, and at pharmacological sanctuary sites including the blood-brain barrier, blood-testis barrier, and the blood placenta barrier [15-18]. MRP1 localizes at the plasma membrane and governs the absorption and deposition of a broad spectrum of substrates [19]. Substrates of MRP1 include heavy metals, toxins, drugs, and metabolites [20, 21]. The pharmacological aim of administering an anticancer drug is to ensure the maximum delivery of its active component to the desired therapeutic target in tumor cells, to initiate enough cellular destruction to cause cell death [9]. However, the overexpression of ABC transporters like MRP1 facilitates the efflux of such administered anticancer drugs leading to decreased bioavailability and therapeutic potency of these drugs. As such, overexpression of MRP1 has been implicated in MDR of many carcinomas and has been reported to be associated with the elevated risk of treatment failure leading to cancer relapse and low survival rates among cancer patients [22]. In addition to conferring resistance to anticancer drugs like paclitaxel, etoposide, and doxorubicin, MRP1 also affects the bioavailability and efficacy of antivirals, antimalarials, antibiotics [23]. Aside the critical role MRP1 plays in MDR, it also aids in maintaining physiological homeostasis by regulating redox homeostasis, steroid metabolism among others [24]. MRP1 also acts as a key player in the etiology of neurodegenerative diseases like Alzheimer's disease and cardiovascular diseases [24].

Biochemical modulation plays a key role in chemotherapy. It is described as the process in which pathways or molecular targets are biochemically modified by therapeutic agents to

enhance the selective cytotoxic effect of anticancer agents on cancer cells but decrease their toxic side effects on normal cells [25, 26]. Biochemical modulation offers a means of regulating the activity of ABC transporters like MRP1 without perturbing the physiological balance in normal cells. Moreover, modulators also have the added advantage of boosting oral availability and enhancing the penetration of drugs that are transported by MRP1 in tissues [27]. In this present study, we screened 30 drugs consisting of both anticancer and recently approved FDA drugs to ascertain their effect on MRP1 protein expression levels using In-Cell Enzyme-Linked Immunosorbent assay (In-Cell ELISA) assay. We identified 4 novel drugs that modulated MRP1 protein expression in MRP1-overexpressing cells. These drugs included 3 novel ATP competitive inhibitors that down-regulated MRP1 protein expression and one FDA approved drug that increased MRP1 protein expression. The ability of test compounds to modulate MRP1 activity and reverse MRP1-mediated resistance was further explored using established methods.

2.0 Materials and methods

2.1 Chemicals

Test compounds consisting of anticancer and FDA approved drugs were procured from Selleck Chemicals (Houston, TX), and APExBIO Technology LLC (Houston, TX). MK571 was acquired from APExBIO Technology LLC (Houston, TX), thiazolyl blue tetrazolium bromide (MTT) was procured from Sigma-Aldrich (St. Louis, MO) and Calcein acetoxymethyl ester (Calcein AM) was obtained from Corning Life Sciences (Corning, NY).

2.2 Cell lines and cell culture

HEK293/pcDNA3.1 and HEK293/MRP1 cells were a kind gift from Dr. Suresh V. Ambudkar (NIH, Bethesda, MD). Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) was used to grow the HEK293 cell lines. Cell lines were cultured in a humidified incubator maintained at 5% CO₂ at 37 °C. This incubation condition was retained in all subsequent cell culture procedures.

2.3 Screening of compounds using In-Cell ELISA assay

In-Cell ELISA assay was used to screen the test compounds for modulators of MRP1 protein expression. HEK293/pcDNA3.1 and HEK293/MRP1 cells were seeded at a cell density of 5×10^4 cells per well in 96-well plates with DMEM containing 10% FBS, treated

with drugs (10 μM), and 0.1% DMSO (controls) after 24 hours, Cells were incubated for 48 hours after drug treatment at 37 °C. At the end of the incubation period, treatments were removed and cells were rinsed twice with 150 μl of PBS. Cells were fixed with 3.7% paraformaldehyde and permeabilized with Triton X-100. The cells were blocked using fish gel (MB-066-0100, Rockland) and incubated overnight at 4 °C with monoclonal anti-MRP1 antibody (1:500; IU5C1, MA516079, Thermo Fisher Scientific) or anti- α tubulin antibody (1:1000; T5168, Sigma -Aldrich) used as the internal control. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific) was incubated for an hour at room temperature. Target proteins were detected with chemiluminescence using Super signal West Dura® Extended Duration Substrate (21EAPI34076, Thermo Fisher Scientific) and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Experiments were conducted in two independent studies, with treatments performed in duplicates.

2.4 Determination of the concentration-dependent activity of selected hit compounds on MRP1 protein levels using In-Cell ELISA assay

HEK293 MRP1 overexpressing cells and HEK293/pcDNA3.1 cells were seeded in 96-well plates as described earlier. Cells were treated with varying concentration of test compounds; 1 μM , 5 μM , 10 μM , 20 μM and incubated at 37 °C for 48 hours. Final DMSO concentration was maintained at less than 0.2% (v/v). Cells were fixed, permeabilized, and blocked as detailed earlier. Incubation with monoclonal anti-MRP1 antibody (1:250; IU5C1, MA516079, Thermo Fisher Scientific) and anti- α tubulin antibody (1:1000, T5168,

Sigma-Aldrich) was conducted overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody incubation was performed for an hour at room temperature. Target proteins were detected using western blotting luminol reagent (Santa Cruz Biotechnology, sc-2048), and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were done in triplicates.

2.5 Time-dependent activity of selected hit compounds on MRP1 Protein levels using In-Cell ELISA assay.

HEK293/MRP1 and HEK293/pcDNA3.1 cells were seeded as detailed above and treated with 10 μ M of test compounds and incubated for various time points; 12, 24, and 48 hours at 37°C. Controls were treated with 0.1% DMSO. Cells were fixed, permeabilized, and blocked as indicated earlier. After which, cells were incubated with monoclonal anti-MRP1 antibody (1:250; IU5C1, MA516079, Thermo Fisher Scientific) and anti- α tubulin antibody (1:1000, T5168, Sigma-Aldrich) was conducted overnight at 4 °C. Followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) for an hour at room temperature. Western blotting luminol reagent (Santa Cruz Biotechnology, sc-2048) was used for detection of target proteins, and plates were read using Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicates.

2.6 Flow cytometric measurements of intracellular Calcein accumulation

Flow cytometry was conducted to determine the effect of selected drugs on MRP1 mediated efflux of calcein-AM. Calcein-AM was used as the fluorescent substrate for the

accumulation assay, with MK571 as the positive control. HEK293/MRP1 cells were prepared in serum-free medium at a cell density of 7×10^5 cells/ml and treated with test compounds (10 μ M final concentration), MK571 (25 μ M final concentration), and DMSO (0.1% final concentration for controls). Cells were incubated at 37 °C for 10 minutes. After incubation, the cells were treated with Calcein-AM (0.25 μ M) and incubated for additional 30 minutes. Ice-cold PBS buffer (3 ml) was added to halt the reaction, after which cells were centrifuged and washed twice with cold PBS. Cells were then resuspended in a cold PBS buffer containing 1% paraformaldehyde. Detection of intracellular accumulation of calcein-AM was done using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) with excitation at 480 nm, and emission and 533/30 nm. Fluorescence intensities are representative of the mean value collected from 10,000 events. Treatments were done in duplicates and conducted in three independent experiments.

2.7 Cytotoxicity of selected test compounds

The sensitivity of HEK293/MRP1 and HEK293/ pcDNA3.1 cells towards the selected test compounds were determined using the MTT colorimetric assay. Cells were seeded at a cell density of 5×10^3 per 100 μ l of culture medium in 96 well plates. After 24 hours, cells were treated with 100 μ l of test compounds at varying concentrations. The final DMSO concentration was kept at 0.05%. Cells were then incubated for 72 hours, after which 100 μ l of the spent culture medium was carefully removed. Cells were then treated with MTT (0.5 mg/ml) for 4 hours. The formazan crystals were dissolved by the addition of 100 μ l of 15% SDS containing 10 mM HCl and absorbance at 570 nm was recorded using a Hidex

Sense Beta Plus plate reader (Turku, Finland). Treatments were done in triplicates, and experiments were performed in two independent studies.

2.8 Resistance reversal assay

The ability of test compounds to reverse MRP1 mediated resistance towards the chemotherapeutic drug, vincristine was analyzed using the MTT colorimetric assay. Cells were seeded in 96 well plates at a cell density of 5×10^3 per 100 ul of culture medium. After 24 hours, cells were treated with 50 ul of test compounds at selected concentrations prepared in a culture medium. After an hour, 50 ul of vincristine at varying concentrations was added to the cells. Final DMSO was maintained at 0.2%, and cells were incubated for 72 hours. At the end of the incubation period, 100 ul of the spent culture medium was carefully removed. Cells were treated with MTT (0.5 mg/ml) for 4 hours. The formazan crystals were dissolved by the addition of 100 ul of 15% SDS containing 10 mM HCl and absorbance at 570 nm was recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were done in triplicates, and experiments were performed in two independent studies.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism™ software (GraphPad Software version 8.4.3, San Diego, CA, USA). The differences between mean values were analyzed using linear mixed model analysis. Sidak correction was applied for multiple comparisons, statistical testing was performed at a 5% level of significance.

3.0 Results

3.1 Screening for modulators of MRP1 protein expression using In-Cell ELISA assay

In-Cell ELISA also known as In-cell western assay or Cytoblot is a cell-based immunocytochemistry assay that allows quantification of target proteins in cultured cells. It merges the specificity of western blot, the replicability, and the high-throughput nature of ELISA. To identify modulators of MRP1 protein expression levels, we screened 30 drugs (consisting of both clinically tested anticancer and recently approved FDA drugs) using In-Cell ELISA assay in HEK293 MRP1-overexpressing cells. The MRP1 protein expression in HEK293/MRP1 treated with 0.1% DMSO was considered as the baseline for computing the percent modulation for the test compounds. The screening was conducted in 96-well format in two independent studies. The percent modulation of MRP1 protein expression by the test compounds from the two independent studies is represented in Figure 2.1. A test compound was considered as a “Hit compound” if its calculated percent modulation is $\geq 30\%$ in the positive (+) or negative (-) direction. Test compounds that showed percent modulation $\geq 30\%$ are presented as red dots in Figure 2.1, whereas drugs that showed percent modulation $< 30\%$ are represented by black dots. The screening process identified a total of 11 hit compounds that modulated the protein expression of MRP1 $\geq 30\%$. The identified hit compounds, their therapeutic targets, and specific percent modulation are listed in table 2.1.

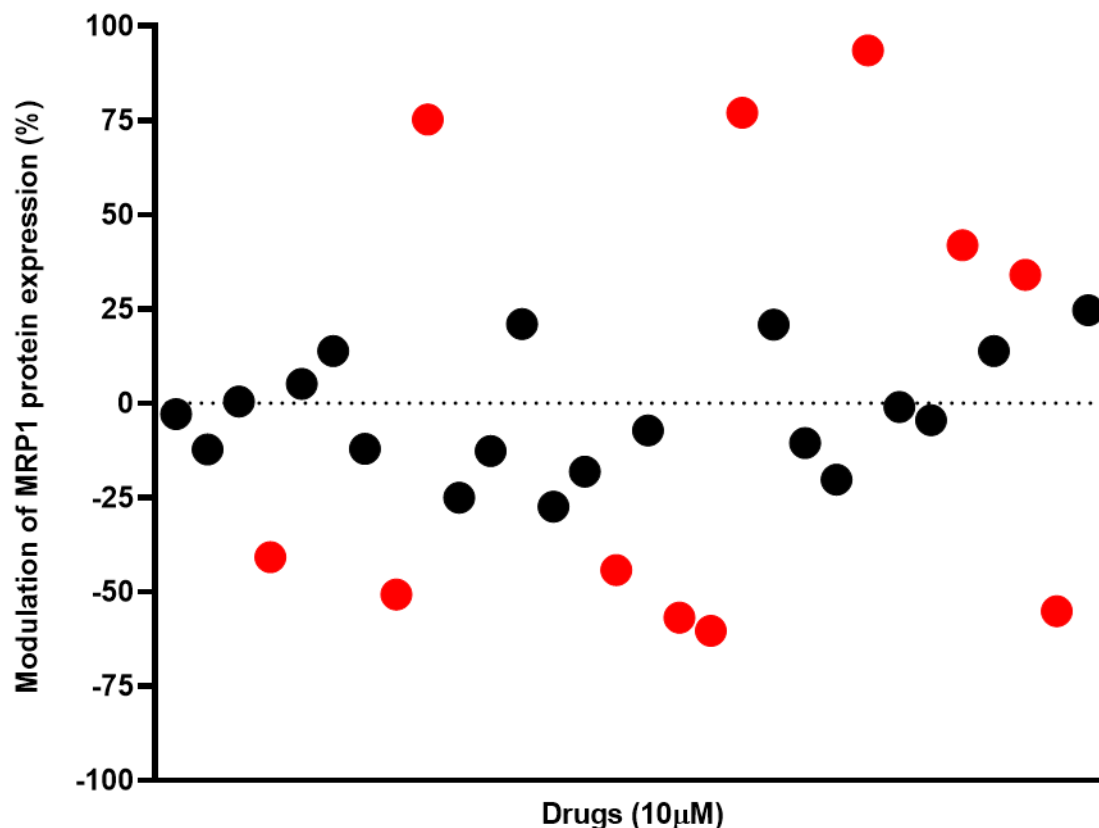


Figure 2. 1. Screening of compounds using In-Cell ELISA assay.

HEK293/MRP1 cells were treated with 10 μ M of test compounds and incubated for 48 hours. MRP1 protein expression was detected using a monoclonal anti-MRP1 antibody (1:500; IU5C1, MA516079, Thermo Fisher Scientific). Alpha-tubulin protein (used as an internal control) was detected using mAb α -tubulin (1: 1000; T5168, Sigma – Aldrich). Secondary antibody; mAb-goat-anti-mouse (1:1000) was used. Treatments were performed in duplicates and experiments were conducted in two independent studies. Red dots represent drugs that showed $\geq 30\%$ modulation of MRP1 protein expression. Black dots represent drugs that showed $< 30\%$ modulation of MRP1 protein expression.

Table 2.1 Modulatory effect of drugs on MRP1 protein expression

Compound	Target	% Modulation on MRP1 protein expression ^a
Saracatinib (AZD0530)	Bcr-Abl	-2.88 ± 0.08
Linifanib (ABT-869)	CSF-1R	-12.23 ± 0.14
Zonisamide	T-type calcium channels	0.47 ± 0.16
Axitinib*	c-Kit	-40.80 ± 0.09
XMD8-92	ERK	5.17 ± 0.09
PI-103	Autophagy	13.88 ± 0.04
Vandetanib (Zactima)	VEGFR	-12.09 ± 0.12
Amuvatinib***	c-Kit	-50.70 ± 0.01
Epirubicin HCl*	Topoisomerase	75.19 ± 0.07
Trichostatin A (TSA)	HDAC	-25.00 ± 0.10
LY294002	Autophagy	-12.67 ± 0.06
Sunitinib Malate (Sutent)	c-Kit	21.04 ± 0.05
SU11274	c-Met	-27.38 ± 0.02
Nutlin-3	E3 Ligase	-18.15 ± 0.07
PCI-24781*	HDAC	-44.22 ± 0.1
Iniparib (BSI-201)	PARP	-7.239 ± 0.11
Vismodegib (GDC-0449)*	Hedgehog	-56.79 ± 0.12
SB 743921 HCl***	Kinesin	-60.30 ± 0.05
Irinotecan*	Topoisomerase	76.98 ± 0.09
Atazanavir sulfate	HIV protease	20.85 ± 0.12
Bumetanide	Na ⁺ -K ⁺ -Cl ⁻ cotransporter	-10.51 ± 0.02
VER-50589	HSP	-20.25 ± 0.09
Felbamate***	NMDA receptor	93.54 ± 0.20

^a Mean ± SEM of n ≥ 2 independent experiments.

* Hit compounds – showed ≥ 30% modulation

***Hit compounds characterized – showed ≥ 50% modulation

Table 2.1 (Continued) Modulatory effect of drugs on MRP1 protein expression

Compound	Target	% Modulation on MRP1 protein levels^a
Tadalafil (Cialis)	PDE	-0.97 ± 0.02
PF-04217903	c-Met	-4.50 ± 0.06
Telaprevir (VX-950)*	HCV Protease	41.90 ± 0.03
Saxagliptin (BMS-477118 Onglyza)	DPP-4	13.84 ± 0.04
Pimobendan (Vetmedin)*	PDE	34.03 ± 0.04
TG101348 (SAR302503)***	JAK	-55.11 ± 0.06
Vatalanib 2HCl (PTK787)	c-Kit	24.68 ± 0.01

^a Mean ± SEM of n ≥ 2 independent experiments.

* Hit compounds – showed ≥ 30% modulation

***Hit compounds characterized – showed ≥ 50% modulation

Out of the 11 hits identified, 5 test compounds increased the protein expression levels of MRP1 and 6 test compounds decreased the protein expression of MRP1. For detailed characterization, hit compounds that modulated protein expression of MRP1 ≥ 50% were considered. Seven of the hit compounds showed modulation of MRP1 protein expression ≥ 50%. Among these hits, three compounds (Vismodegib, Epirubicin HCl, and Irinotecan) have already been reported to modulate MRP1 in other studies [28-30]. Thus, these compounds were not further characterized. In this study, we focused on characterizing the other 4 novel test compounds that to the best of our knowledge have not been reported for their activity as modulators of MRP1 protein expression (Table 2.2). This included 3 ATP competitive inhibitors; SB743921 HCl, Amuvatinib, TG101348 (SAR302503), and an FDA approved drug, Felbamate. SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) decreased MRP1 protein expression levels by 60.30%, 50.70%, and 55.11%

respectively, with SB743921 HCl eliciting the highest negative modulation of MRP1 protein levels. Felbamate on the other hand increased MRP1 protein expression levels by 93.54 % in the HEK293 overexpressing MRP1 cells. The chemical structures of the selected compounds are shown in Figure 2.2.

Table 2.2 Chemotherapeutic targets, % modulation of MRP1 protein expression for selected and characterized Hit compounds

Compound	Chemotherapeutic targets	% Modulation MRP1 protein levels^a	Effect on MRP1 protein levels
SB743921 HCl	Kinesin	60.30 ± 0.05	Decrease
Amuvatinib	C-kit	50.70 ± 0.01	Decrease
TG101348 (SAR302503)	JAK	55.11 ± 0.06	Decrease
Felbamate	N-Methyl-D-aspartate (NMDA) receptor	93.54 ± 0.20	Increase

^aMean ± SEM of n ≥ 2 independent experiments

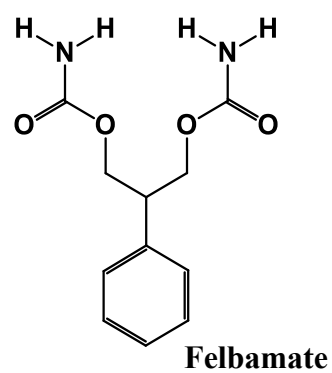
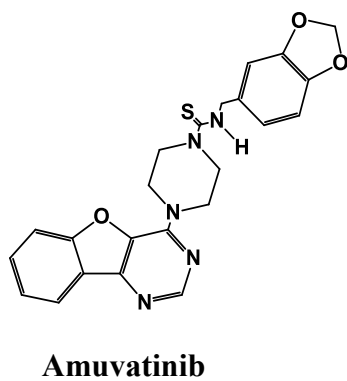
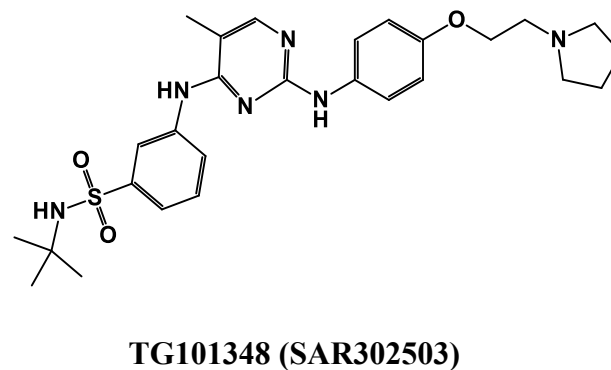
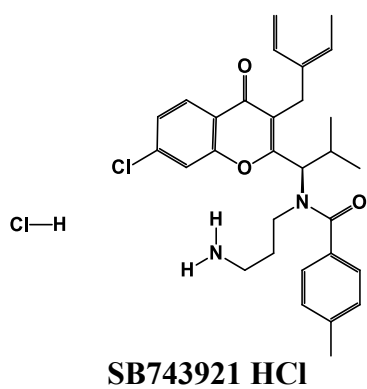


Figure 2.2 Chemical structures of selected hit compounds

3.2 Concentration and time-dependent activity of selected hit compounds for MRP1

In order to determine if the modulatory effects observed for our selected compounds were concentration and time-dependent and to identify the conditions that produce the maximum modulatory effect, we conducted concentration and time-dependent studies using the In-Cell ELISA assay. HEK293 MRP1 overexpressing cells were treated with varying concentration (1 μ M, 5 μ M, 10 μ M, 20 μ M) of the test compounds for the concentration-dependent studies. In the time-dependent studies, cells were treated with 10 μ M of test compounds, and were incubated at various time points; 12 hours, 24 hours, and 48 hours. As shown in Figure 2.3A; expression levels of MRP1 were further reduced by increasing concentrations of SB743921 HCl and Amuvatinib. Drug treatment of 20 μ M exhibited strongest modulatory effect and resulted in 75.62 % and 85.28% reduction in MRP1 expression by SB743921 HCl and Amuvatinib, respectively. TG101348 (SAR302503) showed highest downregulation of MRP1 protein expression at 10 μ M. Treatment with Felbamate also showed concentration dependence and highest modulatory effect on MRP1 expression levels was observed in case of 10 μ M drug treatment. Regarding time-dependence studies, as presented in Figure 2.3B, all drug treatments demonstrated time-dependence and the highest modulatory effect was observed with 48 hour treatment. Therefore, in case of SB743921 HCl, Amuvatinib and TG101348, MRP1 expression levels were reduced by 62.83%, 49.29% and 49.28% respectively by 48 hour drug treatment. In the case of Felbamate, MRP1 expression levels were increased by 77.90% by 48 hour drug treatment.

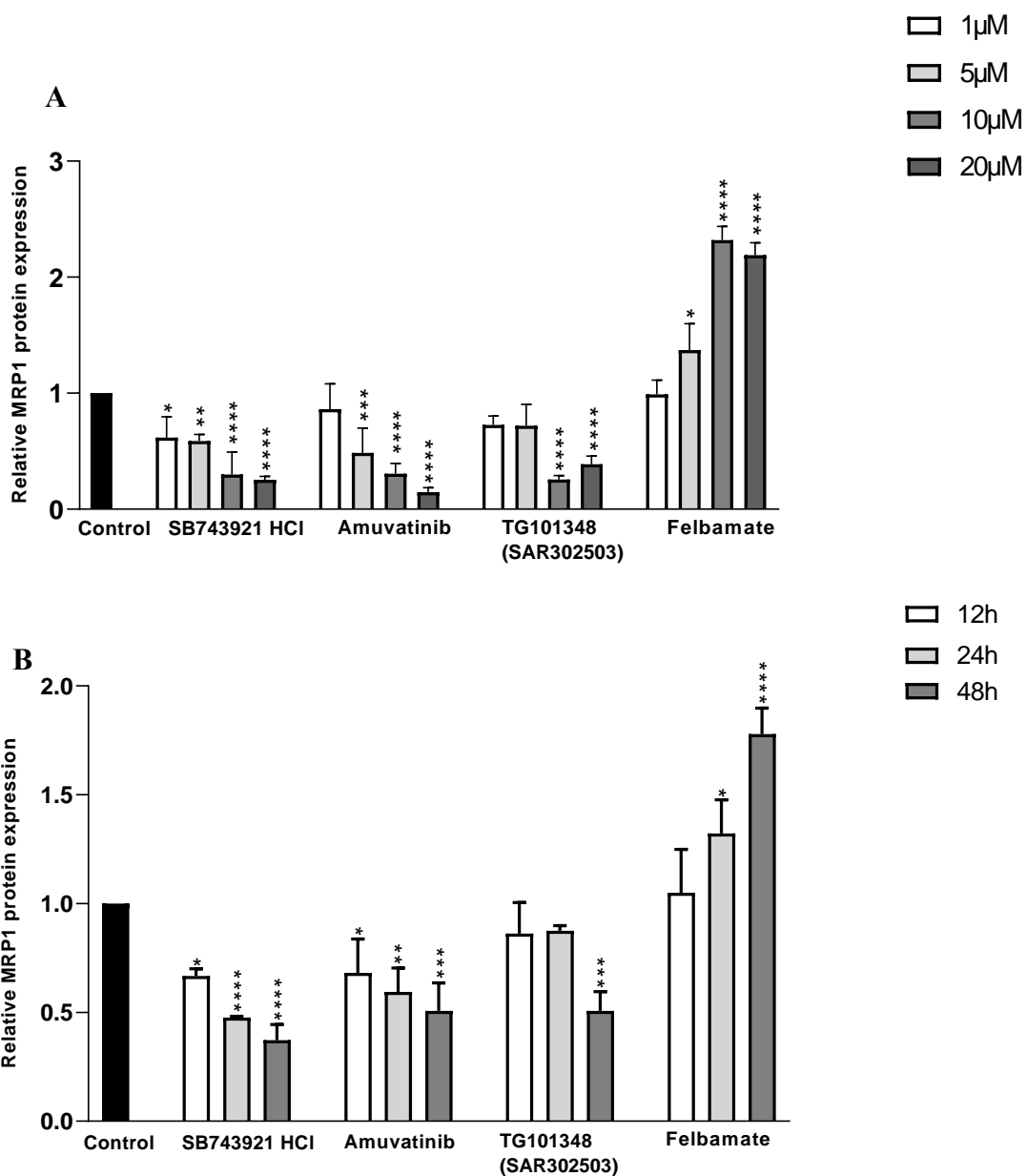


Figure 2.3 Concentration and time-dependent activity of selected hit compounds on MRP1 protein expression using In-Cell ELISA assay.

[A] Concentration dependent activity of test compounds on MRP1 protein expression; HEK293/MRP1 cells were treated with 1 μ M, 5 μ M, 10 μ M and 20 μ M of test compounds and incubated for 48 hours. [B] Time dependent activity of selected hit compounds on MRP1 protein expression, HEK293/MRP1 cells were treated with 10 μ M of test compounds and incubated for 12, 24, 48 hours. Treatments were performed in triplicates and data analyzed using linear mixed model and Sidak post hoc test. Data is represented as mean \pm S.D. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 compared to control.

3.3 Effect of test compounds on MRP1 efflux activity using calcein accumulation assay

Overexpression of MRP1 has been associated with the increased efflux of multiple classes of therapeutic agents across biological membranes. This results in the low bioavailability and reduced pharmacological potency of such compounds. We investigated the effect of the selected compounds on MRP1 efflux activity of calcein-AM using a flow cytometry-based assay. Calcein-AM is a well-known substrate of MRP1. This non-fluorescent compound is converted into a highly fluorescent molecule when its acetoxymethyl ester (AM) component is cleaved off by cellular esterases. Using flow cytometry, the intracellular calcein fluorescence accumulation can be ascertained. In this assay, HEK293/MRP1 cells were treated with 10 μ M of the test compounds for 10 minutes before the treatment with calcein-AM was conducted for an extra 30 minutes. Our results as presented in Figure 2.4 shows that 25 μ M of MK571 (commonly used MRP1 inhibitor) increased intracellular calcein-AM by 3.85-fold compared to the no treatment control. Among the selected compounds; SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) significantly increased the calcein accumulation by 3.51-fold, 1.75-fold, and 2.52-fold respectively with SB743921 HCl exhibiting highest modulatory effect on MRP1 activity compared to no treatment control. Felbamate, on the other hand, did not have any significant impact on calcein accumulation as compared to the no treatment control.

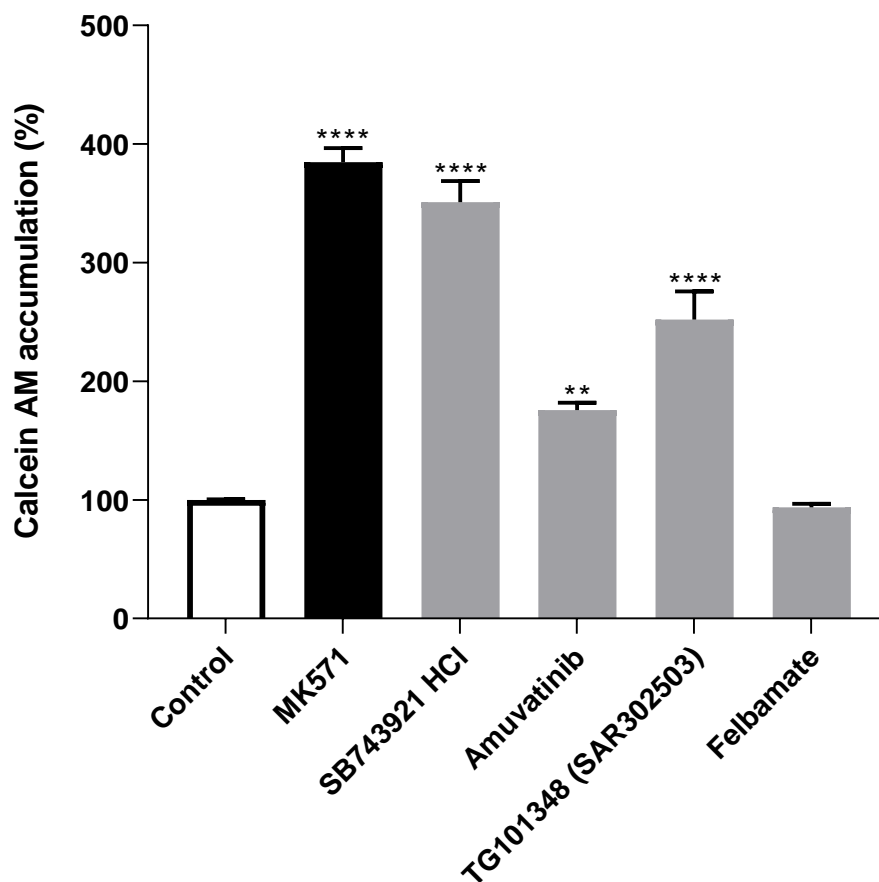


Figure 2.4 Effect of selected hit compounds on MRP1 mediated calcein efflux.

HEK293/MRP1 cells were treated with 10 μM of the test compounds, and 25 μM of MK571 (positive control) for 10 minutes at 37°C before treatment with 0.25 μM calcein-AM for 30 minutes. Flow cytometric measurements of intracellular calcein-AM was conducted at 488 nm and 533/30 nm for excitation and emission wavelengths, respectively. Experiments were done as duplicates in three independent experiments and presented as mean \pm S.E.M. Data was analyzed using a linear mixed model and Sidak post hoc test. *P < 0.05; **P < 0.01; ****P < 0.001 compared to control.

3.4 In vitro cytotoxicity of selected test compounds

The half-maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a specific compound to inhibit a specific biochemical or biological function. Results from the cytotoxicity studies using MTT as shown in table 2.3, reveals that Amuvatinib was the most potent in inhibiting the growth of HEK293/MRP1 cells with an average IC_{50} of 2.64 μ M. TG101348 (SAR302503) with an average IC_{50} of 3.26 μ M was also very potent in inhibiting the growth of HEK293/MRP1 cells. SB743921 also showed an inhibitory effect on HEK293/MRP1 cells with an average IC_{50} of 9.35 μ M. Felbamate had the least growth inhibitory effect on HEK293/MRP1 cells with an average IC_{50} of 47.23 μ M. Thus from table 2.3, we can infer that most HEK293/MRP1 cells survived when treated with felbamate hence requiring very high concentration of the drug to inhibit 50% of the cell growth. As expected HEK293/pcDNA 3.1 was very sensitive to the cytotoxic activity of all the test compounds as compared to HEK293/MRP1 cells since they do not overexpress MRP1, thus these cells can easily be impacted by the cytotoxicity of the drugs. Overall, the three ATP competitive inhibitors that decreased MRP1 protein expression were also very potent in inhibiting the cell growth of HEK293 MRP1-overexpressing cells.

Table 2.3 Cytotoxicity of selected hit compounds on HEK293/pcDNA3.1 and HEK293/MRP1 cells

Cell Line	Drug	IC ₅₀ ^a (μM)
HEK293/pcDNA3.1	SB743921 HCl	6.84 ± 0.73
	Amuvatinib	0.45 ± 0.25
	TG101348 (SAR302503)	2.05 ± 0.34
	Felbamate	14.92 ± 2.06
HEK293/MRP1	SB743921 HCl	9.35 ± 0.12
	Amuvatinib	2.61 ± 0.64
	TG101348 (SAR302503)	3.26 ± 0.21
	Felbamate	47.23 ± 0.64

^a Mean ± SEM of n ≥ 2 independent experiments

3.5 Effect of selected test compounds on MRP1-mediated drug resistance

One of the major setbacks of the current modulators of MRP1 is the fact that they exhibit low MDR reversal effects. Thus, we investigated the ability of the selected compounds to reverse MRP1 mediated resistance towards vincristine. HEK293 MRP1-overexpressing cells were treated with varying concentrations of vincristine in the presence or absence of non-cytotoxic concentrations of the test compounds. MK571 was used as positive control. As shown by the IC₅₀ and fold resistance listed in table 2.4. HEK293 MRP1-overexpressing cells in the absence of MK571 demonstrated very low sensitivity to the cytotoxicity effect of vincristine, giving a high fold resistance of 20.51 fold. HEK293/pcDNA3.1

contrastingly exhibited very high sensitivity towards vincristine, thus gave a very low fold resistance. In the presence of MK571 inhibitor (25 μM), the fold resistance in the MRP1-overexpressing cells drastically decreased from 20.51 to 3.64-fold resistance. From the results obtained, two of the selected compounds; Amuvatinib and TG101348 (SAR302503) at 1 μM reversed MRP1-mediated resistance towards vincristine in HEK293/MRP1 cells. TG101348 (SAR302503) strongly decreased the fold resistance to 7.16-fold, whilst Amuvatinib also reduced the fold resistance to 9.13-fold. This indicated that our selected test compounds may be able to reverse MRP1 mediated resistance. Contrastingly, treatment of HEK293/MRP1 with SB743921 HCl elevated the resistance of the cells against vincristine.

Table 2.4 Effect of selected hit compounds on the IC_{50} values of vincristine in HEK293/MRP1 cells

Cell line/Treatment	IC_{50} ^a (nM)	
	Vincristine	Fold resistance ^b
HEK293/pcDNA3.1	3.11 \pm 0.23	1
HEK293/MRP1	62.25 \pm 7.21	20.51
HEK293/MRP1 + MK571 [25 μM]	11.30 \pm 1.00	3.64
HEK293/MRP1 + Amuvatinib [1 μM]	28.42 \pm 2.85	9.13
HEK293/MRP1 + TG101348 [1 μM]	22.26 \pm 4.78	7.16
HEK293/MRP1 + SB743921 HCl [0.5 μM]	151.57 \pm 20.75	48.78

^a Mean \pm SEM of at least three independent experiments performed in triplicates.

^b Fold resistance determined by dividing the IC_{50} value for each treatment by the IC_{50} value of HEK293/pcDNA3.1 with vincristine alone.

4.0 Discussion

Recent advancement in chemotherapy has contributed to the improvement of survival rates of several cancers. However, MDR has been a prime opponent to this treatment modality. The occurrence of MDR in several carcinomas has limited the effectiveness of chemotherapy. The efflux activity of overexpressed MRP1 in tumor cells has been a key contributor to this phenomenon. The substrate family of MRP1 spans multiple drug classes, this includes conventional chemotherapeutic drugs such as etoposide, doxorubicin, paclitaxel among others [28]. It also affects the efficacy of antivirals, antibiotics, antimalarials among others [23]. As such, the overexpression of MRP1 by tumor cells is used as a survival and protection strategy to reduce the intracellular drug concentration and accumulation of such drugs to render them less potent by reducing their bioavailability. This goes a long way to reduce the cytotoxic effect of such drugs on cancer cells. This phenomenon in cancer cells has led to the elevated risk of treatment failure, and decreased survival rates of patients, thus pose a huge challenge to the pharmaceutical industry and clinical oncology researchers.

Modulating the expression and function of MRP1 via biochemical modulation has become one of the powerful tools used by cancer researchers to overcome MRP1-mediated MDR. Biochemical modulation provides the platform for scientists to modulate the function and the transport in tumor cells without perturbing physiological homeostasis in normal cells. By using the biochemical modulation toolbox, two or more pharmacological agents that may work via varying molecular mechanisms and may have different molecular targets can be combined at their respective effective doses to achieve a common goal without eliciting any unacceptable side effects. Based on this principle, an inhibitor of MRP1 can be

combined with another chemotherapeutic agent with superior potency against tumor cells in one formulation. In this instance, the inhibitor can impede the efflux activity of MRP1 to ensure that the other anticancer drug is bioavailable to yield the desired response and effect. Although some modulators of MRP1 have been identified in recent times, challenges like non-specific toxicity, low multidrug reversal effects, and undesirable off-target effects limit their effectiveness. Thus, there is the need to identify more potent, well-tolerated modulators with significant MDR reversal effects and limited non-specific toxicity.

In this present study, we successfully screened 30 drugs which consisted of both anticancer and FDA drugs using an In-cell ELISA assay to identify modulators of MRP1 in HEK293 MRP1-overexpressing cells. We identified 7 hit compounds (drugs that modulated MRP1 protein expression above 50%) representing 23.33% of the total compound screened. Three of the hit compounds; Epirubicin HCl, Felbamate, and Irinotecan increased the protein expression of MRP1. Four of the hit compounds; Vismodegib (GDC-0449), TG101348 (SAR302503), Amuvatinib, and SB743921 HCl decreased MRP1 protein expression in HEK293/MRP1 cells. Epirubicin HCl is an anthracycline that targets topoisomerase II [31]. Epirubicin HCl prevents DNA segregation and DNA synthesis by stabilizing the DNA – topoisomerase complex [32-36]. This antineoplastic agent is used in the treatment of breast cancer, and a known substrate of MRP1 [29]. Irinotecan is a derivative of Camptothecin that elicits its antitumor activity by inhibiting topoisomerase I (a nuclear enzyme that regulates the unwinding of DNA during replication) [37]. It is used in the treatment of metastatic colorectal cancer [38-41] and has been reported to be a substrate of MRP1 [28]. Vismodegib is a recently approved FDA drug that inhibits the hedgehog signaling pathway

[42]. This orally bioavailable small molecule is used in the treatment of locally advanced and metastatic unresectable basal cell carcinoma (BCC) [42]. Vismodegib is among the few small molecules that have been reported to inhibit the activity of MRP1 [30]. Since the impact of Epirubicin HCl, Irinotecan, and Vismodegib on MRP1 have already been reported in other studies, we focused on characterizing other hit compounds whose modulatory effects on MRP1 had not been reported.

The novel drugs identified in our initial screening included; SB743921 HCl, Amuvatinib, TG101348 (SAR302503), and Felbamate. Except for Felbamate; SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) based on their mode of action have been reported to be ATP competitive inhibitors. ATP competitive inhibitors are inhibitors that act by competing with ATP to block the activity of their targets [43]. These inhibitors are also known as Type 1 inhibitors. SB743921 HCl is a novel kinesin spindle inhibitor that elicits its function by impeding functional mitotic spindle formation in cell mitosis by hydrolyzing ATP [44], thereby regulating cell division. SB743921 HCl has been reported to show a strong inhibitory effect on ERK and AKT activity in chronic myeloid leukemia (CML) cells [45]. Although SB743921 HCl has been reported to have a strong inhibitory effect on tumor cells, its interaction with MRP1 as well as its effect on MRP1 activity and expression is yet to be reported. Amuvatinib on the other hand is an orally bioavailable small molecule that is reported to inhibit the activity of the MET receptor tyrosine kinase, c-KIT, and platelet-derived growth factor receptor (PDGFR) by competing with ATP for binding at the catalytic site [46]. This multi-targeted tyrosine inhibitor is currently in phase II clinical trials for the treatment of solid tumors [47, 48]. A recent study in our lab group that aimed at identifying novel inhibitors of MRP1 using a doxorubicin-based screening

assay revealed that Amuvatinib may inhibit MRP1 activity, however, its impact on MRP1 protein expression as well as its ability to reverse MRP1 mediated resistance has never been reported. TG101348 (SAR302503) is an orally bioavailable selective inhibitor of Janus-associated kinase 2 [49-51]. It elicits its inhibitory effect by competing with JAK2 for ATP binding, this results in JAK2 inactivation and subsequent inactivation of the JAK-STAT pathway. Thereby inducing apoptosis in tumor cells. This ATP competitive inhibitor of JAK2 was recently approved for the treatment of adult patients with intermediate-2 or high-risk primary or secondary myelofibrosis in the United States, and it is currently in phase III clinical trial for myelofibrosis treatment globally [49]. TG101348 (SAR302503) has also been reported to enhance the cytotoxic effect of imatinib (a well-known drug for the treatment) of Chronic myeloid leukemia (CML) in residual CML cells [52]. It is also reported to impede growth of Hodgkin lymphoma and mediastinal large-cell lymphoma in both in vitro and in vivo studies [53]. Despite the success of TG101348 (SAR302503) in treatment of tumor cells, its interaction with MRP1 is yet to be reported.

In this study, we demonstrated using In-Cell ELISA assay that the ATP competitive inhibitors; SB743921 HCl, Amuvatinib, TG101348 (SAR302503) significantly downregulated the protein expression of MRP1 in HEK293 MRP1-overexpressing cells. For the first time, we report that SB743921 HCl and Amuvatinib demonstrate a concentration and time-dependent activity in modulating MRP1 protein expression with greater significance observed at concentrations above 10 μ M and incubation periods above 24 hours. We also showed that TG101348 (SAR302503) exhibited significant downregulation of MRP1 protein expression at 10 μ M and after 48 hours incubation period. Felbamate, an antiepileptic FDA approved drug, was also reported for the first time

to significantly increased the expression levels of MRP1 in HEK293 MRP1-overexpressing cells in this study. Significant upregulation of MRP1 protein expression was observed at concentrations above 10 μ M and after 48 hours of incubation when cells were treated with felbamate. Although these drugs modulated MRP1 protein expression, further research must be conducted to evaluate if SB743921 HCl, TG101348 (SAR302503) are inhibitors of MRP1 and whether Felbamate is a substrate of MRP1. Moreover, further studies to determine the mechanism of interaction between these novel modulators and MRP1 would be a step in the right direction. Nonetheless, it is possible to speculate that SB743921 HCl may downregulate MRP1 protein expression by obstructing the PI3/Akt signaling pathway. This is because the PI3/Akt signaling pathway has been reported to modulate MRP1 expression in human acute myeloid leukemia [54], and SB743921 HCl has also been reported to strongly inhibit this pathway [45]. However, further studies are needed to verify the involvement of the PI3/Akt pathway in the modulatory effect of SB746921 HCl on MRP1.

We also evaluated the effect of these novel modulators on MRP1 efflux activity using the flow cytometry-based calcein accumulation assay. Our results demonstrate that SB743921 HCl, TG101348 (SAR302503) can strongly inhibit MRP1 mediated calcein efflux. Amuvatinib which was previously reported to inhibit MRP1 mediated doxorubicin efflux in small cell lung cancer cells (H69AR) [55], also inhibited MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells in this study. These results indicate that Amuvatinib may be able to inhibit efflux of several substrates of MRP1, making it an interesting therapeutic agent to explore in further studies. Contrastingly, we did not observe any significant inhibition in MRP1-mediated calcein efflux when cells were treated with

felbamate and compared to the no treatment control. This may be to the fact that the presence of felbamate upregulates MRP1 protein expression in these cells, as such increase the overall efflux of calcein as observed.

We also showed that the three novel modulators that decreased MRP1 protein expression in this study are also able to inhibit the growth of HEK293 MRP1-overexpressing cells at clinically achievable concentrations (Table 2.3). This observation may be in synchrony with their ability to decrease MRP1 protein expression in these cells. Felbamate (average IC_{50} – 47.23 μ M) on the other hand, was the least potent in inhibiting the growth of HEK293/MRP1 among the novel modulators identified. This may be because felbamate increases MRP1 protein expression as observed earlier in this study, thus it is easily effluxed by the transporter as such enhancing the survival of these cells and requiring a higher dose of drug treatment to achieve the half-maximal inhibitory effect. However, further research is needed to elucidate and verify the mode of interaction and action between felbamate and MRP1.

One of the major limitations of current modulators of MRP1 is the inability of these modulators to reverse MRP1-mediated resistance. Thus, we investigated the ability of our identified novel modulators (SB743921 HCl, Amuvatinib, TG101348 (SAR302503)) to reverse MRP1-mediated resistance against vincristine in HEK293 MRP1-overexpressing cells. Amuvatinib and TG101348 (SAR302503) were most effective in reversing MRP1-mediated resistance in HEK293 MRP1 overexpressing cells (Table 2.4). TG101348 (SAR302503) sensitized HEK293/MRP1 cells to the cytotoxic effects of vincristine, thereby reducing the average IC_{50} from 62.25 μ M to 22.26 μ M with a fold reduction from 20.51 to 7.16-fold resistance. Amuvatinib also reversed MRP1 mediated resistance towards

vincristine by decreasing the average IC_{50} from $62.25\mu\text{M}$ to $228.42\mu\text{M}$ with a fold reduction from 20.51 to 9.13-fold resistance. Contrastingly, we observed that SB743921 HCl enhanced resistance of HEK293 MRP1-overexpressing cells towards vincristine. Our results demonstrate that Amuvatinib and TG101348 (SAR302503) can reverse MRP1 mediated MDR in MRP1 overexpressing cells. It would be of interest to investigate the ability of Amuvatinib, TG101348 (SAR302503) to reverse MDR mediated by MRP1 and other ABC transporters in other cell lines.

Findings from this project indicate that the test compounds; SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) decreased MRP1 protein expression and its efflux activity, with Amuvatinib and TG101348 (SAR302503) exhibiting high potency in reversing MRP1-mediated MDR. Therefore these aforementioned drugs can be used in the development of combinatorial drug therapy with an anticancer drug that is potent in targeting and treating cancer cells but is a substrate of MRP1. As such in cancer cells overexpressing the MRP1 transporter, administration of such formulations allow the modulator to downregulate the MRP1 expression levels enabling the anticancer drug to accumulate at the appropriate concentration and be bioavailable to elicit its desired effect. These interventions would go a long way to aid in our fight against tumor chemoresistance mediated by MRP1. Our novel modulator, Felbamate which increased MRP1 protein expression in this study can also be used in the treatment of diseases whose etiology involves the down-regulation of MRP1 expression levels. For instance, Alzheimer's disease is a neurodegenerative disorder that is pathologically characterized by the accumulation of beta-amyloid peptide ($A\beta$) in the brain of its patients [56]. Studies by Krohn and his colleagues revealed that deficiency of MRP1/ABCC1 in mice models that

expressed Swedish human A β precursor protein (APP^{swe}) and mutant presenilin-1 (PS1) (APP/PS1 mice x Abcc1^{-/-}) resulted in elevated levels of cerebral beta-amyloid peptide (A β) but did not affect the expression levels of enzymes responsible for the production of A β from APP [57]. However, treatment with an MRP1 inducer, thiethylperazine resulted in decreased A β levels in APP/PS1 mice brains. These results demonstrated the role of MRP1 in the clearance of A β and its sequential accumulation in the brain. Thus in disease states like Alzheimer's disease, drugs with a high potency of inducing and increasing MRP1 expression levels are desirable. Therefore, our novel modulator, Felbamate which increased MRP1 protein expression in HEK293 MRP1 overexpressing cells in this study and is currently used for the treatment of Epilepsy can be a good candidate for treating such disorders. It would be a step in the right direction to investigate how Felbamate can be used in targeting and treating disorders in which MRP1 expression levels are down-regulated.

In summary, we investigated the modulatory effect of a unique set of drugs on MRP1 protein expression. We identified four novel modulators of MRP1 protein expression in HEK293 MRP1-overexpressing cells. We report for the first time that novel ATP competitive inhibitors; SB743921 HCl, Amuvatinib, TG101348 (SAR302503) downregulate MRP1 protein expression and activity. Findings from our work suggest that the identified modulators may limit toxicity and increase the effectiveness of overcoming MRP1 mediated MDR. Thus the drugs can be explored in combinatorial drug therapy aimed at targeting tumors with the MDR phenotype conferred by MRP1 overexpression. Drugs that showed little to low modulatory effect on MRP1 in our initial screening may have a lower risk of being interfered by MRP1-mediated MDR.

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Chapter 3

Tie2 kinase and mTOR targeted agents modulate MRP1 activity in MRP1-overexpressing cells

Abstract

Chemotherapy is the only systemic treatment for many cancers. Overexpression of MRP1 in cancer cells facilitates the efflux of administered chemotherapeutic drugs, thereby reducing their intracellular drug concentration and bioavailability. This results in tumor cells becoming unresponsive and resistant to therapeutic agents. The development of multidrug resistance in cancer cells leads to increased risk of treatment failure and reduced survival chances of cancer patients. Finding ways of regulating the activities of MRP1 in overexpressed cells is of great pharmacological importance. We report that Tie2 kinase inhibitor and mTOR inhibitor, Everolimus modulate MRP1 activity in MRP1-overexpressing cells. Tie2 kinase inhibitor and Everolimus decreased MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells. However, these compounds did not affect the protein expression of MRP1 in HEK293 MRP1-overexpressing cells. In resistance reversal studies, Tie2 kinase inhibitor and Everolimus reversed MRP1-mediated resistance towards vincristine in these cells. Overall, data from this study indicates Tie2 kinase inhibitor and Everolimus holds great potential for the development of therapeutics targeting MRP1-mediated multidrug resistance.

Keywords: ABC transporters; multidrug resistance; MRP1; ABCC1; MRP1 modulators; anticancer drug; drug profiling; drug-transporter interactions, Tie2, mTOR

1) Tie2 Kinase Inhibitor (PubChem CID: 23625762); 2) Everolimus (PubChem CID: 6442177)

1.0 Introduction

Cancer is a global public health challenge with high morbidity and mortality. The potential of tumor cells to develop resistance to mechanistically and structurally discrete chemotherapeutic agents has become one of the major hindrances to the chemotherapy regime. This phenomenon is described in clinical cancer research as Multidrug resistance (MDR). The ATP binding cassette (ABC) transporters have been reported to be key players in the MDR development of several carcinomas. This superfamily of transporters is responsible for the efflux of a wide range of substrates in eukaryotes. These substrates include xenobiotics, toxins, drugs, and their metabolites [1]. Due to the essential role played by ABC transporters, cancer cells take advantage of their pivotal function to ensure their survival against administered anticancer drugs. This they achieve by overexpressing ABC transporters like P-glycoprotein (P-gp), Multidrug resistance protein 1 (MRP1), and Breast cancer resistance protein (BCRP). As such administered chemotherapeutic drugs are challenged by the efflux activity of these transporters as they serve as the first line of defense in tumor cells. The removal of drugs and their metabolites by the overexpressed ABC transporters across the plasma membrane has been associated with decreased bioavailability and reduced therapeutic efficacy of anticancer drugs on tumor cells.

ABC transporters are a type of ATP-binding cassette pumps encoded by the ABC genes [2]. This superfamily of transporters is grouped into seven subfamilies, subfamilies A-G in humans. Most ABC transporters are characterized by a core unit consisting of membrane-spanning domains (MSDs) which consist of six hydrophobic α -helices, and nucleotide-

binding domains (NBDs). The NBDs form the powerhouse of the transporter, as it is responsible for ATP binding and hydrolysis for the generation of energy. The MSDs use the energy generated to facilitate substrate recognition and substrate translocation across the plasma membrane [3]. In recent times, the overexpression of the MRP1 transporter has been associated with the development of MDR in several carcinomas including ovarian cancer [4], childhood neuroblastoma, acute lymphoblastic leukemia[5]. MRP1 is a member of the ABCC subfamily of ABC transporters. It is encoded by the gene ABCC1 [6]. MRP1 localizes at the basolateral membrane and is expressed in the epithelial cells of organs like the adrenal gland, testes, kidney, gastrointestinal tract, and at pharmacological sacred sites like the blood-brain barrier, blood-cerebrospinal fluid barrier, and the blood-testes barrier [7, 8]. Substrates of MRP1 include; heavy metals, leukotrienes, prostaglandins, glutathione (GSH), and glucuronide-conjugates of steroids [9, 10]. MRP1 also transports drugs from various drug classes including; anthracyclines (doxorubicin), folate-based antimetabolites (methotrexate), antivirals (saquinavir), antibiotics (difloxacin), plant alkaloids (etoposide, paclitaxel) among others [11]. Due to the critical role MRP1 plays, and its ability to interact with a wide range of drug families; its overexpression has been a destructive tool used by cancer cells to efflux administered drugs out of the intracellular space in cancer patients rendering these drugs less bioavailable to exert the desired effect. This phenomenon has created a huge barrier to the effectiveness of chemotherapy and reduced survival rates of cancer patients [12].

Finding ways of regulating the activities of MRP1 in overexpressed cells is of great pharmacological essence. One approach is to curb this cancer is to completely shut down the efflux transporter, MRP1. Although this strategy may seem laudable; it would be

suicidal for the cells as MRP1 is a key regulator of physiological homeostasis in cells. MRP1 aids to maintain the GSH/GSSG (Oxidized GSH) ratio to ensure redox homeostasis in cells [13]. Moreover, the transport activity of MRP1 helps to prevent the accumulation of toxicants and the buildup of estrogen-like compounds in the testes which aid to prevent testicular feminization and protect developing spermatozoa [8, 14]. MRP1 also functions to protect the heart by facilitating the efflux of toxic products of oxidative stress from the mitochondria and cardiomyocytes [15]. From the aforementioned roles played by MRP1 in maintaining physiological balance in cells, the complete shutdown of the transporter would cause significant perturbations in the physiological balance of cells. An alternative approach that can be utilized to regulate the activity of the transport in tumor cells without disturbing the physiological equilibrium, is to use the biochemical modulation toolbox. Biochemical modulation involves the use of therapeutic agents including small molecules to achieve selective manipulation of tumor cell metabolism or signal transduction pathways to ensure the more selective response of tumor cells to the action of anticancer drugs [16].

The phosphoinositide 3 kinases/Akt (PI3K/Akt) signal transduction pathway has been reported as one of the cellular pathways that regulate the expression levels of MRP1 [17, 18]. PI3K is a lipid kinase involved in the regulation of biological events such as migration, metabolism, survival, and also activates a lot of downstream proteins [19]. Serine (Ser)/Threonine (Thr) kinase also known as Akt or protein kinase B regulate the expression and activity of numerous proteins including MRP1 [20, 21]. This pathway has been reported to be involved in MDR observed in breast cancer, lung cancer, ovarian cancer, melanoma, and hepatocellular carcinoma [18-23]. As such, this pathway offers a great avenue for the development of novel strategies to target MRP1 in MDR. Moreover,

exploring the impact of activators and downstream effectors of this pathway on MRP1 activity and expression would aid in identifying potential therapeutic targets for the development of more efficacious and specific therapeutics for targeting MRP1 mediated MDR and treating cancer patients [18]. Tunica interna endothelial cell (Tie2) tyrosine kinase receptor is an endothelial cell-specific receptor which activates the PI3/Akt pathway in normal endothelial cells [22]. It has been associated with the extravascular compartment of several tumors such as inflammatory breast cancer, leukemia, gastric, and thyroid tumors [23]. mTOR (mammalian target of rapamycin) on the other hand is a serine/threonine kinase that modulates the diverse nutritional and environmental cues like amino acids, growth factors, cellular stress among others in cells [24]. Moreover, mTOR is also reported to function downstream the PI3K/Akt signaling pathway and has been involved in the etiology of several cancers [24, 25]. In this study, we investigated the effect of a novel inhibitor of Tie2 (Tie2 kinase inhibitor) and mTOR inhibitor (Everolimus) on MRP1 activity and protein expression in MRP1-overexpressing cells using established methods. We also determined the ability of these drugs to reverse MRP1-mediated resistance in MRP1-overexpressing cells.

2.0 Materials and methods

2.1 Chemicals

Test compounds were procured from APEX BIO Technology LLC (Houston, TX). MK571 was acquired from APEX BIO Technology LLC (Houston, TX), thiazolyl blue tetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO).and Calcein acetoxymethyl ester (Calcein-AM) was obtained from Corning Life Sciences (Corning, NY).

2.2 Cell lines and cell culture

HEK293/pcDNA3.1 and HEK293/MRP1 cells were a kind gift from Dr. Suresh V. Ambudkar (NIH, Bethesda, MD) respectively. Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) was used to grow the HEK293 cell lines. Cell lines were cultured in a humidified incubator maintained at 5% CO₂ and 37 °C. This incubation condition was retained in all subsequent cell culture procedures.

2.3 Flow cytometry-based calcein accumulation assay

The effect of Tie2 kinase inhibitor and Everolimus on MRP1 mediated efflux of calcein-AM was ascertained using flow cytometry. HEK293 MRP1-overexpressing cells were prepared in serum-free medium at a cell density of 7×10^5 cells/ml and treated with 10 μ M of test compounds for 10 minutes at 37 °C. Cells were then treated with calcein-AM

(0.25 μ M) for 30 minutes. Final DMSO concentration was maintained at 0.1% (v/v). MRP1 mediated efflux activity was stopped using ice-cold PBS buffer (3 ml). Cells were collected, washed twice with PBS, and resuspended in a cold PBS buffer containing 1% paraformaldehyde. Intracellular calcein-AM fluorescence was detected using BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) with excitation and emission at 480 nm and 533/30 nm respectively. Fluorescent intensity was determined as a mean of 10000 events. Treatments were done in duplicates and experiments were performed in three independent studies.

2.4 Determination of concentration-dependent activity on MRP1 protein expression using western blot assay.

HEK293/MRP1 and HEK239/pcDNA 3.1 (parental control) cells were seeded at 7×10^5 cells per well in 6-well plates with DMEM containing 10% FBS and incubated for 24 hours. Cells were treated with varying concentrations of drugs; 5 μ M, 10 μ M, 20 μ M, and 0.1% DMSO for controls, and incubated for 48 hours at 37 °C. At the end of the incubation period, the spent media was removed and cells were rinsed with 1000 μ l of PBS. The cells were lysed with lysis buffer containing radioimmunoprecipitation assay buffer (RIPA Buffer, Thermo Fisher Scientific, Waltham, MA) supplemented with 1 \times halt protease inhibitor cocktail (to inhibit the activity of cell proteases). Protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin as standard. Proteins (20 μ g) were loaded in each well and sodium dodecyl sulfate (SDS) page electrophoresis was conducted on 8.0%

mini SDS gels, after which proteins were transferred to Immobilon PVDF membranes (EMD Millipore, Burlington, MA) for 4 hours. The membrane was blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline -Tween 20 (TBS-T,0.1%), and washed with TBS-T (0.1%; 3× per 10mins). Followed by incubation (4 °C, overnight) with monoclonal MRP1 antibody [EPR21062](ab233383) and anti-GAPDH antibody (Thermo Fisher Scientific, AM4300) dissolved in TBS-T (0.1%) with BSA (1%) at 1:250 and 1:1000 dilutions respectively. The membrane was washed with TBS-T (0.1%, 3× per 10mins) and incubated with secondary antibody using horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) (Thermo Fisher Scientific) for alpha-tubulin detection, and horseradish peroxidase-conjugated goat-anti-rabbit IgG(H+L) (Thermo Fisher, Scientific) for MRP1 detection. Target proteins were detected using Western blotting luminol reagent (Santa Cruz Biotechnology, sc-2048). Signals were quantified using Image Studio Lite version 5.2 and normalized by using the intensity of the corresponding protein band relative to the GAPDH band.

2.5 In vitro cytotoxicity assay

The cytotoxicity effect of the test compounds was investigated by using the MTT colorimetric assay. HEK293/MRP1 and HEK293/pcDNA3.1 cells were seeded in 96 well plates at a cell density of 5×10^3 per 100 ul of culture medium and incubated overnight. Cells were treated with varying concentrations of the test compound diluted in medium and were incubated for 72 hours. At the end of the incubation period, 100 ul of spent media was carefully decanted. MTT (0.5 mg/ml) treatment was conducted for 4 hours. Dissolution of formazan crystals was done by the addition of 100 ul of 15% SDS containing 10 mM

HCl, absorbance was read at 570 nm using a Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicates and repeated in two independent experiments.

2.6 MDR reversal activity of test compounds

Reversal of resistance effect of Tie2 kinase inhibitor and Everolimus was determined using MTT assay. Cells were seeded at a cell density of 5×10^3 per 100 μ l of culture medium in 96 well plates and incubated for 24 hours at 37°C to allow cells to attach. Drug treatment was then conducted by treating cells with 150 μ l of test compounds at selected non-cytotoxic concentrations. The addition of varying concentrations of vincristine (50 μ l) was conducted after an hour. MTT treatment (0.5 mg/ml) was conducted for 4 hours. The formazan crystals were dissolved by the addition of 100 μ l of 15% SDS containing 10 mM HCl and absorbance at 570 nm was recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were done in triplicates, and experiments were performed in two independent studies.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism TM software (GraphPad Software version 8.4.3, San Diego, CA, USA). The differences between mean values were analyzed using linear mixed model analysis. Sidak correction was applied for multiple comparisons, statistical testing was performed at a 5% level of significance.

3.0 Results

3.1 Effect of test compounds on MRP1 efflux activity

Calcein-AM is a common substrate of MRP1 and is widely used to study MRP1 mediated efflux. When the acetoxymethyl ester (AM) moiety of calcein is cleaved by esterases in cells, calcein becomes fluorescent. The impact of MRP1 efflux activity was investigated using flow cytometry. The structures of Tie2 kinase inhibitor and Everolimus are shown in Figure 3.1. As shown in Figure 3.2, HEK293 MRP1-overexpressing cells in the absence of MK571 showed very low retention of calcein whilst in the presence of MK571 (25 μ M), the accumulation of calcein strongly increased by 3.85 fold. HEK293/MRP1 cells in the presence of Tie2 kinase inhibitor and Everolimus also increased the accumulation of calcein in the cells approximately by 3.96-fold and 3.83-fold respectively. The fold increase in calcein accumulation in the presence of the test compounds was very comparable to the positive inhibitor, MK571. This finding suggests that Tie2 kinase inhibitor and Everolimus may decrease MRP1 efflux activity leading to the increase in calcein observed.

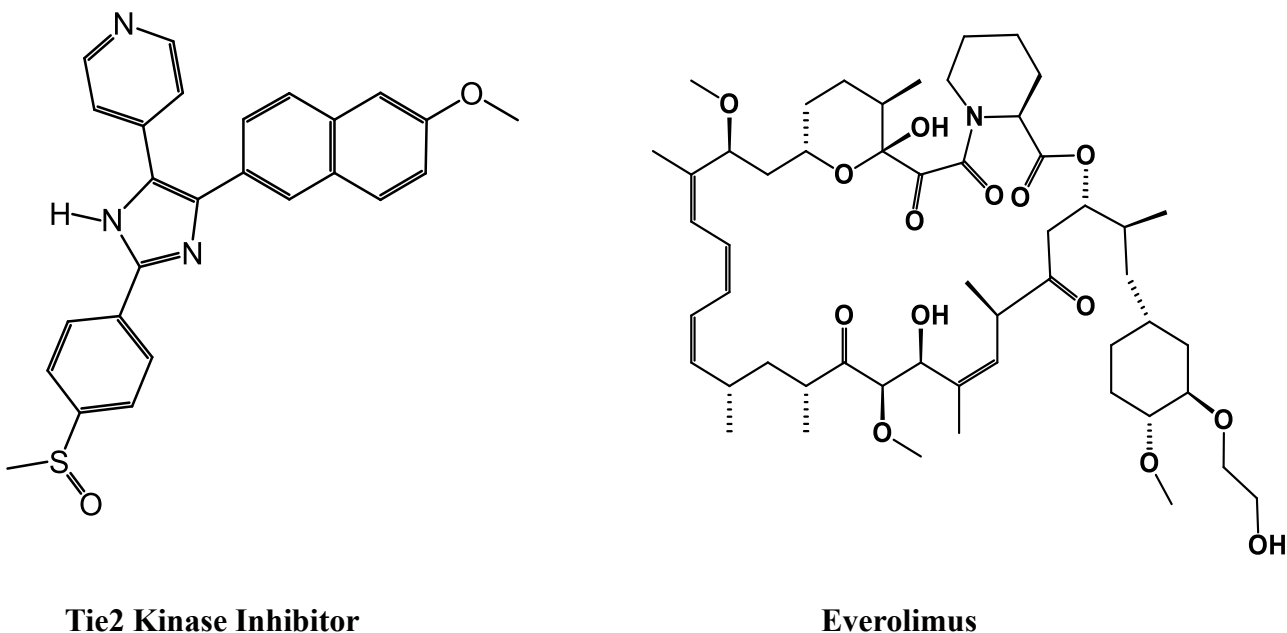


Figure 3. 1 Chemical structures of test compounds

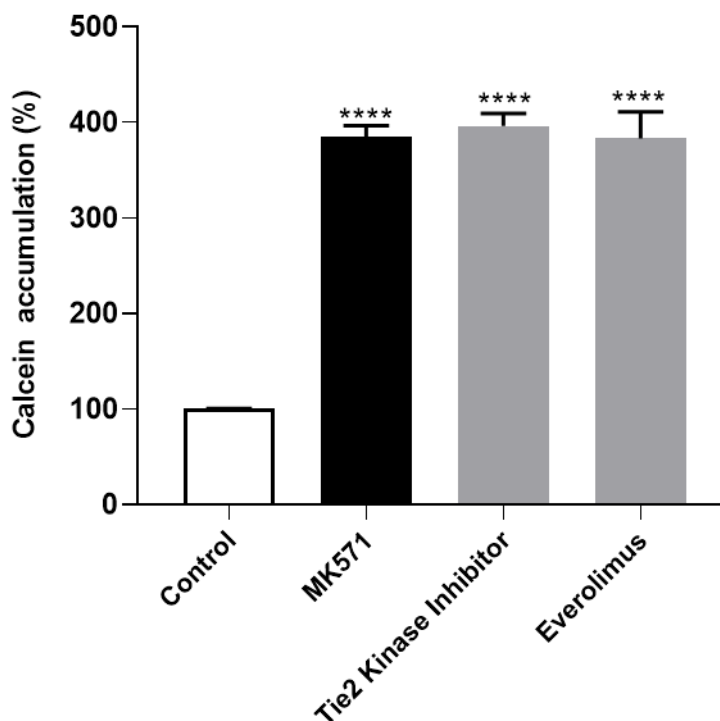


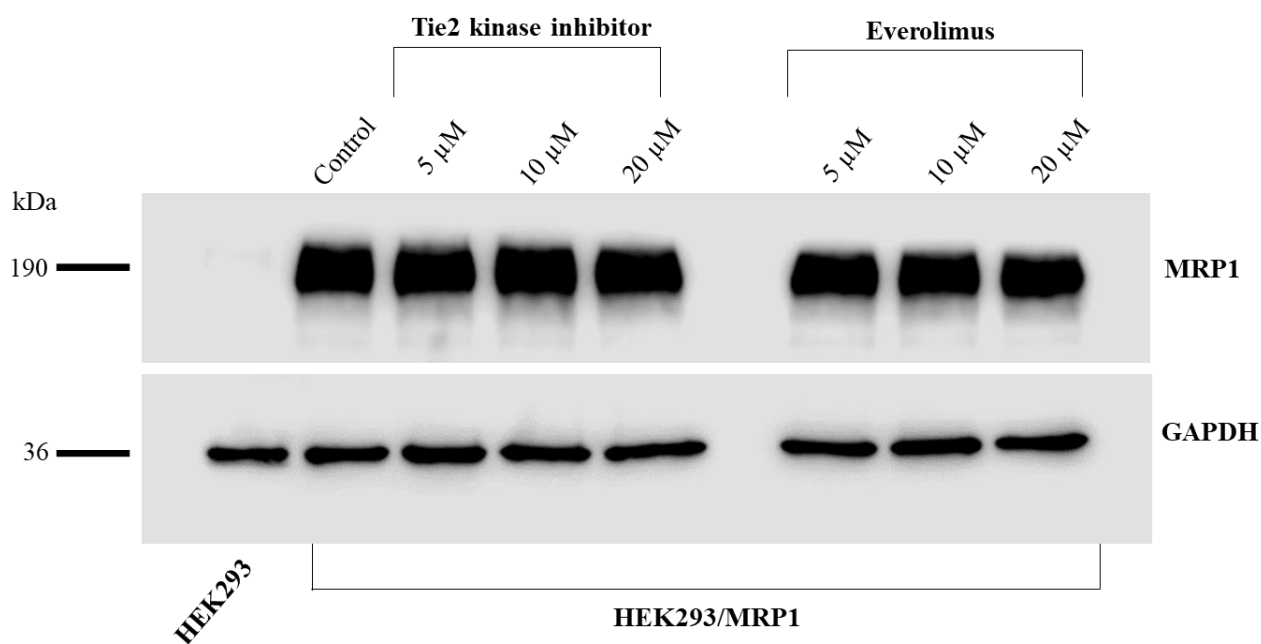
Figure 3.2 Effect of test compounds on MRP1 efflux activity.

HEK293/MRP1 cells were treated with 10 μ M of the test compounds and 25 μ M of MK571 (positive control) for 10 minutes at 37 $^{\circ}$ C before treatment with 0.25 μ M calcein-AM for an hour, Flow cytometric measurements of intracellular calcein-AM was conducted at 488 nm and 533/30 nm for excitation and emission wavelengths, respectively. Experiments were done as duplicates in three independent experiments and presented as mean \pm S.E.M. Data was analyzed using a linear mixed model and Sidak post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001 ; ****P < 0.0001 compared to control.

3.2 Effect of test compounds on MRP1 protein expression levels

To determine the influence of Tie2 kinase inhibitor and Everolimus on MRP1 protein expression, we conducted a western blot assay using varying concentrations of the test compounds (5 μ M, 10 μ M, 20 μ M) (Figure 3.3A). The protein expression levels of MRP1 was not significantly impacted when HEK293 MRP1-overexpressing MRP1 were treated with 5 μ M, 10 μ M, and 20 μ M of test compounds and compared to the protein expression levels of the control (Figure 3.3B).

[A]



[B]

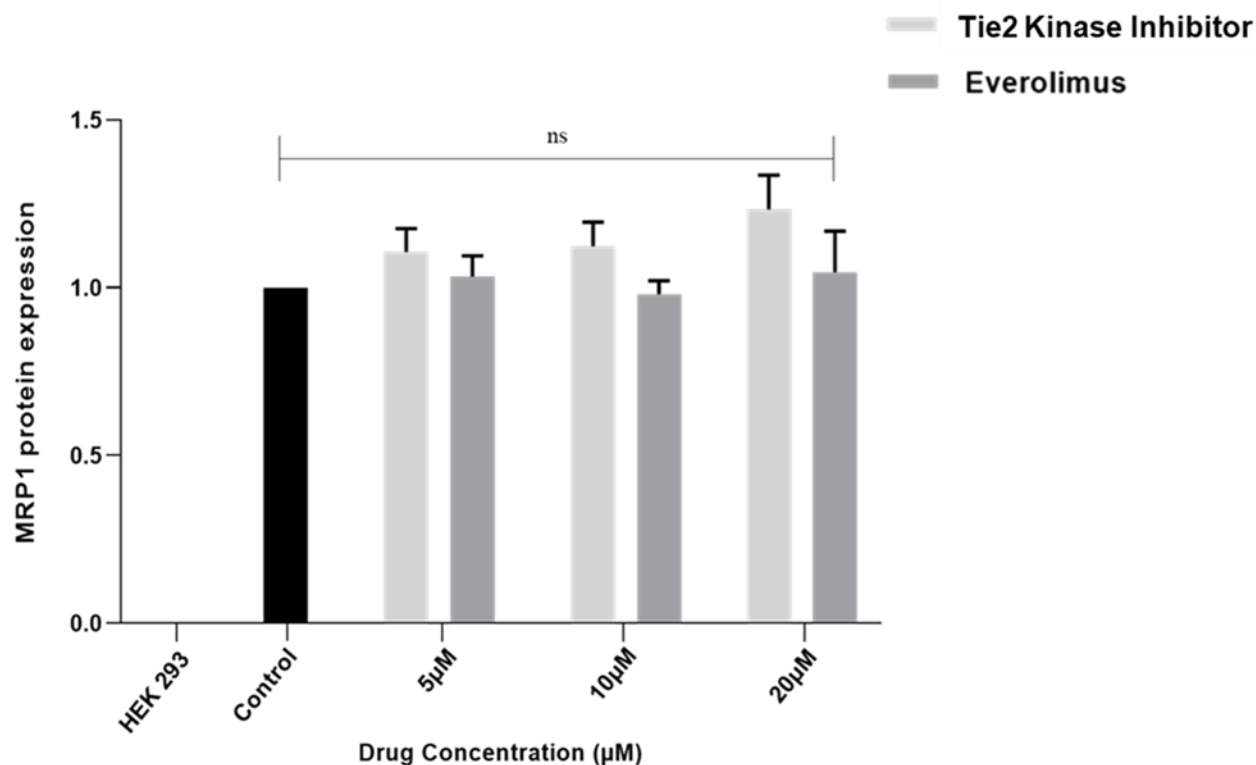


Figure 3.3 Effect of test compounds on MRP1 protein expression.

[A] Shown are representative western blots of whole-cell lysates (20ug of protein/lane) prepared from HEK293/pcDNA3.1 and HEK293/MRP1 cells treated with varying concentrations of test compounds (5 μM , 10 μM , 20 μM), and 0.2% DMSO (controls) for 48 hours. MRP1 proteins were detected with monoclonal anti MRP1 antibody [EPR21062](ab233383, Abcam) and anti-GAPDH antibody (Sigma-Aldrich) at a dilution of 1:250 and 1:1000 respectively. Secondary antibodies; mAb - anti-rabbit and mAb-anti-goat were used at a dilution of 1:1000. Whole cell lysates were run on 8% SDS gel for one hour before being transferred to the pretreated PVDF membrane. Three independent experiments were conducted, and data presented as mean \pm S.E.M. [B] Protein band density was analyzed using the Image Studio Lite (LI-COR Biotechnology) software and corrected for uneven sample loading and transfer using GAPDH as the loading control. Data was analyzed using a linear mixed model and Sidak post hoc test.

3.3 Impact of test compounds on MRP1-mediated resistance

Although recent advancement in chemotherapy has resulted in improved survival rates of cancer patients, multidrug resistance (MDR) in tumor cells possess a great limitation on its success. Thus, we investigated the ability of Tie2 kinase inhibitor and Everolimus to reverse MRP1 mediated resistance towards vincristine. Vincristine is a commonly used anticancer drug, that is used in the treatment of several carcinomas. Unfortunately, MRP1 is reported to mediate the resistance of tumor cells to vincristine. As presented in Figure 3.4, HEK293 MRP1-overexpressing in the absence of MRP1 (solid red) demonstrated very high resistance to vincristine cytotoxic effect giving a fold resistance of 19.96 folds (Table 3.1). The parental cell line HEK293/pcDNA3.1(dotted blue) which does not overexpress MRP1 showed low resistance to vincristine (Figure 3.4). In the presence of MK571 (25 μ M), HEK293/MRP1 cells became more sensitive to the cytotoxic effect of MK571 resulting in reducing the fold resistance observed in these cells to 3.75 fold resistance (Table 3.1). Our test compounds also reversed MRP1 mediated resistance towards vincristine in HEK293/MRP1 cells. Everolimus (dotted purple) and Tie2 kinase inhibitor (dotted orange) decreased vincristine resistance to 7.01-fold resistance and 8.91-fold resistance respectively (Figure 3.4, Table 3.1).

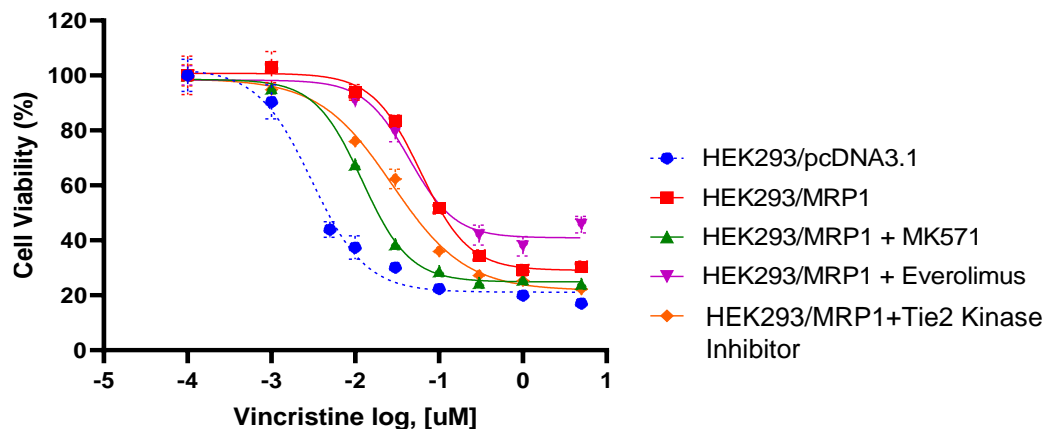


Figure 3.4 Effect of Tie2 kinase inhibitor and Everolimus on drug sensitivity of HEK293 MRP1-overexpressing cells towards vincristine.

Cells were treated with increasing concentrations of vincristine in the absence or presence of a non-cytotoxic concentration of test compounds. MK571 (25 μ M) served as the positive control. MTT assay was conducted to determine cell viability after 72 hours. Data is representative of three independent experiments and expressed as mean \pm SEM.

Table 3.1 Effect of selected Tie2 Kinase Inhibitor and Everolimus on the IC₅₀ values of vincristine in HEK293/MRP1 cells

Cell line/Treatment	IC ₅₀ ^a (nM)	
	Vincristine	Fold resistance ^b
HEK293/pcDNA3.1	3.11 ± 0.23	1.00
HEK293/MRP1	62.02 ± 4.33	19.96
HEK293/MRP1 + MK571 [25 μM]	11.65 ± 1.79	3.75
HEK293/MRP1 + Everolimus [5 μM]	21.78 ± 3.34	7.01
HEK293/MRP1 + Tie2 Kinase Inhibitor [1] μM	27.67 ± 1.09	8.91

^a Mean ± SEM of at least three independent experiments performed in triplicates.

^b Fold resistance determined by dividing the IC₅₀ value for each treatment by the IC₅₀ value of HEK293/pcDNA3.1 with vincristine alone.

4.0 Discussion

Chemotherapy is the standard treatment for systemic cancer in both metastatic and locally advanced carcinomas. Although chemotherapy has been beneficial for treating cancer, patients perpetually experience recurrence after therapy and exhibit a multidrug-resistant phenotype [26]. The development of the multidrug-resistant phenotype in tumor cells is resulted in tumor cells becoming unresponsive and insensitive to a variety of chemotherapeutic agents [27]. This phenomenon is termed Multidrug resistance (MDR). MDR has become one of the major challenges to the success of chemotherapy. The overexpression of ABC transporters like MRP1 has been associated with the development of MDR in tumor cells. MRP1 like other ABC efflux transporters utilizes energy from ATP hydrolysis to facilitate the efflux of its substrate across biological membranes. Overexpression of MRP1 has been associated with increased drug efflux resulting in reduced intracellular effective drug concentration of a wide range of anticancer agents (doxorubicin, vincristine, methotrexate), thereby contributing to MDR and elevated chemotherapeutic failure [17, 28]. Recent studies have aimed at identifying novel strategies to modulate the structure and function of MRP1 in order to regulate its activity in the MDR of tumor cells. Some modulators like MK571, ONO-1078, probenecid, indomethacin have been identified in recent times [29], yet some dreadful side effects and elevated patient toxicities due to these modulators have limited the possibility of translating these promising therapeutics from the bench side to the clinic. Thus there is the need for more potent and safer MRP1 modulators.

Regulating effectors of signaling pathways that have been reported to be associated with MDR can aid discover new and ideal strategies for developing targeted therapeutics for

MRP1-mediated MDR. In this study, we successfully explored the effect of novel Tie2 kinase inhibitor and mTOR inhibitor, Everolimus, on MRP1 activity and expression in HEK293 MRP1-overexpressing cells. To the best of our knowledge there no evidence indicating the interaction between Tie2 kinase inhibitor and MRP1. Thus we report for the first time that Tie2 kinase inhibitor downregulates MRP1 mediated calcein efflux in HEK293 MRP1- overexpressing cells. Tie2 kinase inhibitor is an optimized compound of SB203580 which is highly selective for the Tie2 tyrosine kinase receptor [30]. Tie2 kinase inhibitor is reported to show inhibitory activity against Tie2 and also inhibit angiogenesis in MOPC-315 plasmacytoma xenograft model [30]. Tie2 tyrosine kinase receptor is also known as angiopoietin-1 receptor or Tek. The Ang-Tie2 system is reported to play roles in endothelial cell survival and proliferation, vascular plasticity, and angiogenesis [31]. It is also reported to activate the Akt in the PI3/Akt signaling pathway which is known to modulate the activity of MRP1 expression and activity. Tie2 has been implicated in several tumors [23]. For instance, studies by Martin et al. showed that Tie2 signaling is associated with MDR in human glioma cells by upregulating ABC transporters [23]. Thus the downregulation of MRP1 efflux activity by Tie2 kinase inhibitor observed in this present study may be mediated via disruption of the Tie2 signaling pathway and subsequent deactivation of the PI3/Akt pathway signaling. However, it will be of great interest to ascertain the mechanism of action and interaction of the Tie2 kinase inhibitor and the MRP1 inhibitor. In addition to downregulating the MRP1 efflux activity, Tie2 kinase inhibitor also strongly reversed MRP1- mediated resistance in HEK293 MRP1-overexpressing cells. Treatment of cells with Tie2 kinase inhibitor reversed resistance against vincristine by decrease the average IC_{50} from 62.02 μ M (vincristine only treatment)

to 27.67 μM with the corresponding reduction in fold resistance from 19.96-fold resistance to 8.91-fold resistance. As such, Tie2 kinase inhibitor may be a potent candidate for modulating MRP1 mediated chemoresistance in tumor cells. Treatment of HEK293 MRP1-overexpressing cells with 5 μM , 10 μM , 20 μM of Tie2 kinase inhibitor did not yield any significant alteration on MRP1 expression in this cell line. Studies by Martin and his colleagues also reported that Tie2 upregulation did not have any significant impact on mRNA levels of MRP1/ABCC1 in human glioma cells [26]. Thus considering our results from this study and the observation reported from the previous study [26], modulation of Tie2 signaling may affect MRP1 activity but not modulate its expression levels. However, it would be enlightening to investigate the effect of Tie2 kinase inhibitor on MRP1 expression levels in other cell lines. Findings from this study suggest that the Tie2 kinase receptor may be a potential molecular target for the development of efficacious and specific therapeutics for targeting and treating MRP1 mediated MDR.

The use of Rapamycin and rapalogs (inhibitors of mTOR) have proven promising clinical efficacy in chemotherapy. In this study, we demonstrated that rapamycin analog, Everolimus down-regulates the MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells. Everolimus elicits its function by binding to cyclophilin, FKBP-12 which in turn binds to mTOR and forms the mTORC1 complex when it is associated with raptor and MLST8 and inhibits downstream signaling [32]. mTORC1 complex is implicated in the regulation and ordination of cell cycle progression, growth, and metabolism [33-37]. mTORC1 is a downstream effector of the PI3/Akt pathway which has been reported to modulate ABC transporters including MRP1. Thus Everolimus may elicit its effect on MRP1 by modulating this pathway. In this present study, we demonstrate that

Everolimus can also reverse MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells. Everolimus in this present study successfully decreased the fold resistance of HEK293 MRP1-overexpressing cells towards vincristine from 19.96-fold resistance to 8.91-fold resistance. Although Everolimus has been previously reported to downregulate MRP1 expression levels in cisplatin-resistant gastric cancer cell line [38], treatment of HEK293 MRP1-overexpressing cells with 5 μ M, 10 μ M, 20 μ M of Everolimus did not significantly alter protein expression levels in this study. It is not uncommon to observe cell line-specific effects of a drug, moreover, the HEK293 MRP1-overexpressing cells were generated by transfection as such these cells can possess very different membrane dynamics and molecular profiles.

In summary, we successfully demonstrated the effect of novel Tie2 kinase inhibitor and mTOR inhibitor, Everolimus, on MRP1 activity and expression on HEK293 MRP1-overexpressing cells. We showed that these drugs downregulate MRP1 activity and can reverse MRP1-mediated resistance. Thus these therapeutic agents are good candidates for developing combinatorial therapeutic strategies for the modulation of MRP1 mediated tumor chemoresistance.

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Chapter 4

Identification of FDA approved drugs as modulators of Multidrug Resistance Protein 2 (MRP2/ABCC2) expression levels in MRP2-overexpressing cells

Abstract

Multidrug Resistance Protein 2 (MRP2) is an ATP-dependent transmembrane protein that plays a pivotal role in the efflux of a wide variety of physiological substrates across the plasma membrane. Several studies have shown that MRP2 can significantly affect the absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles of many therapeutic drugs as well as chemicals found in the environment and diet. This transporter can also efflux newly developed anticancer agents that target specific signaling pathways and are major clinical markers associated with multidrug resistance (MDR) of several types of cancers. MDR remains a major limitation to the advancement of the combinatorial chemotherapy regimen in cancer treatment. In addition to anticancer agents, MRP2 also reduces the efficacy of various drug classes such as antivirals, antimalarials, and antibiotics. The unique role of MRP2 and its contribution to MDR makes it essential to profile drug-transporter interactions for all new and promising drugs. Thus, this current research seeks to identify modulators of MRP2 expression levels using cell-based assays. A unique recently-approved FDA library (372 drugs) was screened using a high throughput In-Cell ELISA assay to determine the effect of these therapeutic agents on protein expression levels of MRP2. A total of 49 FDA drugs altered MRP2 expression levels by more than 50% representing 13.17% of the compounds screened. Among the identified hits, fifty-four (54) drugs increased expression levels whereas 12 drugs lowered expression

levels of MRP2 after drug treatment. Our findings from this initial screening showed that modulators of MRP2 peregrinates multiple drug families, and signifies the importance of profiling drug interactions with this transporter. Data from this project provides essential information to improve combinatorial drug therapy and precision medicine as well as reduce drug toxicity of various cancer chemotherapies.

Keywords: ABC transporters; multidrug resistance; MRP2; ABCC2; MRP2 modulators; FDA approved drug; In-Cell ELISA; drug profiling; drug-transporter interactions

1.0 Introduction

MRP2 (ABCC2) is a member of the ATP-binding cassette superfamily of transporters, MRP1 and MRP2 are homologous members of this superfamily [1]. In humans, it is encoded by the gene ABCC2 [2]. Structurally, MRP2 is a 190-kDa membrane protein consisting of 1545 amino acids. The predicted membrane topology consists of 17 transmembrane spanning domains (MSD0, MSD1, MSD2) which are linked together by conserved linker regions, and two highly conserved nucleotide-binding domains (NBD1 and NBD2) that serve as substrate – binding sites [3] as shown in Figure 4.1.

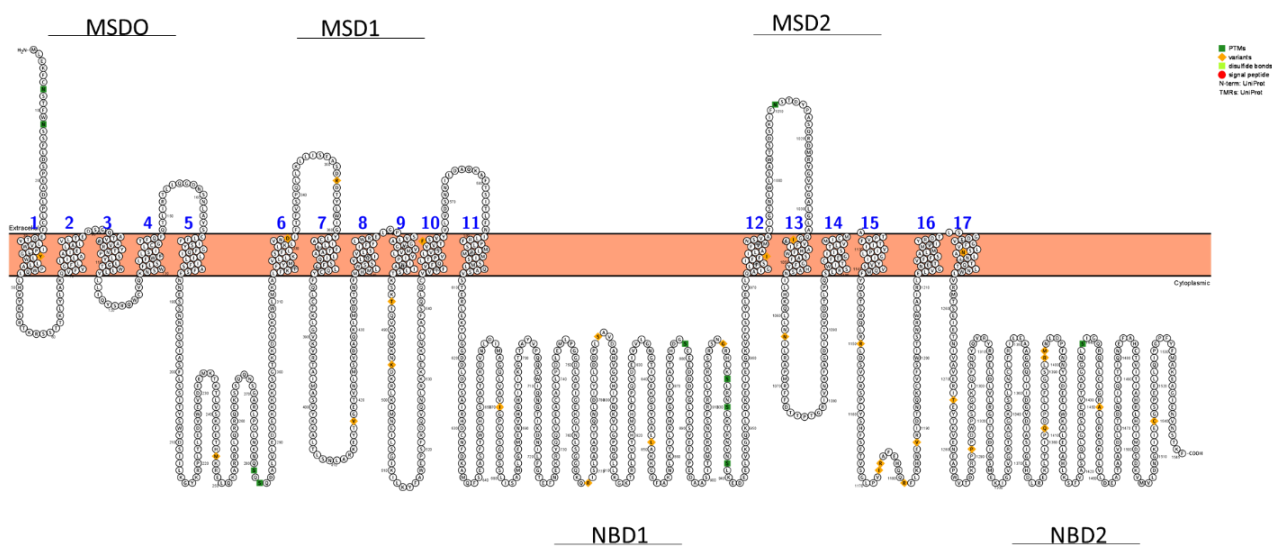


Figure 4.1: The full-length model of the MRP2 protein

The model was generated using the open-source tool Protter(<http://wlab.ethz.ch/protter/start/>)

Whilst MRP1 localizes at the basolateral membrane of endothelial cells, MRP2 localizes at the apical membrane of polarized cells of hepatocytes, renal proximal tubular cells, enterocytes, and syncytiotrophoblasts of the placenta [4]. It is known to play critical roles in the export of conjugated bile salts in the liver as well as transport of physiological important substrates such as glutathione-S-conjugates, 17-beta-glucuronosyl estradiol, leukotriene C4 [5]. Overexpression of MRP2 is associated with multidrug resistance of tumor cells such as hepatocellular, ovarian, colorectal, lung, breast, and gastric carcinomas [6], where it pumps drug conjugates and drug complexes across the plasma membrane into the extracellular space [5]. Thus affecting the bioavailability and efficacy of anticancer drugs like cisplatin and methotrexate. Aside cancer drugs, MRP2 also affects the efficacy of a broad spectrum of drug classes including HIV drugs (lopinavir), antibiotics(ampicillin, azithromycin), and antihypertensives(Olmesartan, Temocaprilate) [7]. With MDR being a major impediment to the chemotherapy regime and the overexpression of the MRP2 transporter being a major factor in this phenomenon, it is of great clinical interest to find ways of addressing this cancer. Two main approaches have been proposed by researchers. One of which, is to completely block the efflux or pump activity of the transporter in these cells [8]. However, this approach would be destructive to the cells since it may also impede some important physiological activities of the transporter, thereby jeopardizing the overall wellbeing and physiological homeostasis of the cell or tissue. Another possible approach that was proposed, was to modulate the activity of this transporter using biochemical modulation. Using biochemical modulation, exogenously supplied metabolites can be used to selectively manipulate the activity of MRP2 in tumor cells to ensure the more selective response of cancer cells to the action of administered anticancer agents [9]. This would go

a long way to improve the bioavailability and efficacy of anticancer drugs in tumor cells. Hence the identification of possible modulators of the MRP2 transporter is of great clinical importance [8]. Moreover, the broad impact of the efflux activity of this transporter on the efficacy of a broad class of drugs makes it essential to investigate the possible interactions between various therapeutic drugs (both approved and those in clinical trials) and this transporter. Thus in this study, a unique set of drugs from the FDA approved drug library was screened using In-Cell ELISA to identify modulators of MRP2.

2.0 Materials and methods

2.1 Chemicals

FDA(Food and Drug Administration) approved drug library was procured from Selleck chemicals (Houston, TX). Super signal West Dura® Extended Duration chemiluminescence substrate (21EAPI34076) was obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 Cell lines and cell culture

MDCKII, MDCKII/MRP2 cells were kindly provided by Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCKII cell lines were grown in Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) enriched with 10% fetal bovine serum (FBS). Cell lines were cultured in a humidified incubator maintained at 5% CO₂ and 37 °C. This incubation condition was retained in all subsequent cell culture procedures.

2.3 Screening of FDA approved drug library using In-Cell ELISA assay in MDCKII/MRP2 cells

In-Cell ELISA assay development and optimization were performed with MDCKII and MDCKII/MRP2 cells and used to screen the FDA approved drug library for modulators of MRP2 protein expression. MDCKII/MRP2 cells were seeded at 7×10^4 cells per well in 96-well plates with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and incubated for 24 hours. Cells were then treated with drugs (10 μ M), and

0.1% DMSO (control and Parental) and incubated for 48 hours at 37°C. At the end of the incubation period, treatment was removed and cells were rinsed twice with 150 µl of PBS. Cells were fixed with 3.7% paraformaldehyde and permeabilized with Triton-X 100. The cells were blocked using fish gel (MB-066-0100, Rockland) and incubated overnight at 4 °C with monoclonal anti-MRP2 antibody (MABN1545, EMD Millipore) or anti- α tubulin antibody (T5168, Sigma-Aldrich) in a 1:1000 dilution. Secondary antibody incubation was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific, Waltham, MA) for an hour at room temperature. Target proteins were detected using Super signal West Dura® Extended Duration Substrate chemiluminescence substrate (21EAPI34076, Thermo Fisher Scientific) and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicates and expressed as means. Drugs that showed modulation of MRP2 protein levels above 50% were selected as hit compounds.

3.0 Results

3.1 Screening of FDA approved drug library for modulators of MRP2

The FDA approved drug library containing 372 drugs was successfully screened using In-Cell ELISA assay using MRP2-overexpressing MDCKII cells. Treatments were performed in triplicates, and experiments were done using the 96 – well format. The relative MRP2-modulation by the FDA approved drugs is presented in Figure 4.2. Data obtained was statistically analyzed and expressed as means. Drugs showing more than 50% modulation on MRP2 protein expression were selected as “Hit compounds”. The results revealed 49 hit compounds that changed the MRP2 protein expression by more than 50%, representing 13.17% of total compounds screened. Among the identified hits for MRP2, 39 drugs increased expression levels whereas 10 drugs lowered expression levels of MRP2 after drug treatment as shown in Figure 4.3. Details on the hit test compounds identified from screening are listed in Table 4.1 – 4.4.

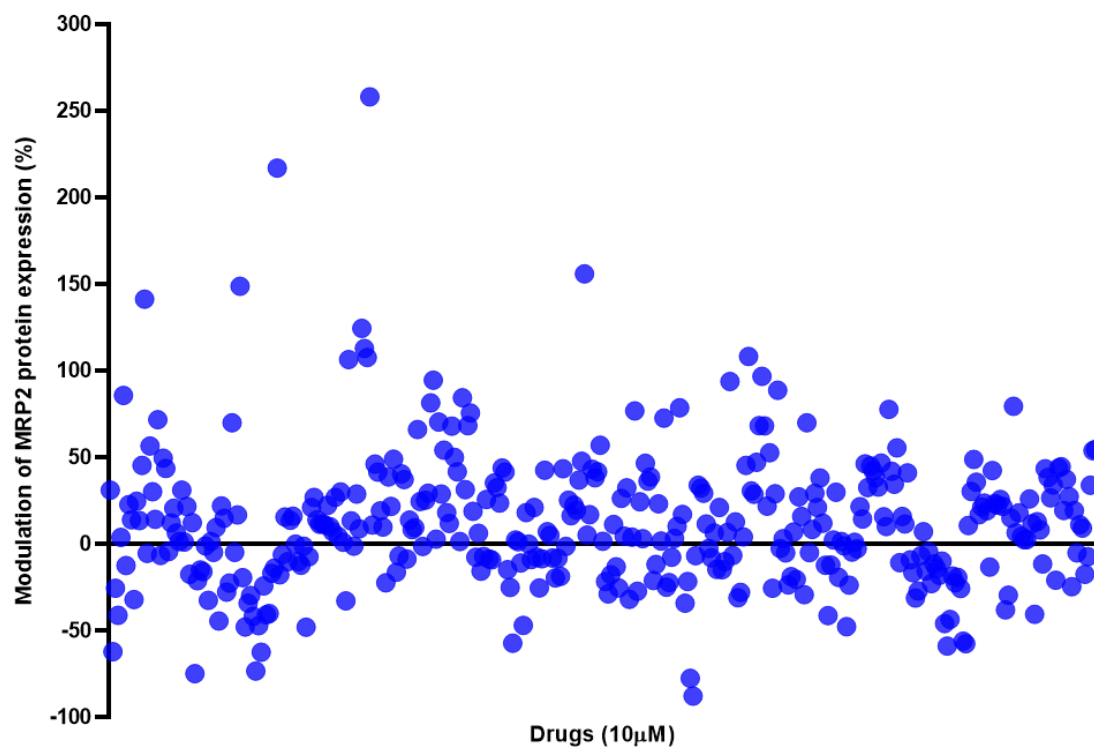


Figure 4.2: In-Cell ELISA assay screening for modulators of MRP2 protein expression from a unique FDA approved drug library.

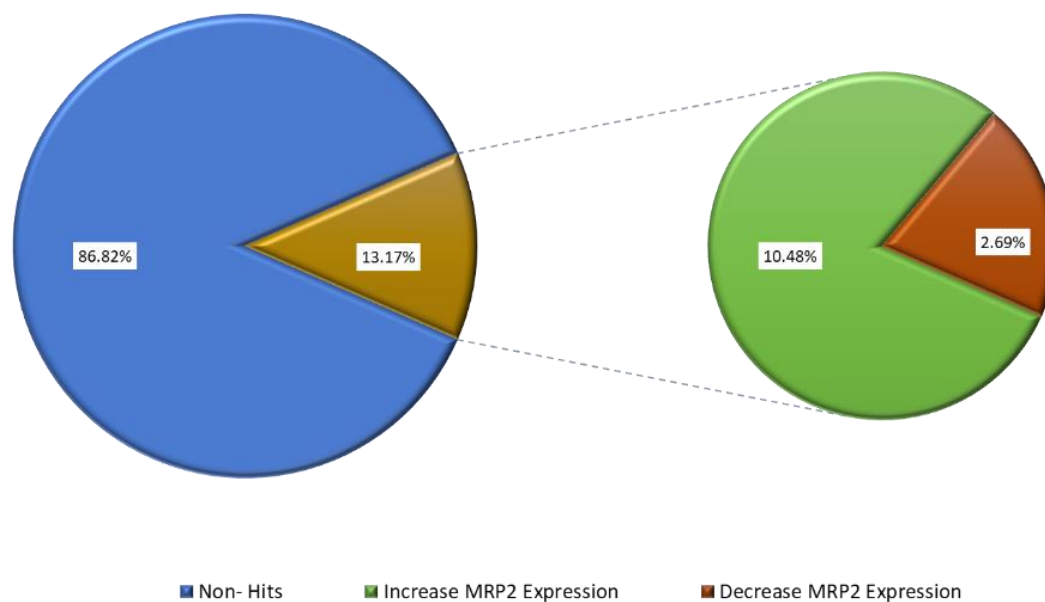


Figure 4.3: Screening of 372 FDA approved drug library using In-Cell ELISA assay

Forty-nine (13.17%) hit compounds altered the MRP2 expression levels by more than 50%. Thirty-nine (10.48%) drugs increased expression levels whereas 10 (2.69%) drugs lowered expression levels of MRP2 after drug treatment.

Table 4.1 List of hit compounds identified from FDA screening on MRP2 protein expression

Drug	Target	% Modulation on MRP1 protein levels ^a
Pralatrexate (Folotyn)	DHFR	-57.39 ± 3.85
Cetirizine Dihydrochloride	Histamine Receptor	106.29 ± 22.62
Mercaptopurine	DNA/RNA Synthesis	112.77 ± 15.36
Streptozotocin (Zanosar)	Nicotinamide adenine dinucleotide	107.57 ± 13.87
Dexamethasone	IL Receptor	257.86 ± 10.58
Megestrol Acetate	Androgen Receptor	124.35 ± 26.22
Trilostane	Dehydrogenase	66.04 ± 4.96
Ranolazine dihydrochloride	Calcium Channel	93.67 ± 39.42
Repaglinide	Potassium Channel	94.45 ± 12.23
Sildenafil Citrate	PDE	70.30 ± 17.82
Gestodene	Estrogen/progestogen Receptor	54.07 ± 4.05
Isotretinoin	Hydroxylase	67.87 ± 5.05
Nafamostat Mesylate	Proteasome	84.25 ± 14.38
Ondansetron hydrochloride (Zofran)	5-HT Receptor	68.23 ± 7.83
Oxcarbazepine	Sodium Channel	75.54 ± 22.66

^a Mean ± SD

Table 4.2 List of hit compounds identified from FDA screening on MRP2 protein expression

Drug	Target	% Modulation on MRP1 protein levels ^a
Afatinib (BIBW2992)	EGFR, HER2	-62.24 ± 20.67
Gefitinib (Iressa)	EGFR	85.68 ± 14.81
Crizotinib (PF-02341066)	c-Met	56.41 ± 9.37
Sunitinib Malate	VEGFR, PDGFR, c-Kit, Flt	71.59 ± 11.40
Cladribine	DNA/RNA Synthesis	-74.96 ± 2.30
Evista (Raloxifene Hydrochloride)	Estrogen/progestogen Receptor	69.87 ± 7.06
2-Methoxyestradiol	HIF	148.42 ± 46.91
Asenapine	Adrenergic receptor, 5-HT receptor	-73.49 ± 5.67
Adrucil (Fluorouracil)	DNA/RNA Synthesis	-62.54 ± 1.78
Vincristine	Microtubule Associated	216.85 ± 46.37
Oxaliplatin (Eloxatin)	DNA/RNA Synthesis	100.88 ± 10.98

^a Mean ± SD

Table 4.3 List of hit compounds identified from FDA screening on MRP2 protein expression

Drug	Target	% Modulation on MRP1 protein levels ^a
Glyburide (Diabeta)	Potassium channel	-77.59 ± 3.61
Adefovir Dipivoxil (Preveon, Hepsara)	reverse transcriptase	-87.87 ± 5.68
Sulfadiazine	Anti-infection	69.784 ± 19.01
Suprofen (Profenal)	COX-1/COX-2	108.14 ± 22.69
Cefditoren pivoxil	5-alpha Reductase	68.25 ± 9.12
Rifabutin (Mycobutin)	Antineoplastic and Immunosuppressive Antibiotics- Anti-infection	96.66 ± 30.90
Esomeprazole Magnesium (Nexium)	proton pump	68.11 ± 20.06
Ethionamide	Anti-infection	52.59 ± 27.15
Vidarabine (Vira-A)	5-alpha Reductase	88.61 ± 18.51
Deferasirox (Exjade)	Ferroptosis P450 (e.g. CYP17)	78.46 ± 19.75
Methylprednisolone	Immunology and Inflammation related, Glucocorticoid Receptor, Interleukins, ACE, Apoptosis related, Autophagy	93.67 ± 25.54
Metolazone (Zaroxolyn)	Treatment congestive heart failure and high blood pressure	155.75 ± 34.37
Darunavir Ethanolate (Prezista)	HIV Protease	56.94 ± 18.04
Prednisone (Adasone)	Glucocorticoid receptor	76.69 ± 23.89

^a Mean ± SD

Table 4.4 List of hit compounds identified from FDA screening on MRP2 protein expression

Drug	Target	% Modulation on MRP1 protein levels ^a
Rasagiline Mesylate	MAO	-58.99 ± 4.72
Dronedarone HCl	Anti-infection	-56.17 ± 5.53
Conivaptan HCl (Vaprisol)	vasopressin receptor	-57.59 ± 0.83
Eltrombopag (SB497115-GR)	c-mpl (TpoR) receptor	79.40 ± 18.03
Paeoniflorin	COX, HIF	53.80 ± 20.35
Benserazide	Dopamine Receptor	54.48 ± 22.74
Lovastatin	HMG-CoA Reductase	77.64 ± 17.81
Lafutidine	Histamine Receptor	55.36 ± 19.22
Erythromycin	Anti-infection, Antibiotics	104 ± 18.32

^a Mean ± SD

4.0 Discussion

Test compounds that modulated the protein expression levels of MRP2 in MDCKII/MRP2 cells above 50% (Hit compounds) in this study cuts across a broad spectrum of drug classes and exhibit great diversity in their structure, molecular targets, and mode of action. This included anticancer drugs, antibiotics, antivirals, anti-inflammatory drugs among others. This finding reaffirms the promiscuous nature of the MRP2 transporter, and how important it is to investigate the interaction between both old and newly developed drugs with MRP2. Although several studies have aimed at investigating the impact of various therapeutic agents on MRP2 efflux activity, it is needful that researchers also pay critical attention to how these drugs may affect the protein expression levels of this transporter. From our screening, about 10.48% of the hit compounds increased the expression of MRP2 protein levels, this included drugs like vincristine (an anticancer drug), oxaliplatin (antineoplastic medication), and irinotecan (an anticancer drug). MRP2 has been reported to be one of the major ABC transporters that affect the bioavailability and therapeutic potency of anticancer drugs in both polarized and unpolarized cells [10]. The anticancer drugs; Vincristine, Oxaliplatin, and Irinotecan have earlier been reported in other studies as substrates of MRP2 [7, 11, 12]. This indicates they are actively transported by MRP2/ABCC2 transporter thus the increase in MRP2 protein expression as observed in this study provides the possible explanation that more MRP2 is expressed in these cells to catalyze and ensure successful transport or efflux of these drugs across the plasma membrane.

Glucocorticoids like dexamethasone and prednisone also upregulated MRP2 protein expression in our present screening. The ability of dexamethasone to increase the expression levels of MRP2 protein as observed from the screening also reaffirms the

observation reported by Narang and his colleagues [13] who reported that dexamethasone increased expression and activity of multidrug resistance transporters at the rat blood-brain barrier. Prednisone, on the other hand, has also been reported to induce the activity of the MRP2 promoter [14], thus providing a possible reason for the increase in MRP2 protein levels observed in this study. Methylprednisolone, another glucocorticoid with anti-inflammatory and immunomodulating properties also upregulated MRP2 protein levels in this screening. However to the best of our knowledge, the interaction between MRP2 and methylprednisolone is yet to be reported, and it would be enlightening for further studies to be carried out to investigate how these drugs may affect MRP2 activity. Erythromycin (an antibiotic) and Lovastatin (a hypolipidemic agent and an HMG-CoA reductase inhibitor) which has been reported by other studies to be substrates of MRP2 [15, 16] also elevated the protein levels of MRP2 in this present study. Hence, this finding provides useful information on the modulatory effect of these drugs that can be further explored.

Furthermore, findings from this study also suggest that anticancer drugs like Pralatrexate, Afatinib, and Cladribine (an immunosuppressant) may decrease MRP2 protein expression levels in MDCKII MRP2-overexpressing cells. Pralatrexate has been reported in earlier studies to act as both a substrate and an inhibitor of MRP2 [17], interestingly, results from our present study demonstrate that pralatrexate may downregulate the expression levels of MRP2. Further investigation can be conducted to provide more insight into the effect of pralatrexate on gene expression and other effectors that regulate MRP2 protein expression. Moreover, the effect of pralatrexate on MRP2 protein expression levels in other MRP2-overexpressing cell lines can be explored to confirm this initial finding. Afatinib is a known moderate inhibitor of P-gp [18, 19], and a substrate /inhibitor of BCRP [19]. Results for

this current study shows that Afatinib may reduce MRP2 protein levels. A thorough search of current literature revealed that little is known about the impact of Afatinib on MRP2 activity and protein expression. Thus it would be enlightening to conduct further investigation to confirm and ascertain how Afatinib affects the efflux activity of this transporter in other MRP2 overexpressing cells. Cladribine is an FDA approved drug used in the treatment of multiple sclerosis and hairy cell leukemia. It is a known substrate of BCRP [20, 21] but proved otherwise on MRP2 when its impact on MRP2 membrane vesicles was explored [22]. Nonetheless, cladribine downregulated the protein expression levels of MRP2 in MDCKII MRP2 overexpressing cells in our present study. Thus it would be illuminating to investigate the impact of cladribine on other MRP2-overexpressing cells since the specific interactions between Cladribine and MRP2 remain uncertain. Further probing using cell lines overexpressing this transporter would be a step in the right direction. Adrucil (Fluorouracil), a DNA and RNA synthesis inhibitor that irreversibly inhibits thymidylate synthase, and Asenapine, an antipsychotic medication belonging to the dibenzooxepinopyrrole class [23] downregulated the expression levels of MRP2 in our present study. To the best of our knowledge, the impact of Adrucil and Asenapine on MRP2 activity and expression has not been reported in literature.

Although HIV Protease Inhibitors (HPIs) have been reported to be substrates of MRP1 and MRP2, the majority of tested HPIs are transported by MRP2. As such the overexpression of MRP2 has great pharmacological implications on administered HPIs [24, 25]. Darunavir Ethanolate is the ethanolate form of darunavir and an antiretroviral drug that inhibits the human immunodeficiency virus type-1 (HIV-1) protease. In this present study, Darunavir Ethanolate increased MRP2 protein expression in MDCKII overexpressing MRP2 cells.

Although to the best of our knowledge, Darunavir Ethanolate has not been reported as a substrate of MRP2, its ability to increase MRP2 protein levels suggests that this antiretroviral drug may also be a victim of MRP2 efflux activity. Interestingly, Darunavir the parent compound of Darunavir Ethanolate has been reported in other studies to induce P-gp mRNA activity and expression in vitro as well as induce MRP1 protein expression in CD4 (+) T cells from healthy human volunteers [26, 27]. On the Contrary, Adefovir dipivoxil, a diester prodrug of adefovir and an antiviral medication used in the treatment of chronic Hepatitis B infection in adults, also lessened the protein levels of MRP2 in this screening. This is not surprising since adefovir is reported to be a known inhibitor of MRP2 [28]. However, no information has been reported on the interaction between this diester derivative of adefovir and MRP2. It would be enlightening to investigate the modulatory effect of Darunavir Ethanolate and Adefovir dipivoxil on MRP2 activity and expression in other MRP2 overexpressing cells.

Our screening also identified other novel drugs whose effect on MRP2 activity or expression levels are yet to be reported or explored to the best of our knowledge. This included glyburide (medication for diabetes), Rasagiline mesylate (medication for Parkinson's disease), dronedarone HCl (antiarrhythmic drug), and conivaptan HCl (vasopressin antagonist, endocrine-metabolic agent). These novel drugs downregulated the protein expression levels of MRP2 in MDCKII MRP2-overexpressing cells in this study. Other non-reported drugs that upregulated the expression levels of MRP2 in our present study included Streptozotocin, Megestrol acetate, Gestodene, Trilostane, Ranolazine dihydrochloride among others. This initial data on these novel drugs would provide fore-knowledge that can further be explored. Like most proteins, MRP2 can be regulated at the

transcriptional and post-transcriptional level. Studies have revealed that alterations in the intracellular concentrations of bile acids and of a number of lipophilic compounds that are ligands for nuclear hormone receptors can regulate MRP2/ABCC2 transcription levels [6]. Nuclear hormone receptors for hydrophobic molecules such as steroid hormones (estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, vitamin D3, ecdysone, oxysterols and bile acids), retinoic acids (all-trans and 9-cis isoforms), thyroid hormones, fatty acids, leukotrienes and prostaglandins [29, 30]. Research has shown that the hormone response element in rat MRP2/Abcc2 promoter (ER-8) is bound by heterodimers of the retinoid receptor [31] with the ligand-activated transcription factors, pregnane X receptor (PXR), farnesoid X receptor (FXR) or constitutive androstane receptor (CAR). Thus various xenobiotics that regulate bile acid concentration can activate these receptors which in turn upregulates the promoter region of the ABCC2 transporter [32-36]. This finding provides a possible explanation to the increase in MRP2 protein expression observed in this study after MDCKII/MRP2 cells were treated with Megestrol acetate (androgen receptor), Gestodene (estrogen/progestogen receptor), Methylprednisolone and Prednisone (Glucocorticoid receptor), and Evista (estrogen/progestogen receptor).

In summary, the modulatory effect of 372 drugs from a recently approved FDA drug library on MRP2 protein expression in MDCKII/MRP2 cells was successfully screened using In-Cell ELISA assay. From this study, 49 hits compounds were identified to have altered the MRP2 expression levels by more than 50%, representing 13.17% of total compounds screened. Among the identified hits for MRP2, 39 drugs increased expression levels whereas 10 drugs lowered expression levels of MRP2 after drug treatment. Although these

identified hit compounds may be substrates, inhibitors, inducers, activators of MRP2, or even false hits due to the non-specific interactions of MRP2 due to unknown reasons, findings from this study bring to light the fact that MRP2 protein expression may be affected by several drugs to a greater extent than imagined.

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Chapter 5

Relevant Contributions

1.0 Scope

This chapter focuses on relevant contributions and side projects undertaken towards the general scholarly goals of our research group. After identifying novel modulators of MRP1 protein expression and activity in our initial screening of 30 drugs which comprised of anticancer and FDA approved drugs as described earlier, we set out to screen a larger number of drugs from different libraries to identify more modulators of MRP1 protein expression. The first two projects in this session describe projects that were undertaken in this direction. The first project aimed at the identification of chemotherapeutic drugs as modulators of MRP1 protein expression in MRP1-overexpressing cells. In this project, we screened 383 anticancer drugs from a unique anticancer library for their modulatory effect on MRP1 protein expression in HEK293 MRP1-overexpressing cells using a high throughput In-Cell ELISA assay. Our results from this study showed that some anticancer drugs may modulate MRP1 protein expression and also demonstrated that the In-Cell ELISA assay can be used as an effective high throughput tool for screening purposes. Drugs that were identified can be used in developing therapeutics for treating tumors with the MDR phenotype conferred by MRP1 overexpression.

The goal of the second project was to screen a recently approved FDA drug library to identify modulators of MRP1 protein expression in HEK293 MRP1-overexpressing cells. A total of 440 FDA drugs were successfully screened using In-Cell ELISA assay. These drugs included antibiotics, antivirals, antidepressants among others. Our findings from the

project suggest and affirm the fact that MRP1 interacts with a broad range of drug classes. This signifies the importance of profiling the interaction of drugs with this transporter, and the data obtained would provide essential information to improve drug efficacy and reduce drug toxicity of various cancer chemotherapeutics and in diseases in which MRP1 is implicated. In the third project, the effect of novel cucurbitacin-inspired estrone analogs inhibitors of P-gp and MRP1 on P-gp and MRP1 protein expression was investigated. These inhibitors were identified in an initial screening that was conducted by our research group in another project. These inhibitors were further characterized using established cell-based methods, thus we ascertained the impact of these novel inhibitors on MRP1 and P-gp protein expression in human embryonic kidney overexpressing P-gp cells (HEK293/P-gp) and small cell lung cancer cell line (H69AR) using western blot assay.

Identification of chemotherapeutic drugs as modulators of Multidrug Resistance Protein 1 (MRP1) expression in HEK293 MRP1- overexpressing cells

Introduction

We recently identified some novel modulators from our initial screening of thirty compounds on MRP1 protein expression in HEK293 MRP1-overexpressing cells. These drugs consisted of both clinically tested anticancer drugs and some recently approved FDA drugs. From our initial screening, we identified that anticancer drugs; SB743921 HCl, Amuvatinib, TG101348 (SAR302503), and a FDA-approved drug; Felbamate, may modulate MRP1 protein expression in HEK293 MRP1-overexpressing cells. Further characterization of these compounds using cell-based established assays revealed that SB743921 HCl, Amuvatinib, TG101348 downregulate MRP1 efflux activity, with Amuvatinib and TG101348 being potent reversers of MRP1 mediated MDR in these cells. Based on these interesting findings we decided to screen different drug libraries containing a larger number of drugs to investigate their effect on MRP1 protein expression. Using In-Cell ELISA assay, we explored the effect of 383 clinically-tested anticancer drugs from a unique anticancer drug library for their effect on MRP1 protein expression in HEK293 MRP1-overexpressing cells. These drugs from the anticancer library consisted of small molecules under clinical trials for 12 different types of cancers.

Materials and methods

Chemicals

Anticancer compound library consisting of 383 anticancer small molecules under clinical trials for 12 different types of cancers was procured from Selleck Chemicals (Houston, TX). Super signal West Dura® extended duration chemiluminescence substrate (21EAPI34076) was purchased from Thermo Fisher Scientific (Waltham, MA).

Cell lines and cell culture

HEK293/pcDNA3.1 and HEK293/MRP1 cells were a kind gift from Dr. Suresh V. Ambudkar (NIH, Bethesda, MD) respectively. Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) was used to grow the HEK293 cell lines. Cell lines were cultured in a humidified incubator maintained at 5% CO₂ and 37°C. This incubation condition was retained in all subsequent cell culture procedures.

Screening of anticancer library using In-Cell ELISA assay

Cells were seeded at 5×10^4 cells per well in 96- well plates with DMEM containing 10% FBS and incubated for 24 hours at 37°C. Cells were treated with drugs (10uM), 0.1% DMSO for controls and incubated for 48 hours at 37°C. At the end of the incubation period, treatment was removed and cells were rinsed twice with 150 μ L of PBS. Cells were fixed with 3.7% paraformaldehyde and permeabilized with Triton-X 100. The cells were blocked with fish gel (MB-066-0100, Rockland) and incubated overnight at 4 °C with monoclonal

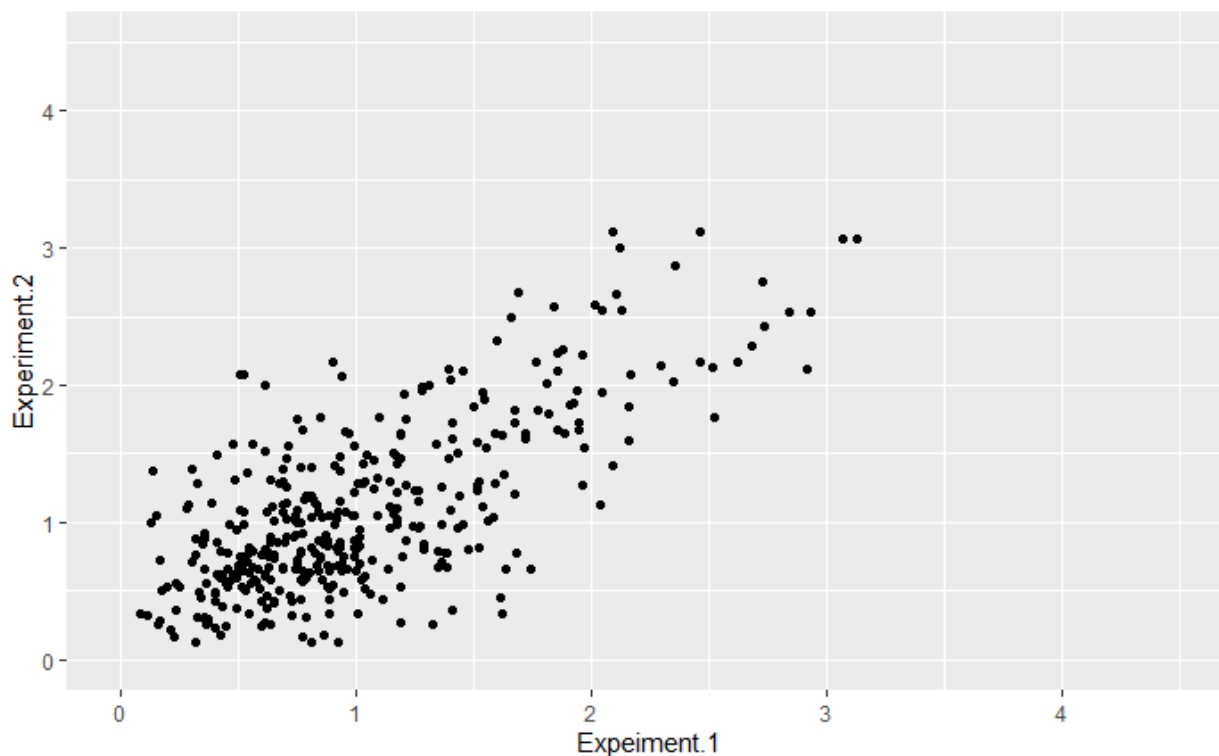
anti-MRP1 antibody (IU5C1, MA516079, Thermo Fisher Scientific) or anti- α tubulin antibody (T5168, Sigma-Aldrich) at 1:500 and 1:1000 dilutions, respectively. Secondary antibody incubation was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific) for an hour at room temperature. Target proteins were detected using Super Signal West Dura® Extended Duration chemiluminescence substrate (21EAPI34076, Thermo Fisher Scientific) and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Experiments were conducted in two independent studies and treatments were performed in duplicates. Dunnett test was applied for multiple comparisons, statistical testing was performed at a 5% level of significance.

Results and Discussion

Screening of anticancer compound library for modulators of MRP1 protein expression

The anticancer library containing 383 drugs was successfully screened using In-Cell ELISA assay. Two independent experiments were conducted in the 96-well format. The relative MRP1-modulation activity of the anticancer drugs from the two independent experiments is represented as a 2D scatter plot (Figure 5.1). As indicated in Figure 5.1(bottom), the assay had good reproducibility with a correlation range of 0.71 between the two given experiments. Differences between the two groups were determined by the Student's t-test using excel, and correlation analysis was evaluated by Pearson's correlation using R studio version 3.5.2. Dunnett test was applied for multiple comparisons, statistical testing was performed at a 5% level of significance. Figure 5.2 shows the effect of the various anticancer drugs screened on MRP1 protein expression in HEK293/MRP1 cells. Screening of the 383 anticancer drugs revealed 89 hit compounds that changed the MRP1 expression by 50% or more, representing 23.2% of total compounds screened. Among the identified hits, 57 drugs increased expression whereas 32 drugs lowered expression of MRP1 after drug treatment as shown in Figure 5.3. The identified hit compounds included known MRP1 substrates like doxorubicin, vincristine, etoposide, and dexamethasone [1, 2]. Some novel MRP1 modulators were also identified in this initial screening whose interaction or relationship with MRP1 have not been reported. These novel modulators may be substrates, inhibitors, inducers, or activators of MRP1. Some may also be false hits due to the non-specific interaction with MRP1 for unknown reasons. However, these novel

modulators identified would be further validated and characterized by our research group in future studies.



Correlation coefficient

Experiment	1	2
1	1	0.71
2	0.71	1

Figure 5.1 Screening of Anticancer drug library using In-Cell ELISA assay

The screening was conducted in two independent studies at a compound concentration of 10uM. The table below the plot shows correlation coefficients between the experiments. Correlation coefficient calculated and 2D graph generated using R studio version 3.5.2.

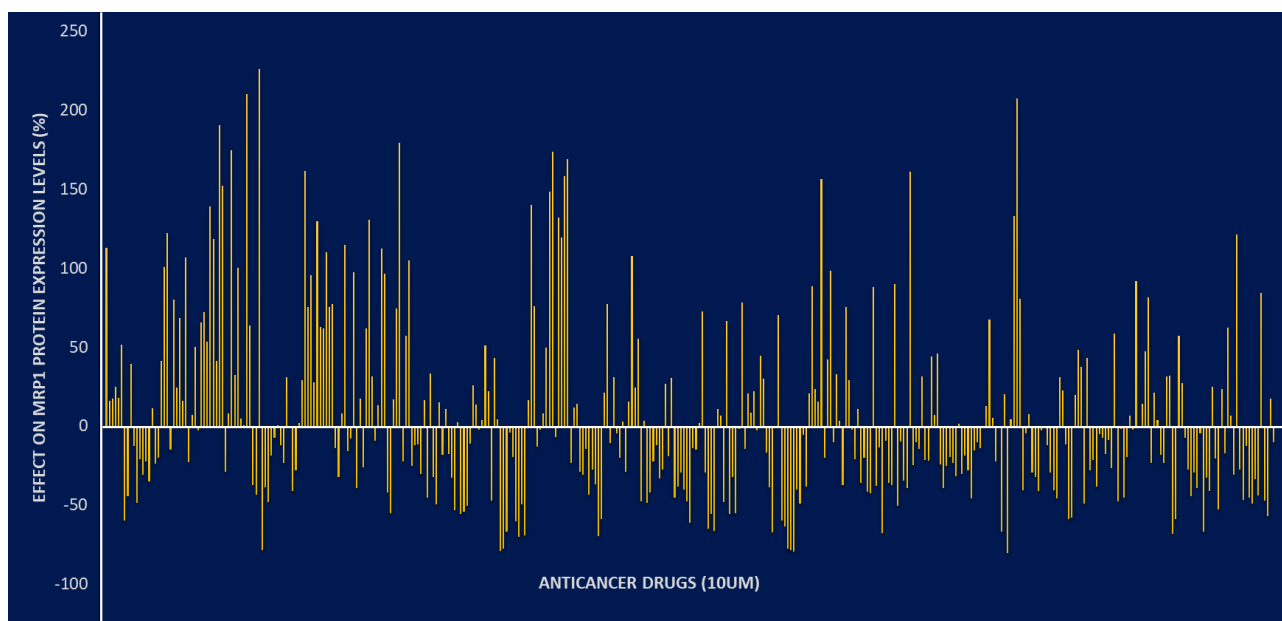


Figure 5.2 Effect of Anticancer drugs on MRP1 protein expression levels in HEK293/MRP1 cells from screening using In-Cell ELISA assay

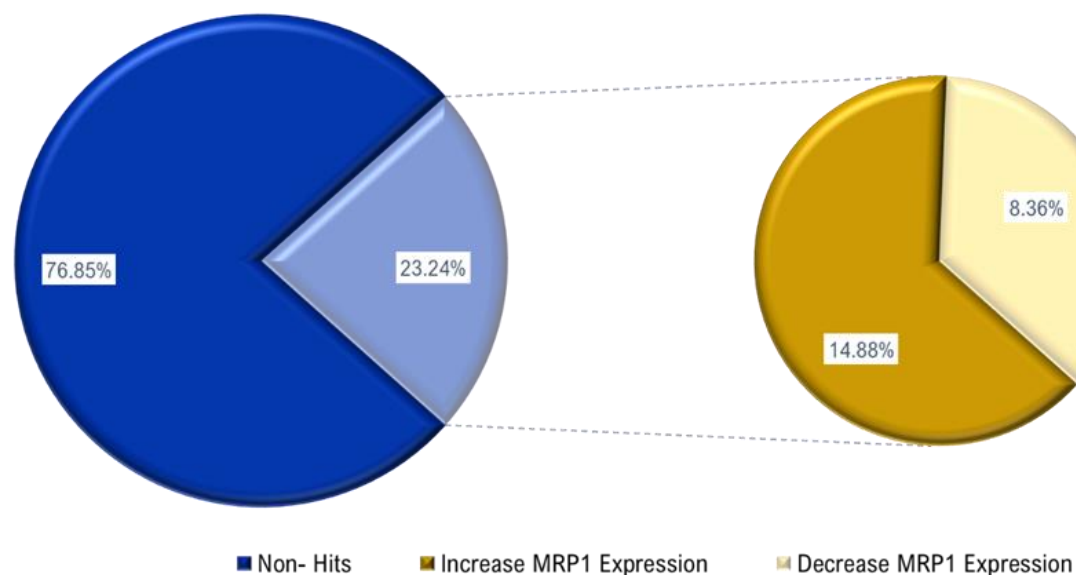


Figure 5.3 Screening of 383 anticancer drug library using In-Cell ELISA assay

Eighty-nine (23.24%) hit compounds modulated the MRP1 expression levels, with 57 (14.88%) drugs increased expression levels whereas 32 (8.36%) drugs lowered expression levels of MRP1 after drug treatment.

Identification of FDA approved drugs as modulators of Multidrug Resistance Protein 1 (MRP1/ABCC1) expression in MRP1-overexpressing cells

Introduction

MRP1 is reported to affect the efficacy and bioavailability of drugs belonging to various drug classes aside from anticancer drugs [3]. We decided to explore the effect of other therapeutic agents from other drug families on MRP1 protein expression in HEK293 MRP1-overexpressing cells. We achieved this by screening 440 FDA drugs from a recently approved FDA drug library to ascertain their effect on MRP1 protein expression. This FDA approved drug library consisted of structurally diverse therapeutic agents that belonged to different drug families. This includes antivirals, antibiotics, antidepressants, anti-inflammatory drugs as well as drugs used in the treatment of cardiology, immunology, neuropsychiatry-related conditions.

Materials and methods

Chemicals

FDA (Food and Drug Administration) approved drug library was procured from Selleck chemicals (Houston, TX). Super signal West Dura® extended duration chemiluminescence substrate (21EAPI34076) was purchased from Thermo Fisher Scientific (Waltham, MA).

Cell lines and cell culture

HEK293/pcDNA3.1 and HEK293/MRP1 cells were a kind gift from Dr. Suresh V. Ambudkar (NIH, Bethesda, MD) respectively. Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) was used to grow the HEK293 cell lines. Cell lines were cultured in a humidified incubator maintained at 5% CO₂ and 37 °C. This incubation condition was retained in all subsequent cell culture procedures.

Screening of FDA approved drug library using In-Cell ELISA assay

In-Cell ELISA assay was performed by seeding HEK293/pcDNA 3.1 and HEK293/MRP1 cells at a cell density of 5×10^4 cells per well in 96- well plates with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were treated with drugs(10uM), DMSO (control and Parental) after 24 hours (95% confluency), and incubated for 48 hours at 37 °C. At the end of the incubation period, treatment was removed and cells were rinsed twice with 150 μ L of PBS. Cells were fixed with 3.7%

paraformaldehyde and permeabilized with Triton-X 100. The cells were blocked using fish gel (MB-066-0100, Rockland) and incubated overnight at 4 °C with monoclonal anti-MRP1 antibody (IU5C1, MA516079, Thermo Fisher Scientific) or anti- α tubulin antibody (T5168, Sigma-Aldrich) at a 1:1000 dilution, respectively. Secondary antibody incubation was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific) at a dilution of 1:1000 for an hour at room temperature. Target proteins were detected using Super Signal West Dura® extended duration chemiluminescence substrate (21EAPI34076, Thermo Fisher Scientific), and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Treatment was performed in triplicates. Data obtained were statistically analyzed to calculate the mean and percentage modulation. Drugs that showed modulation above 50% were considered as Hit compounds.

Results and discussion

Screening of FDA approved drug library for modulators of MRP1

The FDA approved drug library containing 440 drugs was successfully screened using In-Cell ELISA assay. The FDA approved drug library was screened using HEK293 MRP1-overexpressing cells. Treatments were performed in triplicates, and experiments were done using the 96-well format. The relative MRP1-modulation activity of the FDA approved drugs are shown according to plates screened as presented in Figure 5.4. The mean and percentage modulation of the drugs screened were calculated using Microsoft excel. Drugs showing more than 50% modulation on MRP1 were selected as hit compounds. Screening of the 440 FDA-approved drugs on HEK293/MRP1 cells revealed 70 hit compounds that

modulated the MRP1 expression levels by 50% or more representing 15.90% of total compounds screened. Among the identified hits, 56 drugs increased expression levels whereas 14 drugs lowered expression levels of MRP1 after drug treatment as shown in Figure 5.5. Findings from our initial screening showed that aside anticancer drugs, drugs from other drug classes can also modulate MRP1 protein expression. The identified hit compounds included antivirals, anticonvulsants, anti-inflammatory, antiestrogen agents among others. The modulators identified in this study would be further investigated and characterized by our research group in future projects.

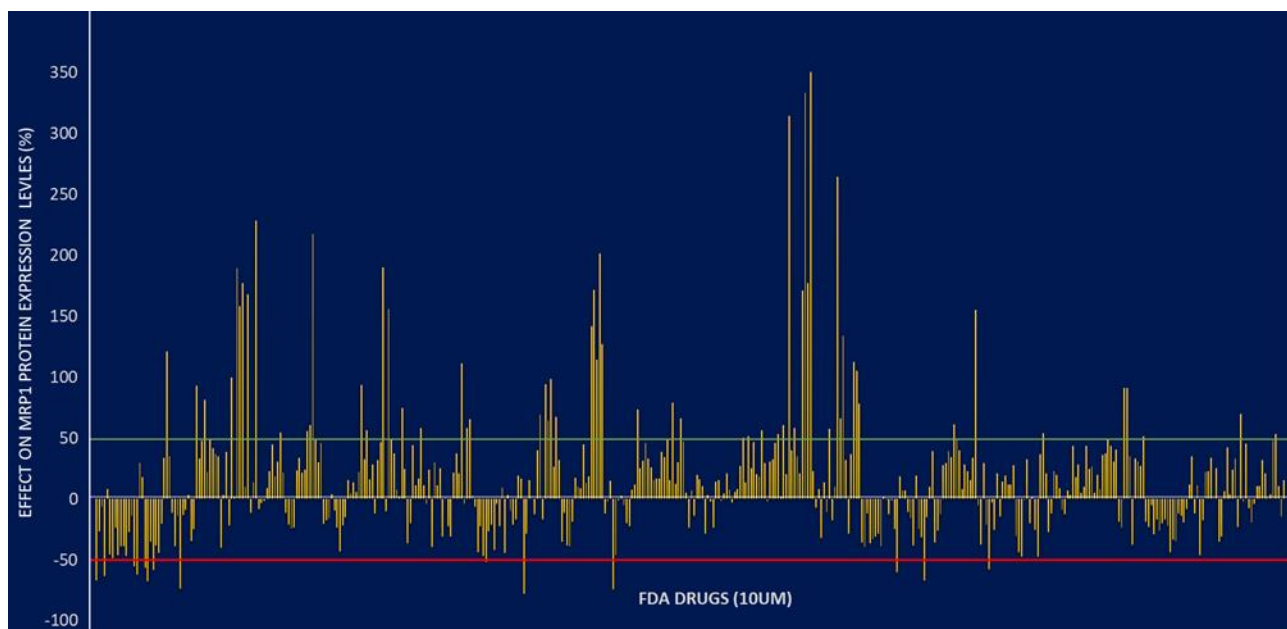


Figure 5.4: Screening of 440 FDA approved drug library using In-Cell ELISA assay

Seventy hits compounds (15.91%) changed the MRP1 protein expression by more than 50%. Fifty-six drugs (12.73%) increased expression levels whereas 14 drugs (3.18%) lowered expression levels of MRP1 after drug treatment.

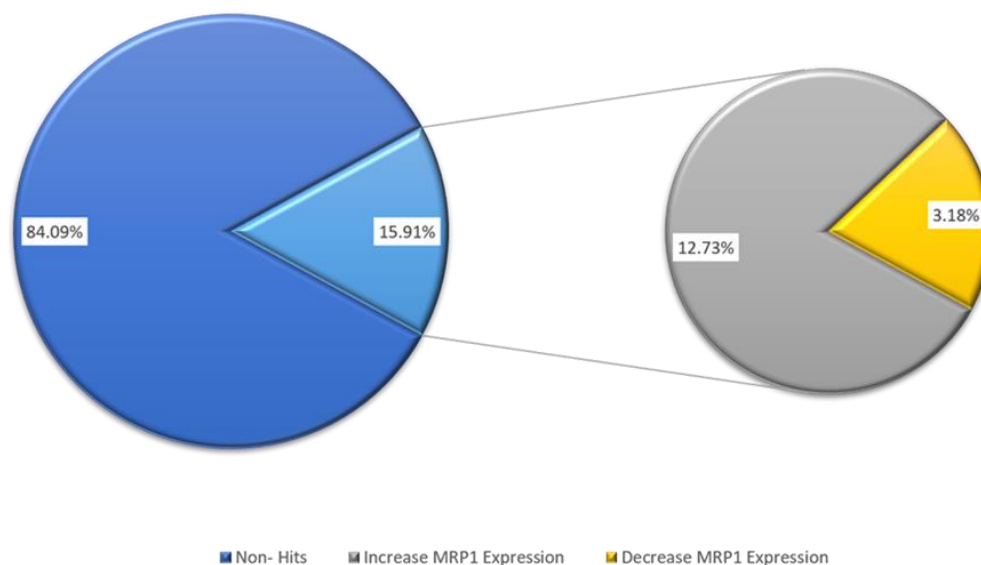


Figure 5.5: Screening of 440 FDA approved drug library using In-Cell ELISA assay

Seventy (70) hit compounds (15.91%) that changed the MRP1 expression levels by more than 50%. 56 drugs (12.73%) increased expression levels whereas 14 drugs (3.18%) lowered expression levels of MRP1 after drug treatment.

Effect of novel cucurbitacin-inspired estrone analogs on the protein expression levels of P-glycoprotein (P-gp) and Multidrug resistance protein 1 (MRP1)

Introduction

Natural compounds including phytochemicals have been established as compounds that can modulate the activity of ABC transporters including MRP1 and P-gp. Polyphenols like curcumin, and bioflavonoids like apigenin, quercetin have been reported to have a significant effect on the transport activity of MRP1 [4, 5]. Recently, in an ongoing project, we screened some cucurbitacin-inspired estrone analogs to identify inhibitors of P-glycoprotein (P-gp) and Multidrug resistance protein 1 (MRP1) in human embryonic kidney overexpressing P-gp cells (HEK293/P-gp) and small cell lung cancer cell line (H69AR) respectively. We identified 8 of these analogs to show inhibitory effects on P-gp activity in HEK293/P-gp whereas 4 of these analogs also strongly inhibited MRP1 in H69AR. These inhibitors have further been characterized using established cell-based assays. We further investigated the influence of these novel inhibitors on P-gp and MRP1 protein expression using western blot assay.

Materials and methods

Cell lines and cell culture

H69 and H69AR cells were purchased from ATCC (Manassas, VA). HEK293/pcDNA3.1 and HEK293/P-gp were kindly gifted by Dr. Suresh V. Ambudkar (NIH, Bethesda, MD). Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) was used to grow the HEK293 cell lines. H69 cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% FBS. H69AR cells were monthly exposed to 0.8 mM doxorubicin and cultured without drug treatment for 1 week before use in experiments. Cells were cultured at 37 °C in a humidified incubator set at 5% CO₂.

Western blot assay

HEK293 and H69 cell lines were seeded at cell densities of 7×10^5 and 1×10^6 cells in 6-well plates respectively in a culture medium. Cells were treated with drugs (10 μ M), and 0.1% DMSO for controls after 24 hours, and incubated for 48 hours at 37 °C after drug treatment. At the end of the incubation period, treatments were removed and cells were rinsed with 1000 μ L of PBS. The cells were lysed with lysis buffer containing RIPA buffer (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 \times halt protease inhibitor. Protein concentration was determined with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Proteins (20 μ g) were loaded in each well on 8.0% mini sodium dodecyl sulfate (SDS) gels, and SDS page electrophoresis was conducted. Proteins were transferred to Immobilon PVDF membranes (EMD Millipore, Burlington, MA). The

membrane was blocked and incubated overnight at 4 °C with rabbit monoclonal anti-MRP1 antibody [EPR21062](1:250; Abcam, ab233383) and anti-alpha-tubulin antibody (1:5000; Sigma-Aldrich) respectively. Followed by incubation with secondary antibody for an hour at room temperature using horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) (1:1000; Thermo Fisher Scientific) for alpha-tubulin, and horseradish peroxidase-conjugated goat-anti-rabbit IgG(H+L) (1:1000; Thermo Fisher, Scientific) for detection of MRP1. Target proteins were developed using a Western blotting luminol reagent (Santa Cruz Biotechnology, sc-2048) and an LI-COR Odyssey Fc imaging system. Protein band densities were quantified and analyzed using Image Studio Lite version 5.2 (LI-COR Biotechnology, Lincoln, NE), and uneven sample loading and transfer was corrected using the intensity of the corresponding protein band relative to the alpha-tubulin (loading control) band. The experiment was conducted in three independent studies. The data obtained was statistically analyzed and the Dunnett test was applied for multiple comparisons, statistical testing was performed at a 5% level of significance.

Results and Discussion

Effect of test compounds on P-gp and MRP1 protein expression levels

We determined the effect of these novel cucurbitacin-inspired estrone analogs which were identified as inhibitors in our initial screening on protein expression of P-gp and MRP1. Our results as shown in Figure 5.6 and Figure 5.7 indicate that these inhibited do not have a significant impact on P-gp and MRP1 protein expression in HEK293/P-gp and H69AR cells.

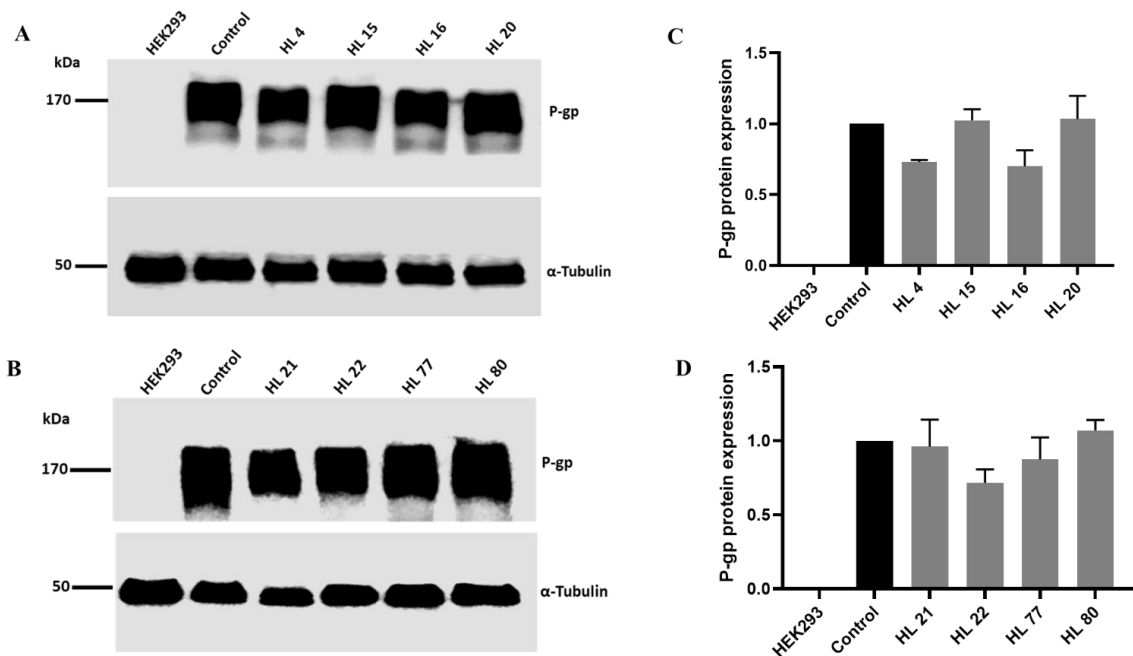


Figure 5.6: Effect of novel cucurbitacin-inspired estrone analogs on protein expression of P-gp in HEK293/P-gp cells

[A, B] Shown are representative western blots of whole-cell lysates (20 μ g of protein/lane) prepared from drug treatment (10 μ M) on HEK293/P-gp cells. P-gp proteins and α -tubulin was detected using monoclonal P-gp antibody [C219] (GTX23364, GeneTex) and anti- α -tubulin antibody (Sigma-Aldrich) at 1:250 and 1:5000 dilutions, respectively. Secondary antibody incubation used performed using GAM (mAb – goat- anti-mouse)

(1:10000). [C, D] Dunnett test was applied for multiple comparisons, statistical testing was performed at a 5% level of significance, and the graph was developed using Graph Pad Prism version 6.

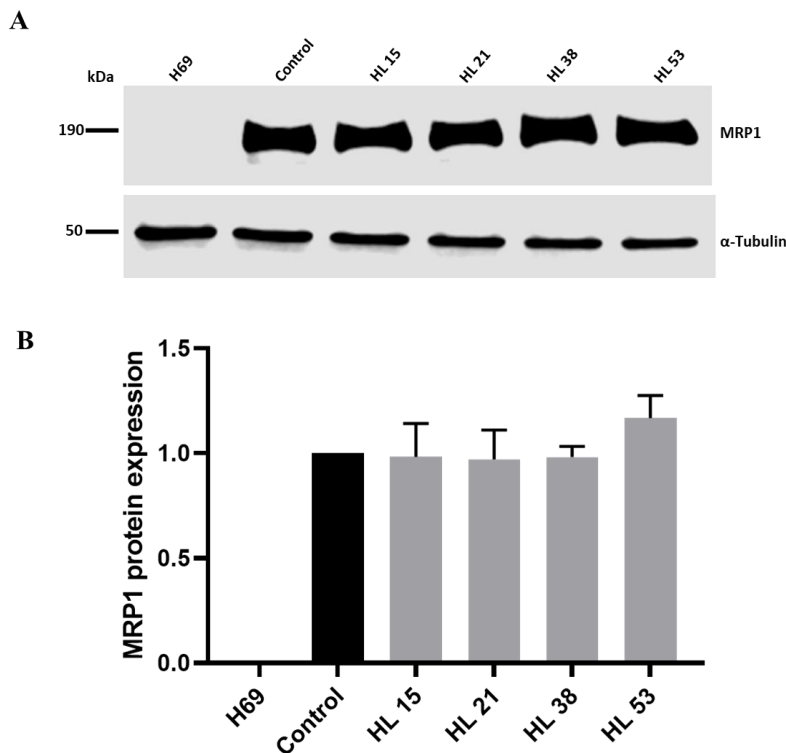


Figure 5.7 Effect of novel cucurbitacin-inspired estrone analogs on protein expression MRP1 in H69AR cells.

[A] Shown are representative western blots of whole-cell lysates (20 ug of protein/lane) prepared from H69AR cells treated 10 μ M of test compounds. MRP1 proteins were detected with monoclonal anti-MRP1 antibody [EPR21062](ab233383, Abcam) and anti-GAPDH antibody (Sigma-Aldrich) at a dilution of 1:250 and 1:1000 respectively. Secondary antibodies; mAb - anti-rabbit and mAb-anti-goat were used at a dilution of 1:1000. [B] Dunnett test was applied for multiple comparisons, statistical testing was performed at a 5% level of significance, and graph was developed using Graph Pad Prism version 6.0.

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Chapter 6

Final discussions and General conclusions

The overexpression of ABC transporters in tumor cells has been reported to be responsible for the multidrug resistance (MDR) phenotype observed in several carcinomas. The role of ABC transporters like P-gp and BCRP in the MDR of tumor cells has been well investigated in clinical cancer research. However, recent studies have revealed that ABC transporters; MRP1 and MRP2 are also major players in the development of MDR in several carcinomas [1, 2]. MRP1 was discovered by Cole and her colleagues when they observed the overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line (H69AR) which did not overexpress P-gp [3]. The plasticity of the binding site of this transporter enables it to interact with a variety of substrates, which mostly include amphipathic organic acids with large hydrophobic groups [4]. This ubiquitous transporter is reported to mediate the transport of heavy metals, organic anions, glucuronide-conjugates of steroids, prostaglandins, drugs, and their metabolites across biological membranes [5, 6]. Due to its transport activity, MRP1 governs the absorption and disposition of drugs and their metabolites across cells. MRP2 which is popularly known as canicular multi-specific organic anion transporter 1 (cMOAT) [7] also facilitates the transport of xenobiotics and endogenous compounds to the bile, urine, or feces [8]. Due to the pivotal roles of MRP1 and MRP2 in the transport and distribution of drugs and their metabolites, their overexpression has been associated with reduced intracellular concentration and bioavailability of various classes of drugs (vinca alkaloids, anthracyclines, antibiotics, protease inhibitors) [8-11] in tumor cells. As such, these transporters have been implicated in MDR of several solid human tumors like kidney,

colon, breast, lung, and ovarian carcinomas [12]. Strategies to overcome the MRP1 and MRP2 mediated MDR in tumors involve the identification of modulators of these transporters which can regulate their activities in tumor cells without interrupting their role in maintaining physiological equilibrium in normal cells. Although some modulators of MRP1 and MRP2 have been identified in recent times, most of the current modulators that have been identified are limited by non-specific toxicity, low MDR reversal effects, and low therapeutic efficacy in in-vivo experiments. Thus there is the need for the identification of more potent and safer modulators of MRP1 and MRP2.

In this present study, we aimed at identifying modulators of MRP1 and MRP2 by screening therapeutic agents from various drug libraries using In-Cell ELISA assay. Our initial screening of 30 compounds, identified a total of 7 test compounds that modulated MRP1 protein expression in HEK293 MRP1-overexpressing cells by 50% or more. Four of the test compounds; Vismodegib (GDC-0449), TG101348 (SAR302503), Amuvatinib, and SB743921 HCl decreased the protein expression levels of MRP1, and three test compounds; Epirubicin HCl, Felbamate, and Irinotecan increased the protein expression of MRP1. Three of these modulators (Epirubicin HCl, Irinotecan, Vismodegib (GDC-0449) had already been reported in other studies [13-15], thus were not considered for further characterization in this study. Four of the identified modulators exerted novel modulatory activity on MRP1 protein expression. This included ATP competitive inhibitors; SB743921 HCl, Amuvatinib, TG101348 (SAR302503), and Felbamate (a recently approved FDA drug). SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) downregulated MRP1 protein expression in HEK293 MRP1-overexpressing cells whereas Felbamate increased MRP1 protein expression. Our findings from this study also showed

that SB743921 HCl and Amuvatinib decreased MRP1 protein expression in HEK293 MRP1-overexpressing cells in a concentration and time-dependent manner. SB743921 HCl, Amuvatinib, and TG101348 (SAR302503) inhibited the growth of these cells at clinically achievable concentrations. Moreover, we report that Amuvatinib and TG101348 (SAR302503) reverse MRP1 mediated resistance against vincristine in HEK293 MRP1-overexpressing cells.

We also demonstrated that for the first time that Tie2 kinase inhibitor can inhibit MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells. Moreover, Tie2 kinase inhibitor was able to reduce the fold resistance of HEK293 MRP1-overexpressing cells towards vincristine. Everolimus, an mTOR inhibitor that was previously reported by our lab group to be an inhibitor of MRP1 in H69AR cells, also inhibited MRP1 mediated calcein efflux in HEK293 MRP1-overexpressing cells. Everolimus also reversed MRP1 mediated resistance in HEK293 MRP1-overexpressing cells. Findings from this study show that these therapeutic agents may be useful for developing combinatorial therapy targeting malignancies involving MRP1.

Furthermore, we also screened a recently approved FDA approved library for modulators of MRP2 using In-Cell ELISA. This unique FDA drug library comprises drugs from different drug classes including antivirals, antibiotics, antidepressants, antihypertensives among others. Our screening of 372 FDA drugs identified 49 modulators of MRP2 in MDCKII MRP2-overexpressing cells. Thirty-nine of these modulators increased MRP2 expression whereas 10 compounds lowered MRP2 expression levels after drug treatment. The ability of MRP2 to be modulated by compounds from different drug families that exhibit great structural diversity in this study indicates that MRP2 is a promiscuous

transporter. As such this transporter can interact with several compounds irrespective of their structure and drug classification. Modulators identified in this study would be further characterized in future projects.

On the whole, we identified modulators of MRP1 and MRP2 protein expression and activity. These modulators can be used in the development of combinatorial drug therapy for MRP1 and MRP2 targeted therapeutics. Our findings indicate the importance of investigating the possible drug-interactions between various therapeutic compounds with these transporters. Research into drug-transporter interactions would provide a better understanding of the physiology of these transporters and the pharmacology of these therapeutic agents. Thereby creating a platform for optimizing drug treatment for chemotherapy and other MRP1/MRP2 related malignancies.

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