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DISCOVERY AND CHARACTERIZATION OF NOVEL SUBSTRATE SELECTIVE INHIBITORS OF HUMAN MRP1 (ABCC1)

BY

ANGELINA SAMPSON

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biochemistry

South Dakota State University

2019

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DISSERTATION ACCEPTANCE PAGE

Angelina Sampson

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 husband, Dr. Emmanuel Blay whose support and advice has helped me tremendously.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADMET	absorption, distribution, metabolism excretion and toxicity
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
CFTR	cystic fibrosis transmembrane conductance regulator
$E_2 17\beta G$	17β-estradiol glucuronide
FDA	Food and drug administration
GFP	green fluorescent protein
GSH	reduced glutathione
GSSG	oxidized glutathione
HEK293	human embryonic kidney 293
HCS	high content screening
MDR	multidrug resistance
MRP1	multidrug resistance protein 1
MSD	membrane spanning domain
NBD	nucleotide binding domain
P-gp	permeability glycoprotein
TMD	transmembrane domain

ABSTRACT

DISCOVERY AND CHARACTERIZATION OF NOVEL SUBSTRATE SELECTIVE INHIBITORS OF HUMAN MRP1 (ABCC1)

ANGELINA SAMPSON

2019

Multidrug resistance protein 1 (MRP1/ABCC1) actively transports a variety of drugs, toxic molecules and important physiological substrates across the plasma membrane. It can confer broad-spectrum drug resistance and can decrease the bioavailability of many important drugs such as anti-cancer agents, antibiotics, antivirals, antidepressants and anti-inflammatory drugs. Calcein-AM, a fluorescent reporter commonly used for studying compound interactions with MRP1 was recently used in the development of a high content imaging-based assay by our group. This assay was robust and had better sensitivity than fluorescent plate readouts. The assay identified 12 MRP1 inhibitors after screening an anti-cancer library of 386 compounds. Due to the multiple distinct substrate binding sites of MRP1, we sought to use different fluorescent probes to identify substrate selective inhibitors which were likely missed by the calcein-AM screening. The high content imaging-based uptake assay was modified using doxorubicin (anticancer drug) and CRO-9 (dye) as fluorescent reporters which vary in structure and function. The doxorubicin assay, after screening the same 386 compound library identified a total of 28 MRP1 inhibitors including 16 inhibitors that have not been previously reported as inhibitors of MRP1. The CRO-9 assay identified a total of 50 MRP1 inhibitors including 19 additional inhibitors that have never been reported as inhibitors of MRP1. These 50 MRP1 inhibitors

included 10 out of 12 hits identified using calcein-AM and 27 out of 28 inhibitors discovered through the doxorubicin assay. MRP1 inhibition was confirmed using flow cytometry, confocal microscopy and membrane-based transport assays. Selected drugs were evaluated for their ability to reverse resistance of MRP1-overexpressing H69AR lung cancer cells against various substrates. From the doxorubicin screening, mifepristone and doramapimod were the most effective in reversing MRP1 mediated resistance whiles celecoxib exhibited selective MRP1 inhibition. From the hits identified through the CRO-9-based screening, LY2603618 and ZSTK474 were the most effective in reversing MRP1 mediated resistance in H69AR cells. Together, our findings signify the effectiveness and value of doxorubicin and CRO-9 based high content screening approach. Anti-cancer agents that exhibit MRP1 inhibition may be used to reverse multidrug resistance or to improve the efficacy and reduce the toxicity of various cancer chemotherapies.

Chapter 1

1.0 Scope of the Study

The significance of this study is to identify novel inhibitors of the multidrug resistance protein 1 (MRP1) from a unique anticancer library using a high throughput imaging-based assay with different fluorescent reporters. This chapter explores literature on ATP Binding Cassette (ABC) transporters, their structure and function, and their role in the development of multidrug resistance in cancer. The chapter also covers modulators of ABC transporters and how they can be used for different therapeutic purposes. The literature regarding some of the common traditional methods used for screening for modulators of key ABC transporters such as P-gp, MRP1 and BCRP is carefully examined. The chapter then highlights the literature regarding high content imaging-based assay using fluorescence reporters, the main technique for the identification of MRP1 inhibitors. The chapter finally concludes by looking at the rationale of the current study and importance of profiling drug interaction with ABC transporters. It also addresses potential utilization of our findings in overcoming multidrug resistance.

1.1 Introduction to ABC transporters

ABC transporters represent a large evolutionarily conserved superfamily of membrane proteins ubiquitous in practically all living organisms. In the human genome, a total of 48 ABC transporter genes have been identified and are classified into seven subfamilies, designated A to G (Figure 1.1) according to sequence homology and structural organization [1].

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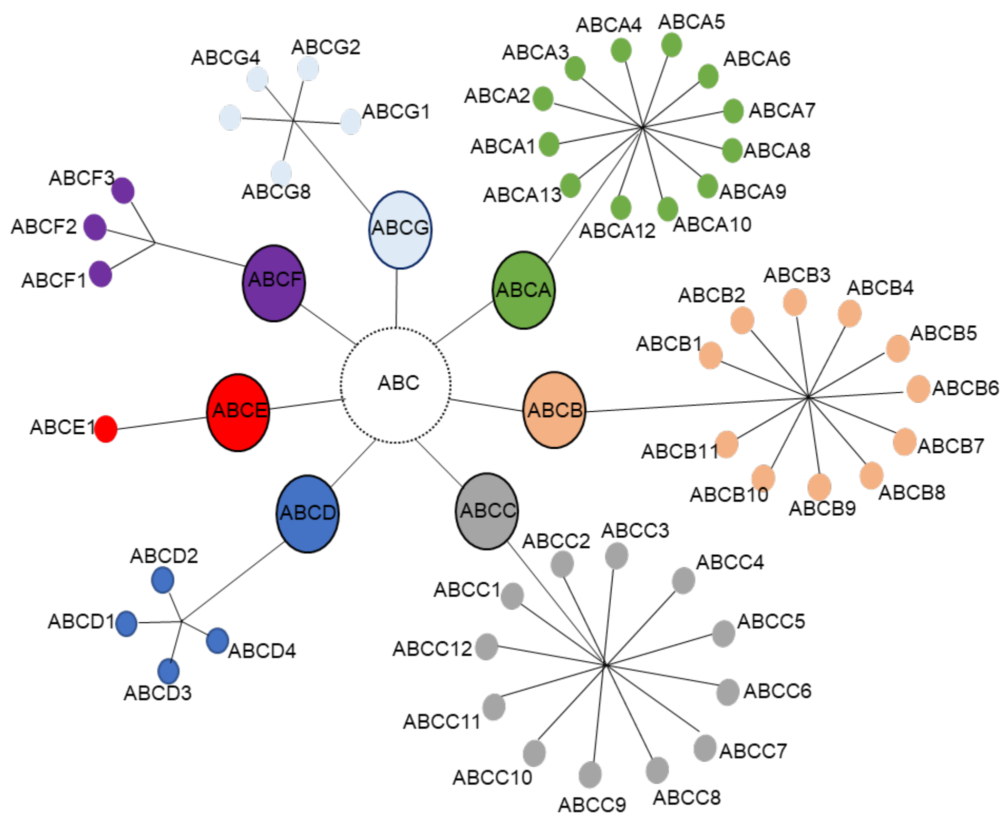

Figure 1.1 Classification of ABC transporter family genes identified in the human genome

Most ABC transporters can shuttle endogenous molecules and xenobiotics across membranes. ABC transporters play a role in physiological functions such as tissue defense against exogenous molecules and maintenance of balance between antioxidant and free radical concentrations (the MRPs), detoxification (P-gp), absorptive and secretory activities (ABCBs and MRPs), antigen presentations (ABCB2 and ABCB3) and lipid metabolism (ABCA1 and ABCGs) [2]. ABC transporters also play a role in the transport of various physiological substrates such as amino acids, peptides, lipids, and inorganic

ions. The importance of ABC transporters is highlighted by the fact that mutations in various ABC transporter genes are associated with human diseases. These diseases include cystic fibrosis (ABCC7), Dubin-Johnson syndrome (ABCC2), gout (ABCG2), intrahepatic cholestasis (ABCB4), schizophrenia (ABCA13), sitosterolemia (ABCG5 and ABCG8), Tangier disease (ABCA1), surfactant metabolism syndrome (ABCA3), Harlequin-type ichthyosis (ABCA12), inflammatory bowel disease (ABCB1), and adrenoleukodystrophy (ABCD1) [2,3]. Nine ABC transporters are associated with drug resistance due to their ability to efflux xenobiotics with majority from the ABCC subfamily These are P-glycoprotein (P-gp/ABCB1), Multidrug resistance proteins (MRP1-MRP7), and breast cancer resistance protein (BCRP/ABCG2) [5]. Accumulating evidence has indicated a key role of these transporters in governing the transit of both endogenous and drugs substrates and their metabolites across major organs and physiological barriers (Figure 1. 2) such as liver, kidney, blood-brain barrier, blood-testis barrier, and non-polarized cells [5, 6]. The cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) and sulfonyl urea receptor (SUR, ABCC8) are ATP-dependent protein channels but do not directly transport drugs [8]. ABC transporters that efflux drugs such as P-gp, MRP1 and BCRP are known to affect drug efficacy and toxicity [8, 9]. Thus, it is essential to assess their interactions with new drugs in the drug discovery and development process.

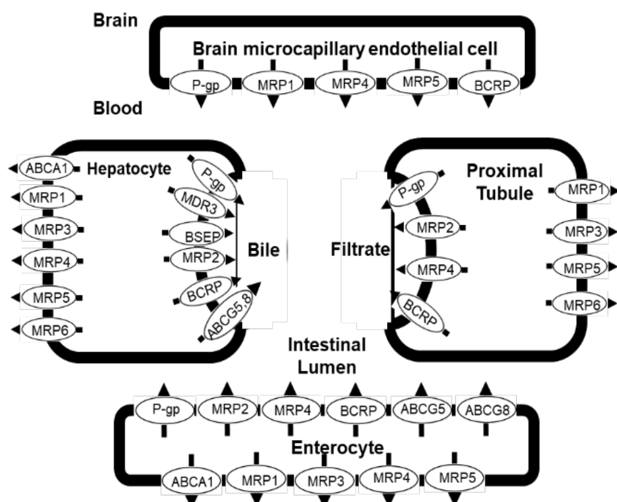

Figure 1.2 Localization of ABC transporters at four pharmacological barriers

Top: blood-brain barrier; mid-left: hepatocyte; mid-right: proximal tubule cell; enterocyte) [10, 11, 12]. Arrows indicate the direction of transport. Common name of transporters is shown.

1.2 Structure and function of ABC transporters

ABC transporters efflux specific substrates across cell membranes against a concentration gradient using energy derived from ATP binding and hydrolysis. The general structure of ABC transporters comprises of nucleotide binding domains (NBD) located on the cytoplasmic side of the membrane and two sets of membrane spanning domains (MSD). The NBDs of all ABC transporters have 3 conserved motifs; Walker A, B and C. Walker A and Walker B motifs are essential for ATP- binding and hydrolysis reactions [14]. The Walker A motif binds to the α- and γ- phosphates of di- or tri- nucleotides whiles Walker B motif helps manage magnesium ions. Walker C (LSGGQ) is a signature to ABC-transporters and has been proposed to play a role in the dimerization of NDB1 and NDB2

[15]. Each MSD contains six transmembrane spanning α-helices [13, 6] which form substrate translocation pathway. ABC transporters with at least two MSDs and two NBDs are considered full transporters whereas those with one of each domain are known as half transporters [17]. MRPs with a four-domain arrangement of two MSDs and two NBDs are known as 'short MRPs', whiles ABC transporters with an additional NH_2-proximal MSD0 are referred to as 'long MRPs' [18] (Figure 1.3).

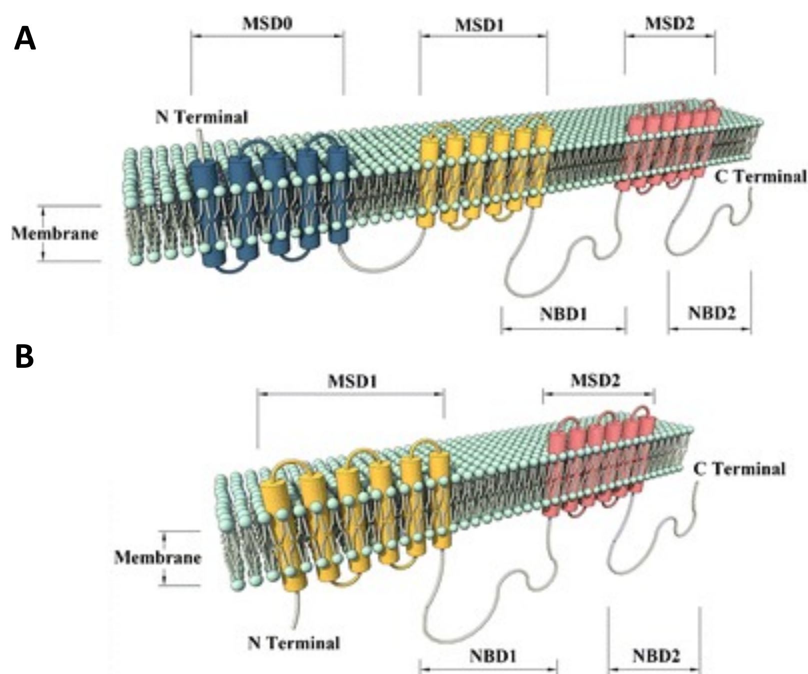

Figure 1.3 The predicted topology of long and short MRPs

Protein backbone is illustrated as thin tubes while transmembrane helices are shown as cylinders. **A**. The predicted topology of the MSDs and NBDs of long MRPs (MRP1, 2, 3, 6, and 7). **B**. The predicted arrangement of short MRPs (MRP4, 5, 8, and 9). [18]

MRP1 has an extra MSD (MSD0) towards the N terminus and comprises of 5 additional transmembrane spanning α-helices located on the extracellular side of the membrane. MSD0 does not play a role in the efflux activity of the transporter, but it is required for efficient retention of MRP1 at the cell surface [19]. The NBDs are directly involved in the binding and hydrolysis of ATP [20]. The MSDs provide substrate binding sites and contribute to transporter specificity [15, 16]. The binding site of MRP1 is bipartite in nature; a positive charged region that binds to GSH moiety (P-pocket) and a large hydrophobic area that encompass a lipid tail (H pocket) [23]. This nature enables it to bind to a more diverse nature of compounds which ae structurally unrelated.

The proposed transport mechanism for substrate transport is the ATP switch model by Higgon and Linton [22]. According to this model, the NBDs of MRP1 alternate between a closed and an open conformation. These movements result in ligand translocation [24]. The closed conformation has two ATP molecules bound to the NBD dimer interface forming a nucleotide pocket. In the pocket, ATP binds to the Walker A and Walker B motifs. Transport is initiated by the binding of substrate to the MSDs in the open NBD conformation which increases affinity to ATP [16, 18]. The binding of ATP induces changes in the formation of closed NBDs dimer which subsequently induces a major conformational change in the MSDs to start substrate transport. ATP hydrolysis occurs which causes changes in the NBD dimer dissolution to be transmitted to the MSDs. The basal state of transporter is restored with sequential release of inorganic phosphate and adenosine diphosphate (ADP) [16, 19]. This substrate transport mechanism was confirmed in studies on the binding of LTC4 (MRP1 substrate) to bovine MRP1 (91% identical to human MRP1). The binding of LTC4 was shown to bring the NBDs closer and aligned

properly. ATP hydrolysis occurs when the two NBDs dimerize to form a complete catalytic site. The binding of LTC4 stabilizes the closed conformation where NDBs are properly aligned [17, 20].

1.3 Multidrug resistance in cancer

Multidrug resistance (MDR) is the resistance of cancer cells to multiple chemotherapeutic agents with different structures, targets and mechanism of action. MDR mechanisms can be categorized into 7 areas as shown in Figure 1.4. These areas include; (1)increased drug efflux by membrane transporters (mainly, ABC transporters); (2) reduction in drug uptake by influx transporters (solute carriers); (3) enhanced drug metabolism; (4) blocking apoptotic signaling pathways; (5) Gene regulation; (6) mutation in drug targets or feedback activation of other targets and signaling pathways; and (7) chemoresistance induced by changes in the microenvironment. However, one of the most common mechanism studied is increased drug efflux due to the overexpression of ABC transporters [21, 22].

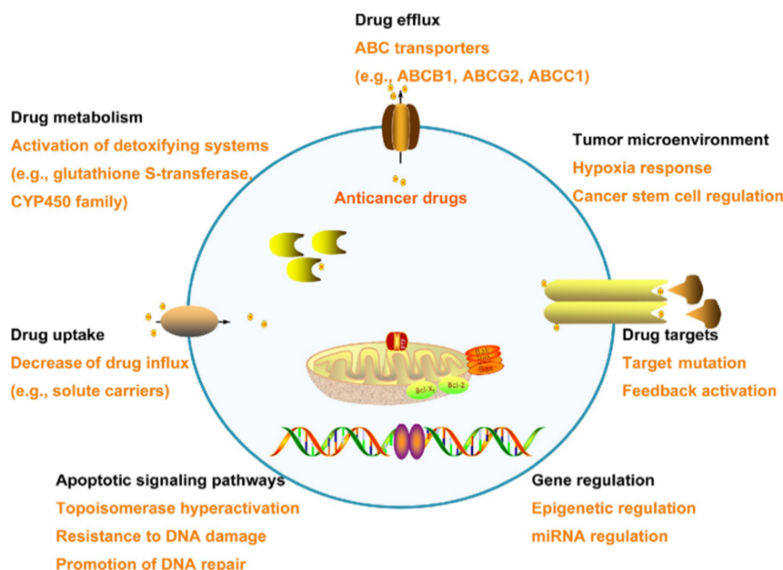

Figure 1.4 Mechanisms of Multidrug resistance in cancer
[29]

The most studied MDR transporters include P-gp, MRP1 and BCRP [30]. The overexpression of these transporters leads to efflux chemotherapeutic agents thereby reducing drug accumulation and causing tumor cells to become resistant. The specific association between membrane transporters and multidrug resistant phenotype was first discovered in the Chinese hamster ovary cell line in the 1970s. This cell line selected for resistance to colchicine, was found to also exhibited resistance to daunomycin and puromycin [31]. P-gp, a 170 kDa glycoprotein, regulates the export of a wide variety of anticancer drugs and other hydrophobic compounds including anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxins (etoposide, teniposide), vinca alkaloids (vincristine, vinblastine), taxanes and the cancer imaging agent tetraphenylphosphonium (TPP) [32]. Intravenous administered drugs such digoxin, cyclosporine, ivermectin, paclitaxel, vinblastine, grepafloxacin, indinavir and nelfinavir also undergo intestinal excretion

mediated by P-gp. P-gp is expressed in normal tissues, such as the epithelium of the kidneys, liver, intestine, pancreas, placenta and adrenal gland, where it excretes toxic metabolites [33]. It is also located at the apical, mucosal and lumenal surface of epithelial cells in organs often involved in drug absorption, distribution and excretion [34]. Literature has shown that tumors originating from tissues with naturally high levels of P-gp expression may be intrinsically drug resistant (e.g., colon, kidney, pancreas, and liver carcinoma) [34]. Several studies have reported the presence of P-gp mRNA and protein in clinical samples cancers such as leukemia, kidney, colon, breast, and lung cancers [31,32]. The elevated expression of P-gp has been linked to poor response to chemotherapy.

MRP1, a 190 kDa protein, was first discovered in an anthracycline-resistant cell line HL60/Adr [37]. Unlike P-gp, MRP1 is localized at the basolateral membrane of epithelial cell layers [68], and thus, it transports substrates towards the basolateral side of the epithelia. A significant number of drug substrates of P-gp overlaps with MRP1. MRP1 confers resistance to a wide range of anticancer drugs such as anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins, methotrexate (MTX), saquinavir, and mitoxantrone (MX); however, in contrast to P-gp, it does not confer resistance to taxanes. Taxanes are an important component of the P-gp resistance profile. Although the drug resistance profile of MRP1 and P-gp overlaps, their physiological substrate profile differs significantly. While substrates for P-gp are neutral or mildly positive lipophilic compounds, MRP1 can efflux of a broad array of physiological organic anions such as leukotriene C_4 (LTC_4) and dinitrophenyl-S-glutathione (DNP-SG) [19]. The discovery that MRP1 was capable of transporting both conjugated and non-conjugated organic anions broadened it pharmacological and physiological relevance. Conjugated xenobiotic organic

anions which are produced from phase II metabolism of drugs and GSH conjugated drugs are also effluxed by MRP1. It can also transport glucuronate conjugates (e.g., $E_2 17\beta G$), dianionic bile salts, and sulfate conjugates [28].

Clinically, MRP1 has been found to be up-regulated in a variety of solid tumors, including those of lung, breast, and prostate [32]. Its expression is a negative prognostic marker for early-stage breast cancer [38] and predicts poor response to chemotherapy and decreased survival in non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) [28, 29]. NSCLC forms majority of lung cancer cases and the disease is commonly aggressive and chemo-resistant. Interestingly, MRP1 is found to be frequently overexpressed in a large percentage of tumors prior to treatment and in the case of prostate cancer, the MRP1 levels is known to increase with disease progression [40]. The most compelling case for the clinical role of MRP1 in multidrug resistance is the case of neuroblastoma where there is a strong association of MRP1 with a negative clinical outcome. A retrospective study showed that MRP1 expression was found in 209 neuroblastoma samples. Multivariate analysis revealed that an increased MRP1 expression was highly predictive of a lower relapse free survival and the overall survival of patients [41]. The study provided evidence of MRP1 as a strong independent prognostic indicator in neuroblastoma. Interestingly, P-gp/ABCB1, expression did not predict clinical outcome [41]. This suggests that MRP1, not P-gp, is the primary mode of MDR in neuroblastoma.

BCRP, a (70 kDa) consisting of a single NBD and MSD, functions as either homodimers or homomultimers bridged by disulfide bonds. It was first cloned based on its overexpression in a doxorubicin-resistant MCF7 breast cancer cell line (MCF-7/AdrVp). BCRP expression is varied among normal tissues with the highest levels found in the

placenta. It is also prominently expressed on the apical membrane of the epithelium in the small intestine and colon and on the canalicular membrane of hepatocytes [42]. BCRP can mediate efflux of doxorubicin, mitoxantrone, topotecan, irinotecan and it active metabolite SN38, methotrexate, and tyrosine kinase inhibitors. In addition to hydrophobic substrates such as mitoxantrone, BCRP can also transport hydrophilic conjugated organic anions like the sulfate conjugates unlike P-gp [43]. BCRP has been found to be overexpressed in several tumors, however majority of work has been in the area of acute myeloid leukemia (AML). Several studies have shown a positive correlation between high levels of BCRP expression and poor clinical outcomes in AML, e.g., a relapsed or refractory disease state, lower response rate, shorter overall survival, and/or no complete remission. Other studies reported no correlation of BCRP expression with clinical outcomes or no expression of BCRP in AML. Factors such as low sensitivity of methods used and the co-expression with P-gp and MRP1 in patients with AML has been attributed as the possible reasons of the disparities in literature [44].

Several *in vitro* studied have demonstrated that TKIs such as imatinib, nilotinib, and dasatinib are modulators of P-gp and BCRP [45]. Clinical studies have also shown a correlation between mRNA expression of P-gp and BCRP in peripheral blood leukocytes and poor clinical outcomes in 118 chronic-phase CML patients receiving a standard dose of imatinib mesylate [46]. BCRP mRNA expression levels were higher in patients who did not respond to treatment before and during imatinib therapy. These data suggest that the elevated expression of BCRP may be associated with imatinib resistance.

1.4 Modulators of ABC transporters

Ligands which interact with ABC transporters can act as substrates (effluxed by transporter), inhibitor (impairs transport of other compounds), inducers (enhance transporter protein expression) or activators (enhance transporter activity). The different properties and modes of action can be explored for diverse therapeutic use. Inducers and activators are not always related as an increase in protein expression may not induce increased protein activity [17]. Whiles inducers promote upregulation of transporter, activators induce conformational changes which stimulates the transport/efflux of a substrate bound to another binding site. Both inducers and activators can be useful in conditions where toxicity to target tissues can be reduced due to transporter overexpression. Dexamethasone [47], rifampicin [47], sunlinac [48], vinblastine and TBHQ are all inducers of MRP1[49]. A compound can have overlapping modes of action. Some substrates can act as inhibitors. Generally, it has been much more difficult to find good small molecule inhibitors for MRP1 than for P-gp. This may be due to MRP1's preference for anionic compounds as substrates or inhibitors. Most anionic compounds enter cells poorly, thus it may be difficult to obtain good intracellular concentrations to aid effective inhibition. A variety of inhibitors of MRP1 have been identified including the LTC_4 analogue MK571, S-decylglutathione, sulfinpyrazone, benzbromarone and probenecid [13, 34, 35]. For specific MRP1 *in vivo* inhibition, general organic anion transporter inhibitors such as sulfinpyrazone, benzbromarone and probenecid are not suitable, as they affect organic anion uptake systems in cells. The main challenges with these inhibitors are their broad range specificity and cytotoxicity. In order to overcome MRP1 mediated resistance in cancer chemotherapy, better MRP1 inhibitors need to be developed, with higher

specificity, cellular penetration properties, and low cytotoxicity. The scope of this study focuses mainly on identifying inhibitors of MRP1. This work seeks to provide methodological alternatives to identify MRP1 inhibitors using high throughput screening based on high content imaging.

1.5 *In vitro* screening methods for evaluating ABC transporter interaction

Determination of a drug candidate as an inhibitor/substrate of ABC transporters is vital in profiling drug-transporter interactions as it helps predict the drug candidate's bioavailability *in vivo*. Drugs and food nutrients that modulate ABC transporters may also affect the bioavailability of a co-administered drug. Generally, *in vitro* assays are categorized as 'cell-based' or 'membrane-based' depending on whether an assay is performed with intact cells or with isolated membranes [51]. Both cell and membrane-based assays are riddled with their intricate advantages and disadvantages and are frequently performed together to verify and validate data derived from each other. Several *in vitro* assays have been developed to probe the interactions between drug candidates and these efflux transporters. ABC transporters are also well known to have a broad range of substrate specificity, due to the existence of multiple substrate binding sites. Thus, assay systems that utilize a single probe compound may not identify all substrates or modulators that bind at different binding sites resulting in false negatives. Cost reduction, effective prediction of drug-transporter interaction *in vivo*, and toxicity are areas that need improvement in the current systems used. In the following sections, some common in vitro assay for studying drug -transporter interactions relevant to this study are discussed.

1.5.1 Fluorescence accumulation and efflux assay

One of the most widely used cell-based assays for assessment of compound interaction with ABC transporters is the fluorescent accumulation assay. This assay measures the intracellular accumulation of a fluorescent substrate in a transporter-overexpressing cell line in the presence or absence of test compounds in order to understand the effect of test compounds on transporter activity. The intracellular accumulation of the fluorescent substrates is inversely proportional to the transporter activity and can be measured by fluorescence spectrophotometry. Thus, an increased intracellular accumulation of a given substrate (higher intracellular fluorescence) can be observed in the presence of an inhibitor, while the opposite (decreased intracellular accumulation) occurs in the presence of an inducer or activator. Discrimination between an inducer or activator is dependent on the incubation period with the test compounds. Activators need lesser time period to elicit their effect as they only induce conformational changes whiles inducer need extended incubation time period with drugs as de novo synthesis of transporter is needed. Generally, assays using a fluorescent probe to track transporter activity are also termed the dye extrusion assay or the uptake assay [52].

In efflux studies, however, the amount of fluorescent substrate in the extracellular environment of cells is measured under various conditions known to influence the transporter activity. Cells are preloaded with the substrate of interest in the presence of an inhibitor of the efflux transporter, the amount of fluorescent substrate expelled from the cells will be less than that observed for cells without inhibitor. In contrast, the amount of fluorescent substrate expelled will be greater in the presence of an inducer or activator. This method is thus based on the altered accumulation and efflux of a fluorescent substrate.

The analysis of the efflux activity of transporters may be based on the evaluation of the fluorescence accumulation, efflux or both.

Table 1.1 shows a list of common fluorescent substrates used for studying drug transporter interactions. Detection and quantification of the intracellular accumulated fluorescent substrate is commonly determined by flow cytometry. Quantitative measurements of the differences in fluorescence levels between non-treated control and drug treatments are thus used for inferences and analysis. Therefore, test compound that exhibit inherent fluorescence at the emission similar to that of the fluorescent substrate used can interfere with quantification. As such, it is important to inspect the background fluorescence of individual test compounds.

Table 1.1 Fluorescent substrates for ABC transporter assays

	ABCB1 (Pgp)	**ABCC1 (MRP1)**	**ABCG2 (BCRP)**	**REFERENCES**
Fluorescent probe				
Green/FITC				
BODIPY FL EDA	✔	✔	✔	[53]
BODIPY FL Forskolin	✔	✔	✔	[53]
BODIPY FL Histamine	✔	✔	✔	[53]
BODIPY FL Prazosin	✔	✔	✔	[54]
BODIPY FL Thapsigargin	✔	✔		[53]
BODIPY FL Verapamil, HCl	✔	✔		[53]
BODIPY FL Vinblastine	✔	✔		[55],
Calcein AM	✔	✔		[56], [57]
CBIC2(3) (JC-1)	✔	✔	✔	[58], [59],
DiNOC1(3) (JC-9)	✔	✔		[53]
DiOC2(3)	✔	✔		[60]
DiOC5(3)	✔	✔		[53]
DiOC6(3)	✔	✔		[61]
eFluxx-ID Green	✔	✔	✔	[62]
ER-Tracker Green	✔	✔		[53]
MitoTracker Green FM	✔	✔		[53]
Phengreen diacetate	✔	✔	✔	[63]
Rhodamine 123	✔			[57], [64]
SYTO 13	✔	✔		[53]
SYTO 16	✔	✔		[53]
SYTO 9	✔	✔		[53]
Yellow/PE				
Alexa Flour 555 hydrazide				[53]
Alexa Fluor 532, 594, 546 C5-maleimide				[61]
CellTracker Orange CMTMR				[53]
eFluxx-ID Gold	✔	✔	✔	[62]
MitoTracker Orange CMTMRos	✔			[53]
Rhodamine 6G chloride	✔	✔		[53]
Rhodamine B, hexyl ester, perchlorate (R6)	✔	✔		[53]
TMRE	✔			[53]
Red				
DiIC1(5)	✔	✔		[53]
Doxorubicin	✔	✔	✔	[65], [66], [60]
Mitoxantrone	✔	✔	✔	[67], [66]
Pheophobide A			✔	[68], [69]
DAPI/ Blue				
Dyecycle violet (DCV)	✔		✔	[70], [63]
Orange				
Tetramethylrosamine Chloride	✔	✔		[71], [53]

1.6 Traditional membrane-based assays

1.6.1 Inside-out Membrane Vesicles

Membrane vesicles prepared from cells expressing ABC transporters are a valuable tool for studying ABC transporter activities. Membrane vesicle preparation exposes the substrate-binding site of ABC transporters and allows better identification of certain classes of compounds due to matrix simplification of the vesicles. Membrane vesicles can be prepared from various sources such as intestinal brush border membranes, hepatic sinusoidal and canalicular membranes, encephalic luminal and abluminal membranes, and any transporter-expressing cell lines[72]. Cell lines engineered to overexpress individual transporters are especially useful for the evaluation of specific interactions between the transporter and compounds of interest. Vesicular transport studies using various types of membranes from different sources (insect cells [10], transfected [73] or selected [74] mammalian cell lines and artificial membrane vesicles [75] have been reported. Mammalian HEK293 and insect Sf9 membrane vesicles expressing ABC transporters are widely used as a screening tool in drug discovery [10] and are commercially available. The preparation of membrane vesicles involves the disruption of cells or tissues and the collection of the membrane fraction through ultracentrifugation. Three types of membrane formations can exist in a crude membrane preparation: lamellar, inside-out, and right-side-out (Figure 1.5) [73].

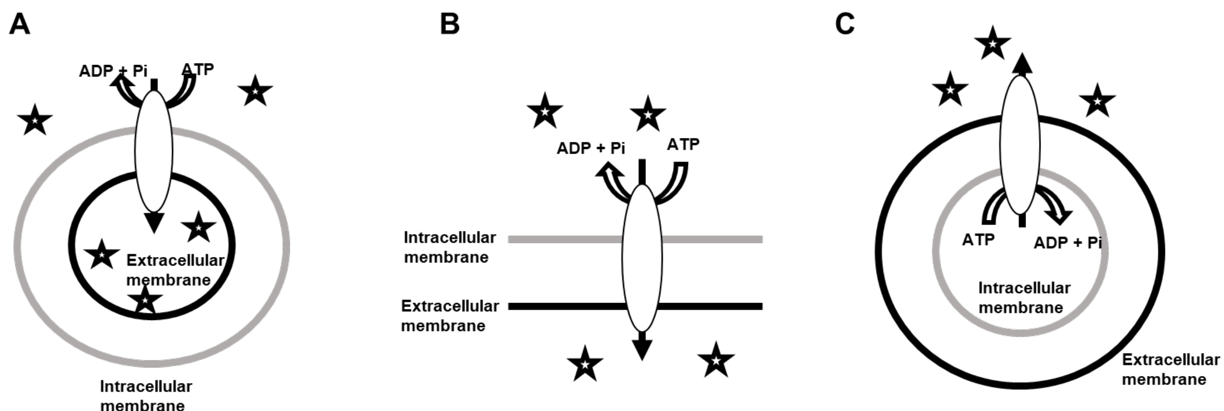

Figure 1.5 Various orientation of crude membrane vesicle

A. Membrane vesicles formed inside-out. Further enrichment of crude membrane vesicle can increase their percentage. B. Membrane vesicles in open lamellar configuration. C. Membrane vesicles in right side-out configuration.

In the membrane vesicular transport assay investigating efflux transporters, only inside-out vesicles contribute to the trapping of target substrates, so it is desirable to enrich vesicles with the inside-out orientation. Traditionally, low ionic strength buffer devoid of bivalent ions has been used to achieve this. Nitrogen cavitation is another feasible method for promoting the formation of inside-out vesicles in the crude membrane preparation [57, 9]. Purification of crude membrane using methods such as sucrose gradient centrifugation [58, 59] and concanavalin-A chromatography [75] can further enrich the concentration of inside-out vesicles [73]. Once the membrane vesicles are generated, they are relatively stable and can be stored at -80 °C for many months [73]. Membrane vesicles are also ideal for studying ABC transporter kinetics.

1.6.2 Membrane vesicular transport

The membrane vesicular transport assay is based on the ATP-dependent transport of a substrate into the inside-out membrane vesicles. It was the first *in vitro* assay developed for ABC transporter studies when Horio *et al*. used membrane vesicles prepared from a cancer cell line to detect the ATP-dependent transport of vinblastine by ABCB1 [76]. To date, it is commonly employed for the determination of transporter substrates and inhibitors, especially in a high throughput format [59, 42]. Recently, this assay has been coupled with targeted metabolomics for the identification of endogenous ABC transporter substrates present in bodily fluids, a novel approach termed 'transportomics' [62, 41].

Functionally, the membrane vesicular transport assay can be performed in a 'direct' or an 'indirect' (inhibition) setup [51]. In the direct setup, the transport of a compound of interest into the membrane vesicles is measured directly, enabling the identification of a substrate-type relationship with a given transporter. This setup, however, requires each test compound to be labeled or analyzed with sensitive analytic methods such as LC-MS/MS, making it rather costly for screening purposes. Furthermore, the direct setup is not robust in terms of identifying substrates with medium-to-high membrane permeability as they diffuse out of the membrane vesicles, resulting in false negative hits [72]. As a result, the direct setup is typically used for studying transport of compounds with low membrane permeability such as methotrexate, bile acids, and sulfate and glucuronide conjugates of various phytoestrogens [62, 41, 63, 64, 65, 66]. The indirect setup, on the other hand, examines the inhibitory effect of a test compound on the transport of a reporter substrate. Because only the reporter substrate needs to be labeled and/or analyzed, the indirect setup is well-suited for high throughput screening of compound-transporter interactions. As a

caveat, this setup will not provide information on whether the test compound is a substrate of the transporter being studied.

The quantity of the transported unlabeled molecules can be determined by HPLC, LC/MS, LC/MS/MS. Alternatively, the compounds are radiolabeled, fluorescent in nature or have a fluorescent tag so that the radioactivity or fluorescence retained on the filter can be quantified. Table 1.2 gives a list of common radiolabeled substrates used for transporter studies.

The vesicular transport assay can also be performed in an "indirect" set-up, where interacting test drugs modulate the transport rate of a reporter compound [53]. This assay type is particularly suitable for the detection of possible drug-drug interactions and drug-endogenous substrate interactions. This assay format is not sensitive to the passive permeability of the compounds and therefore detects all interacting compounds. However, being an indirect method, it will not give information on whether the compound tested is an inhibitor of the transporter or a substrate of the transporter inhibiting its function in a competitive fashion.

Table 1.2 Commonly used radiolabeled substrates for transport assays

Radiolabeled substrate	TRANSPORTER	REFERENCES
[^{14}C]-1-Chloro-2,4-Dinitrobenzene ([^{14}C]-) (CDNB)	MRP1, MRP2	[80],
[^{14}C]-2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*] Pyridine ([^{14}C]-PhIP)	BCRP, MRP2	[82]
[3',5',7'-^{3}H(n)]-Methotrexate	MRP2, MRP4	[83]
[^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)	BCRP	[51]
[^{3}H]-Daunorubicin	P-gp, MRP1	[84]
[^{3}H]-Docetaxel	MRP2	[85]
[^{3}H]-Etoposide	MRP2, BCRP	[86], [87]
[^{3}H]-Leukotriene C4 ([^{3}H]-LTC4)	MRP1	[81]
[^{3}H]-Methotrexate	BCRP	[79]
[^{3}H]-Mitoxantrone	BCRP	[79]
[^{3}H]-Paclitaxel	P-gp, MRP2	[61], [88]
[^{3}H]-Vinblastine	MRP2	[88]
[6,7-^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)	MRP1, MRP2, MRP3, MRP4	[88], [89]
[7-*methoxy*-^{3}H]-Prazosin	BCRP	[90]
[G-^{3}H]-Digoxin	P-gp	[57]
[G-^{3}H]-Vinblastine Sulfate	P-gp	[91]
[ring C, *methoxy*-^{3}H]-Colchicine	P-gp	[57]

1.7 Current/alternate methods for assessment of compound interactions

Relatively new assays are currently available for high-throughput screening of compound interaction with ABC transporters. Although not as widely used, they offer unique advantages over the abovementioned traditional assay systems. Some of these new assays

include high content imaging, bioluminescent imaging, fluorescence resonance energy transfer (FRET), and antibody binding shift assay. For the scope of this study we will focus mainly on high content imaging.

1.7.1 High Content Imaging

In recent years, imaging-based high content screening (HCS) using an automated microscope platform has gained increasing popularity in drug discovery and biomedical research. HCS allows high-throughput imaging of single or multiple biological activities measured as intensity or spatial localization of fluorescent dyes or proteins in cells or whole organisms. HCS platform offers unique advantages over traditional high throughput screening (HTS). The major advantage is the ability to provide multiple readouts. The readout is typically a fixed endpoint based on object segmentation, which minimizes background noise and facilitates automated analysis.

HCS methods have been used to investigate compound interactions with P-gp and BCRP. Ansbro *et al*. screened a kinase inhibitor library for P-gp inhibitory activity with an uptake assay using multidrug-resistant P-gp-overexpressing KB-V1 carcinoma cells and the parental KB-3-1 cells with calcein AM as the fluorescent probe [92]. Phase-contrast and fluorescent images were acquired which allowed for segmentation masks. This helped identify individual cells constructed based on phase-contrast images and used for the quantification of intracellular fluorescence intensity[92]. Using the HCS platform, several P-gp modulators were identified. The assay also provides greater sensitivity over plate reader, which is more suitable for homogeneous assay. We recently set up an uptake assay

for studying compound interaction with MRP1 using a similar method [93]. Example for cell segmentation is shown in Figure 1.5. An additional, advantage of HCS is that unlike assays using a flow cytometer, cells can be imaged *in situ* without the need of preparing cell suspension and performing multiple washing steps, which increase the processing time and limit the assay throughput. Furthermore, cell viability and density before and after treatment can be visually inspected in bright-field or phase-contrast images. This assists in the evaluation of compound cytotoxicity, which may interfere with the assay.

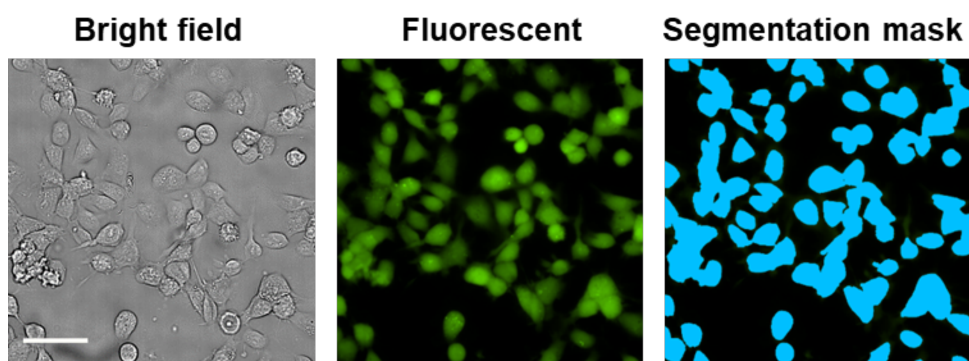

Figure 1.6 Example of high content images and segmentation
[93]

The examples discussed above improve the sensitivity and processing time of traditional uptake assay. However, HCS platform can offer additional analysis capability studying multiple functions simultaneously. In a recent study conducted by Antczak *et al.*, an HCS platform was used to simultaneously investigate BCRP-inhibitory and cytotoxicity of test compounds [94]. Using human glioblastoma cell line U87MG stably expressing BCRP, uptake of a fluorescent BCRP substrate, JC-1 (J-aggregate–forming lipophilic cation

5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl benzimidazole carbocyanine iodide), after a 16-h incubation was used to probe compound inhibition of BCRP, while Hoechst accumulation in the nuclei was used to reflect cell number, which indicates cytotoxicity of test compounds.

1.8 Rationale of Study

ABC transporters are involved with the uptake of small molecules thus recognized as the key drug transporters for assessment in the drug discovery and development process. This recommendation was made by the International Transporter Consortium (ITC), a team of experts in pharmacology and pharmaceutical sciences from academia, industry, and the US Food and Drug Administration (FDA) in 2007 [10]. Currently, recommendations for the appropriate *in vitro* and *in vivo* studies for drug-transporter interaction are only available for ABCB1 (MDR1) and ABCG2 (BCRP) [60]. We anticipate that with an increased appreciation for the relevance of ABC transporters, interactions between drug candidates and ABC transporters will become a routine assessment.

Drug discovery and development is an intricate, time-consuming, and costly process. The cost of successfully developing an approved drug from a new molecular entity (NME) is estimated to be between $800 million and $1.2 billion, with a development timeline of 8–12 years [95]. Between 2007 and 2010, the combined success rate at Phase III clinical trial and submission fell to approximately 50%, with major causes of attrition being lack of efficacy and safety issues [96]. In Phase II clinical trials, apart from strategic failures, lack of efficacy, safety issues, and unfavorable bioavailability are reasons drug candidates fail

[97]. As the key efflux transporters regulating the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of xenobiotics, ABC transporters can influence the bioavailability and toxicity of a substrate drug through direct efflux, drug-drug and drug-nutrient interaction (DDI and DNI) [10], [98]. Efflux by ABC transporters in organs such as intestines, livers, and target organs can limit the bioavailability of drugs, resulting in sub-therapeutic concentrations of drugs [98]. Conversely, the efflux activity of ABC transporters can be crucial in liver toxicity [98]. Considering the importance of ABC transporters in determining drug absorption, disposition, and toxicity, there is a need for profiling the interactions of leading drug candidates with ABC transporters.

Developing high content screening assays for ABC transporters may provide an effective pathway against drug-induced toxicity. With new test compounds and pharmacological libraries being developed against a wide range of diseases, it is important to develop screening methods with high specificity and reliability for identifying modulators of ABC transporters especially for transporters that are implicated in multidrug resistance phenotype (P-gp, MRP1, and BCRP). The use of just one traditional in vitro screening method is fraught with challenges such as the possibility of false negative results because of the relatively low sensitivity of these methods.

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Chapter 2

Doxorubicin as a Fluorescent Reporter Identifies Novel MRP1 (ABCC1) Inhibitors Missed by Calcein-Based High Content Screening of Anticancer Agents

Abstract

Multidrug resistance protein 1 (MRP1/ABCC1) actively transports a variety of drugs, toxic molecules and important physiological substrates across the plasma membrane. It can confer broad-spectrum multidrug resistance and can decrease the bioavailability of many important drugs. Substrates of MRP1 include anti-cancer agents, antibiotics, antivirals, antidepressants and anti-inflammatory drugs. Using calcein as a fluorescent reporter in a high content efflux assay, we recently reported the identification of 12 MRP1 inhibitors after screening an anti-cancer library of 386 compounds. Here, we describe the development of a new high content imaging-based efflux assay using doxorubicin as a fluorescent reporter. Screening the same anti-cancer library of 386 compounds, the new assay identified a total of 28 MRP1 inhibitors including 16 inhibitors that have not been previously reported as inhibitors of MRP1. Inhibition of MRP1 activity was confirmed using flow cytometry, confocal microscopy and membrane vesicle-based transport assays. Six drugs (afatinib, celecoxib, doramapimod, mifepristone, MK-2206 and rosiglitazone) were evaluated for their ability to reverse resistance of MRP1-overexpressing H69AR lung cancer cells against vincristine, doxorubicin and etoposide. Mifepristone and doramapimod were most effective in reversal of resistance against vincristine while mifepristone and

rosiglitazone were most successful in resensitizing H69AR cells against doxorubicin. Furthermore, resistance towards etoposide was completely reversed in the presence of celecoxib or doramapimod. Selected drugs were also evaluated for resistance reversal in HEK cells that overexpress P-glycoprotein or breast cancer resistance protein. Our results indicate mifepristone and doramapimod as pan inhibitors of these three drug transporters while celecoxib exhibited selective MRP1 inhibition. Together, our findings signify the importance of MRP1 in drug discovery and demonstrate the effectiveness and value of doxorubicin-based high content screening approach. Anti-cancer agents that exhibit MRP1 inhibition may be used to reverse multidrug resistance or to improve the efficacy and reduce the toxicity of various cancer chemotherapies. On the other hand, anti-cancer drugs which did not interact with MRP1 carry low risk for developing MRP1-mediated resistance.

Keywords: MRP1; ABCC1; ABC transporter; multidrug resistance; MRP1 inhibitors; drug absorption and disposition; high content screening; anti-cancer agent; drug-transporter interactions; doxorubicin; drug profiling

1) Afatinib (PubChem CID: 10184653); 2) Alisertib (PubChem CID: 24771867); 3) Alvespimycin (PubChem CID: 5288674);; 4) Amuvatinib (PubChem CID: 11282283); 5) Celecoxib (PubChem CID: 2662); 6) Doramapimod (PubChem CID: 156422); 7) Flavopiridol (PubChem CID: 5287969); 8) GSK2126458 (PubChem CID: 25167777); 9) GSK461364 (PubChem CID: 15983966); 10) GW4064 (PubChem CID: 9893571); 11) LY2228820 (PubChem CID: 11570805); 12) LY294002 (PubChem CID: 3973); 13) Mifepristone (PubChem CID: 55245); 14) MK-2206 (PubChem CID: 24964624); 15)

NVP-BSK805 (PubChem CID: 57339395); 16) OSI-420 (PubChem CID: 18924996); 17) Rosiglitazone (PubChem CID: 77999); 18) Saracatinib (PubChem CID: 10302451)

1.0 Introduction

Cancer is the second leading cause of death worldwide with 8.8 million deaths occurring in 2015 and an estimated 20 million new cases expected annually till 2025 [1, 2]. Combinatorial therapeutic approach of using multiple cytotoxic drugs for cancer treatments has led to improvements in survival rates for various types of cancers [3] . However, the emergence of drug resistance in tumor cells in many cancers over the years have been a challenge to treatment. Tumor cells when exposed to cytotoxic drugs can develop cross-resistance to a wide range of compounds [4, 5]. This phenomenon, known as multidrug resistance (MDR) makes tumors unresponsive to a variety of drugs irrespective of their differences in structure and molecular targets [3] and can lead to failure in chemotherapy. Conventional chemotherapeutic drugs such as doxorubicin, vincristine, actinomycin-D, and paclitaxel have been previously shown to induce MDR [6].

Development of resistance is a major obstacle in the success of chemotherapy. MDR is reportedly responsible for over 90% of chemotherapy failures of metastatic cancers involving surgery or radiation [7]. MDR can be acquired through various mechanisms such as alteration of drug target, decreased drug absorption, increased drug efflux, increased cell repair activity, alteration in lipid membrane composition and altered cell cycle check points [3, 7]. However, the overexpression of ATP-binding cassette (ABC) drug efflux transporters is the most frequent mechanism of MDR and is widely studied [3, 8, 9, 10]. A total of 48 ABC transporter genes have been reported in the human genome and they are grouped into seven subfamilies, designated A-G [11]. In particular, ABC drug transporter proteins such as P-glycoprotein (P-gp/ABCB1), multidrug resistance protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) are frequently

upregulated in cancer [12, 13]. These drug transporters actively pump out chemotherapeutic drugs reducing the intracellular levels of drugs and thereby attenuating their cytotoxic actions.

The prototypical eukaryotic ABC proteins have a four-domain core structure, containing two hydrophobic membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs) that are cytosolic [14, 15]. Structural studies of the ABC transporter proteins revealed that the two cytosolic NBDs coordinate in a head-to-tail orientation to form a "sandwich" dimer that generates two composite nucleotide binding sites. Furthermore, the two membrane-spanning domains are intertwined to form the substrate-binding site/s and the substrate translocation pathway and extend into the cytoplasm making contacts with the NBDs. The binding and hydrolysis of ATP which is coupled to substrate binding provides the energy required for the transport process [16]. The structure of MRP1 and several "C" subfamily members contain an additional MSD at the amino-terminus of the protein, whose specific biological role is poorly understood.

MRP1 is an integral membrane protein that functions as an ATP-dependent drug efflux pump. It localizes at the plasma membrane and can efflux a wide variety of endogenous and exogenous substrates including toxic chemicals, drugs and their metabolites out of cells. Overexpression of MRP1 can confer resistance against commonly used cytotoxic anti-cancer agents like doxorubicin, vincristine, methotrexate and etoposide. MRP1-overexpression has been associated with MDR of several types of cancers in clinics. In addition to anti-cancer agents, MRP1 can reduce the efficacy of many commonly used drugs including antibiotics, antivirals, antidepressants, anti-inflammatory and anti-HIV drugs [8, 9, 10, 17, 18] Therefore, MRP1 is considered an important therapeutic target and

may be used to improve the efficacy of various therapies including cancer chemotherapy. On the other hand, MRP1 plays an important role in tissue defense and in regulation of various biochemical processes such as redox homeostasis, steroid metabolism, lipid metabolism, and the etiology of neurodegenerative, immunological, and cardiovascular pathologies [19, 20]. It effluxes a remarkable variety of xenobiotics and organic anions from endogenous sources, which are mostly conjugated to glutathione, glucuronide, or sulfate. Physiological substrates of MRP1 include organic anions such as cysteinyl leukotriene (LTC_4), estradiol glucuronide ($E_217\beta G$), glutathione (GSH), and cobalamin. Automated fluorescence imaging-based high content screening platform is becoming very popular in drug discovery and development research due to its capability to provide high resolution *in situ* visual data that allows to simultaneously study multiple phenotypes. We have recently demonstrated the development and validation of a high content imaging-based efflux assay using calcein-green as a substrate reporter of MRP1 transport activity [9]. Using this assay in screening a unique library of 386 anti-cancer compounds, we identified a total of 12 inhibitors of MRP1 that included 10 novel inhibitors not previously reported to interact with MRP1. Doxorubicin, a cytotoxic anti-cancer drug, is a well-known substrate of MRP1 and P-gp. However, it has not been successfully used in high-throughput screening assays using flow cytometry or fluorescent microplate reader-based methodologies likely due to its dimness and relatively poor affinity for the transporter protein. Due to the polyspecificity and the promiscuous nature of MRP1 substrate binding, we hypothesized that doxorubicin-based screening assay may identify novel inhibitors of MRP1. In this study, we describe the development, optimization, and validation of a high content imaging-based efflux assay using doxorubicin as a fluorescent reporter substrate.

The value and effectiveness of this assay is demonstrated by screening the library of 386 anti-cancer drugs. The screening process identified a total of 28 inhibitors, which included 10 of 12 inhibitors discovered previously with calcein-green assay as well as 18 MRP1 inhibitors that were missed by the calcein-based screening. We verified the inhibitory activity of the identified compounds using established methods and discovered 16 compounds that have not been previously known to inhibit MRP1. The ability of some selected compounds to reverse the resistance of an MRP1-overexpressing MDR cancer cell line is also demonstrated.

2.0 Materials and methods

2.1 Chemicals

Doxorubicin, adenosine monophosphate (AMP), adenosine triphosphate (ATP), estradiol 17-(β-D-Glucuronide) ($E_2$17βG), poly-D-lysine, thiazolyl blue tetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO). MK571 was acquired from Cayman Chemical (Ann Arbor, MI) and [6,7-^{3}H]$E_2$17βG (49.9 Ci mmol^{-1}) from PerkinElmer (Waltham, MA). Anti-cancer compound library consisting of 386 anti-cancer small molecules under clinical trials for 12 different types of cancers was procured from Selleck Chemicals (Houston, TX).

2.2 Cell lines and cell culture

H69 and H69AR cells were purchased from ATCC (Manassas, VA). HEK293T cells were kindly provided by Dr. Adam Hoppe (South Dakota State University, Brookings, SD)

while HEK293/pcDNA3.1, HEK293/BCRP and HEK293/P-gp (MDR1) were a kind gift from Dr. Suresh V. Ambudkar (NIH, Bethesda, MD). HEK293 and H69 cell lines were cultured in DMEM (GE Healthcare, Marlborough, MA) and RPMI 1640 (ATCC), respectively, supplemented with 10% fetal bovine serum. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37 °C. H69AR cells were exposed to 0.8 μM doxorubicin once a month and cultured drug-free for a week before use.

2.3 MRP1 inhibition screening with automated image acquisition and analysis

Assay development and optimization were performed with H69 and H69AR cells with doxorubicin as the fluorescent substrate and MK-571 as the positive inhibition control. Cells were seeded at 6×10^4 cells per well in 100 μL culture medium in 96-well optical-bottom plates with polymer base (Thermo Fisher Scientific, Waltham, MA) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) and incubated overnight. Culture medium was removed prior to drug treatment and replaced with 80 μL of serum-free medium. For pretreatment, 10 μL of test compounds (10 μM final concentration), DMSO (0.2% final concentration) as negative control, or MK-571 (50 μM final concentration) as positive control were added. Following the 30 min incubation, 10 μL of doxorubicin (10 μM final concentration) was added and the cells were incubated for 2 h. At the end of the incubation period, treatment was removed, cells were rinsed once and 100 μL of PBS containing 10mM HEPES and 4.5% glucose was added.

Images were obtained using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA) equipped with a 0.70 numerical aperture 60×

objective. A total of 8 images (4 bright field and 4 fluorescent) were taken for each well. Fluorescent images were acquired using a texas red filter with excitation and emission wavelengths of 562/40 nm and 624/40 nm, respectively, with an exposure time of 100 ms. As the intracellular accumulation of doxorubicin slowly dropped over time, a negative and positive control were included in every two columns. Screening experiments were performed three independent times. Fluorescent images were analyzed using the MetaXpress software (version 5.10.41, Molecular Devices). Segmentation of fluorescent objects on the texas red channel was done using a custom application module based on the 'Find Blobs' module. The custom module differentiates fluorescent accumulation from background and artifacts by applying segmentation masks based on set parameters for object size.

2.4 Flow cytometry-based doxorubicin accumulation assay

Flow cytometry was used to confirm the ability of drugs to inhibit MRP1 mediated efflux of doxorubicin. H69AR cells were prepared in serum-free culture medium to a density of 7×10^5 cells/mL. For this assay, 1 mL of cells were incubated with test compounds (10 μM) at 37 °C for 1 hour after which 10 μM of doxorubicin was added for an additional 2 hours. Total DMSO concentration was maintained at 0.2% (v/v). Efflux activity of MRP1 was stopped by the addition of ice-cold PBS buffer. Cells were then collected, washed with cold PBS and resuspended in ice-cold PBS containing 1% formaldehyde. Intracellular fluorescence of doxorubicin was detected by BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) equipped with 488nm and > 670nm for excitation and emission

respectively. Fluorescence intensity was collected as mean of 10,000 events. Treatments were performed in duplicate and repeated in 3 independent experiments.

2.5 Doxorubicin accumulation assay using confocal microscopy

HEK293T cells were plated on poly-D-lysine-coated cover glass placed in a 6-well plate at a density of 5×10^5 cells/well in 2 mL culture medium. Cells were transiently transfected with an MRP1-GFP expression vector after 24 hours using jetPRIME Transfection Reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's protocol. After 48 hours, cells were pre-treated with 10 μM test compound for 30 min, before incubation with doxorubicin (10 μM) for 1 h. Cells were maintained in buffer (4.5 % glucose, 10mM HEPES, PBS containing Ca^{2+} and Mg^{2+}) as intracellular fluorescence was visualized using an iMIC digital microscope (TILL Photonics GmbH, Gräfelfing, Germany) equipped with a 1.35 numerical aperture 60x oil-immersion objective. Excitation was done at 488 nm for GFP and doxorubicin, with emission bands of 475/42 and 605/64 nm, respectively. Images were processed using ImageJ (NIH, Bethesda, MD).

2.6 Membrane vesicle preparation

Membrane vesicles were prepared as described in [21] with modifications. Frozen cell pellets of HEK293/pcDNA 3.1 and HEK293/MRP1 were thawed and suspended containing 50 mM Tris.HCl, pH 7.4, 250 mM sucrose, 0.25 mM $CaCl_2$, and 1x complete protease inhibitors (Santa Cruz Biotechnology, Dallas, TX). Cell disruption was achieved *via* nitrogen cavitation at 450 psi for 5 min. The resulting lysates were supplemented with

1mM EDTA and centrifuged at 500 × *g* at 4 °C for 10 min. The supernatant was collected twice by resuspending cell pellets and repeating centrifugation. Pooled supernatant was layered over 35% (w/w) sucrose containing 10 mM Tris.HCl, pH7.4, and 1 mM EDTA and centrifuged at 25,000 rpm at 4 °C for 1 h in a SW28 rotor (Beckman Coulter, Brea, CA). The opaque membrane interface formed was collected and washed twice by ultracentrifugation. The membrane pellet obtained was further resuspended in transport buffer (50 mM Tris.HCl, pH 7.4, and 250 mM sucrose) and passed 20 times through a 27-gauge needle for vesicle formation. Protein concentration was determined using Quick Start Bradford Protein Assay (BioRad, Hercules, CA).

2.7 Membrane vesicular transport assay

A rapid filtration technique was used to measure the ATP-dependent transport of [3H]$E_2$17βG into MRP1 enriched inside-out membrane vesicles [22]. The vesicles (2 μg protein) were incubated with 400 nM/20 nCi [3H]$E_2$17βG for a minute at 37°C in a 30-μL reaction mixture containing 4 mM AMP or ATP, 10 mM $MgCl_2$, and test compound in transport buffer (250 mM sucrose and 50 mM Tris-HCl, pH 7.4). Total DMSO concentration was kept at 0.29%. Reaction was stopped by the addition of ice-cold buffer and resulting mixture transferred to a 96-well MultiScreenHTS-FB plate (EMD Millipore, Billerica, MA). Filter membranes were washed 4X with 200 μL ice-cold suspension buffer under vacuum aspiration. Radioactivity retained on the membranes was measured using a Tri-Carb 4810TR liquid scintillation counter (PerkinElmer, Waltham, MA). ATP-

dependent uptake was calculated by subtracting the uptake measured in the presence of AMP from the uptake in the presence of ATP. Treatments were performed in triplicates.

2.8 Drug sensitivity assay

The sensitivity of H69 and H69AR cells towards multiple chemotherapeutic drugs (vincristine, doxorubicin, etoposide) and the ability of test compounds to reverse the resistance of H69AR cells against these drugs were analyzed using the MTT colorimetric assay. The sensitivity of HEK293, HEK293/BCRP and HEK293/P-gp towards mitoxantrone and vincristine respectively was also tested. H69 and HEK293 cells were seeded in 96-well plates (CellBIND®, Corning) at 2.5×10^4 and 5×10^3 cells per well in 100 μL culture medium. After 24 h, cells were pretreated with 50 μL of test compounds in culture medium and incubated for an hour. An additional 50 μL of cytotoxic drugs (vincristine, doxorubicin, etoposide or mitoxantrone) at varying concentrations was then added to the cells. Final DMSO concentration was maintained at 0.2%. Cells were further incubated for 96 h (H69 cell lines) or 72 (HEK293 cell lines). At the end of the incubation period, 100 μL of culture medium was carefully removed and cells were treated with MTT (0.5 mg/mL) for 4 h. The formazan crystals were dissolved by the addition of 100 μL of 15% SDS containing 10 mM HCl and absorbance at 570 nm were recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicate.

2.9 High content screening data analysis

The MetaXpress software was used for analysis. The software derives the mean fluorescent intensity of each well from averaging the fluorescent intensities of the segmentation mask

of 4 captured images. The use of segmentation mask prevents the inclusion of background fluorescence. The relative inhibition of each test compound on doxorubicin efflux was determined for each well using the following equation:

$$\% \, inhibition = \frac{X_T - X_{doxorubicin}}{X_{MK\text{-}571} - X_{doxorubicin}} \times 100 \,,$$

where X represents the average fluorescent intensities and T represents the test compound. Both positive and negative controls were placed in every two columns and used for the determination of the percent inhibition for compounds within the same columns. The Z'-factor, a parameter commonly used to infer the versatility and variation of an assay [23] was determined with the following equation:

$$Z'\text{-}factor = 1 - \frac{3(\sigma_{MK\text{-}571} + \sigma_{doxorubicin})}{|\mu_{MK\text{-}571} - \mu_{doxorubicin}|},$$

where σ and μ represent the standard deviations and means, respectively.

2.10 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). The differences between mean values were analyzed using linear mixed model analysis. Dunnet correction was applied for multiple comparisons. For all analyses, differences were considered significant at *P* value lower than 0.05.

3.0 Results

3.1 Assay development and optimization

Calcein-AM, a well-known substrate for MRP1 and P-gp, is commonly used to study the activity of these transporters in cell-based assays. We recently reported the development of an imaging-based high-throughput efflux assay for MRP1 using calcein-AM in high content screening system which offers a much more robust and efficient system compared to a fluorescent microplate reader or flow cytometer [9]. Doxorubicin, a fluorescent compound and a well-known substrate of MRP1 and P-gp, is a very commonly used cytotoxic chemotherapeutic agent for various types of cancer. However, due to its relatively low affinity for MRP1 and P-gp the efflux assay produces a very narrow dynamic range between positive and negative controls. In addition, high non-specific background fluorescence gives many false hits and has not worked well for MRP1 inhibitor screening using fluorescence intensity-based microplate reader method. Consequently, doxorubicin has not been successfully used as a substrate reporter in high-throughput efflux assays. Here, High-Content Screening System which contains advance image analysis and quantitation tools was used to develop an imaging-based high-throughput efflux assay for MRP1 using doxorubicin as a fluorescent substrate reporter. We used parental H69 cell line and its MRP1-overexpressing derivative H69AR cell line for the development of this assay. The basic idea is that H69 cells are expected to show higher levels of fluorescence due to very low expression levels of endogenous MRP1 and other transporters capable of effluxing doxorubicin. In contrast, H69AR cells over-expressing MRP1 will show much lower fluorescence accumulation under similar experimental conditions due to active doxorubicin efflux.

Fluorescent intensities of images were quantified using the 'Find Blobs' option in the custom module of the MetaXpress software. Cells were identified using object segmentation in the texas red channel. To develop and optimize the assay conditions, both time and concentration dependent accumulation of doxorubicin in H69 and H69AR cells were studied. As shown in Fig. 2.1A, increased concentrations of doxorubicin yielded an increase in intracellular fluorescence in both cell lines. However, intracellular accumulation of doxorubicin was much higher in the parental H69 cells as compared with MRP1-overexpressing H69AR cells for all tested concentrations. To determine if the difference observed between the two cell lines is specifically due to MRP1 transport activity, effect of MRP1 inhibitor, MK571, was observed at different doxorubicin incubation times. As shown in Fig. 1B, MRP1 inhibition by MK571 did not affect the doxorubicin accumulation in H69 cells irrespective of the doxorubicin incubation time. In contrast, MRP1-overexpressing H69AR cells showed much higher doxorubicin accumulation with MK571 treatment compared with untreated H69AR cells at all doxorubicin incubation time points (Fig. 2.1C). These data provide the basis and feasibility of using H69AR cells to develop a high-throughput screening assay to identify inhibitors of MRP1 using doxorubicin as a fluorescent substrate reporter.

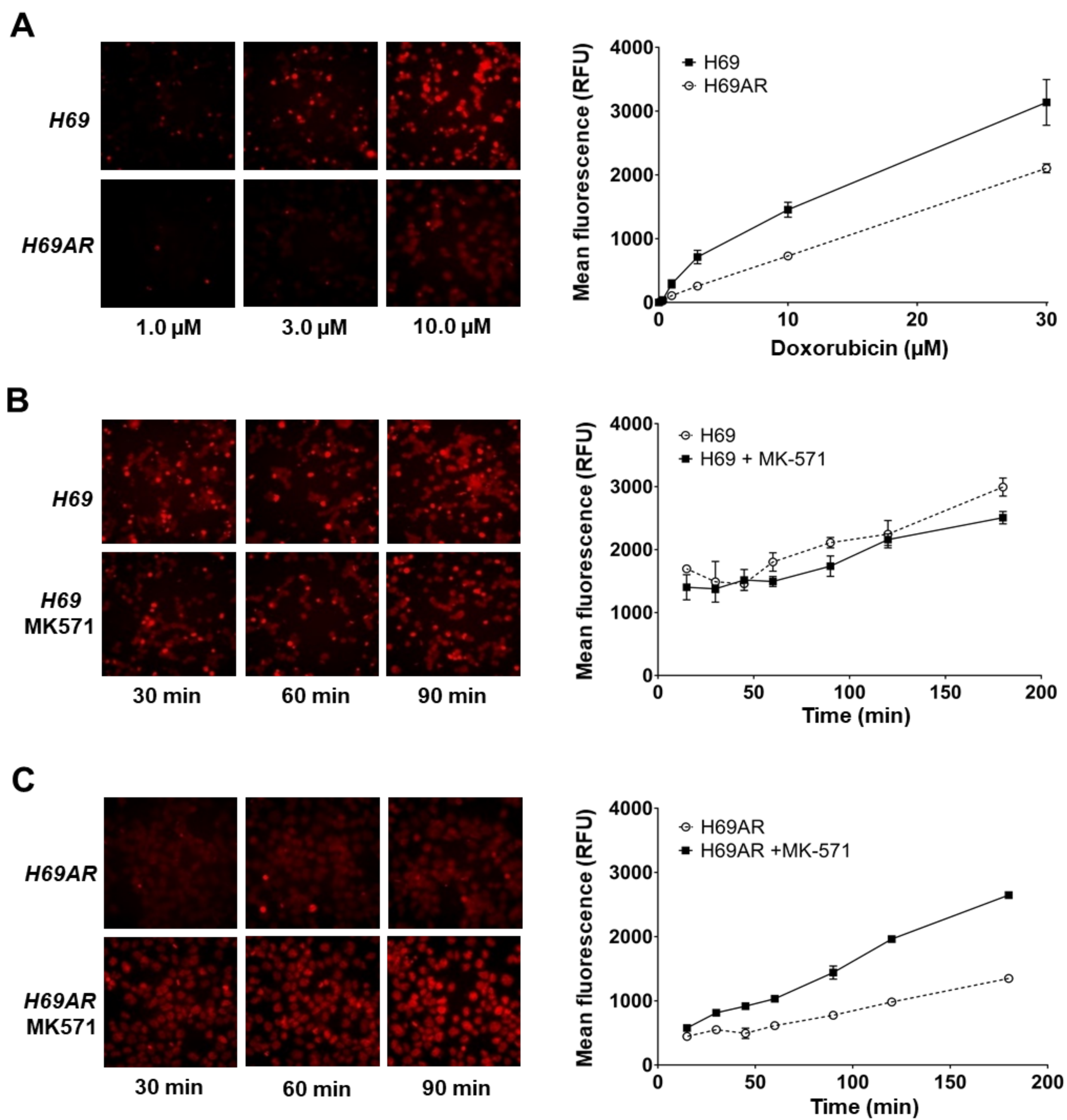

A
H69
H69AR
1.0 µM
3.0 µM
10.0 µM
H69
H69AR
Mean fluorescence (RFU)
Doxorubicin (µM)
B
H69
H69
MK571
30 min
60 min
90 min
H69
H69 + MK-571
Mean fluorescence (RFU)
Time (min)
C
H69AR
H69AR
MK571
30 min
60 min
90 min
H69AR
H69AR +MK-571
Mean fluorescence (RFU)
Time (min)

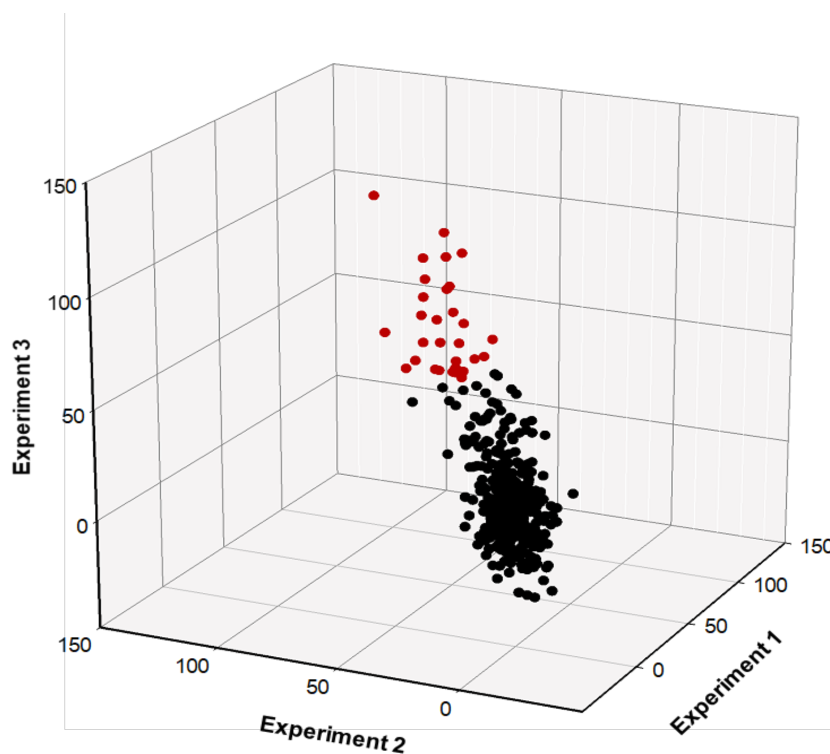
Figure 2.1 Concentration- and time-dependent accumulation of doxorubicin in H69 and H69AR cells

A. H69 and H69AR cells were treated with doxorubicin at various concentrations (1–30 μM) for 1 h. Representative images of doxorubicin treatment at 1, 3, and 10 μM are shown. The average fluorescence intensities derived from the fluorescent images were graphed and shown on the right. **B.** H69 cells were treated with 10 μM doxorubicin in the absence and presence of 50 μM MK571 for 15–180 min. Representative images of doxorubicin treatment at 30, 60, and 90 min are shown. **C.** H69AR cells were treated with 10 μM doxorubicin in the absence and presence of 50 μM MK571 for 15–180 min. Representative images of doxorubicin treatment at 30, 60, and 90 min are shown. Data are representative of two experiments and shown as mean ± SD (n = 3).

3.2 Screening of anti-cancer compound library for MRP1 inhibitors

After the development and optimization of high-content imaging-based MRP1 efflux assay using doxorubicin, we sought to identify novel MRP1 inhibitors within a library of 386 anti-cancer small molecules under clinical trials for 12 different types of cancers. The anti-cancer library was screened using MRP1-overexpressing H69AR cells and doxorubicin as a fluorescent substrate reporter. Treatment with 50 μM MK571 (a commonly used MRP1 inhibitor) was considered as 100% MRP1 inhibition for calculating percent inhibition for the test compounds. Three independent screening experiments were done using the 96-well format. The relative MRP1-inhibitory activities of the test compounds from three independent experiments are represented as a 3D scatter plot (Fig. 2.2). As indicated in Fig. 2.2 (bottom), the assay had good reproducibility with a correlation range of 0.73-0.85

between any two given experiments. The quality and performance of the assay was also assessed by deriving the Z'-factor from the experiments. The average Z'-factor across all plates was 0.58, indicating a good assay performance. A positive hit was defined as a compound with ≥ 40% mean percent inhibition. Using this threshold value, we identified a total of 33 hit compounds. Five of the hits were autofluorescent compounds and were discarded. The remaining 28 hit compounds are displayed as red dots in the 3D scatter plot (Fig. 2). Identified MRP1 hits, together with their therapeutic targets and percent inhibition are shown in Table 1. Ten of the 28 hits were recently identified by our group by screening the anti-cancer library using calcein as a fluorescent substrate and are listed in Table 1 as well. Among the remaining 18 hit compounds, celecoxib and LY294002 have been reported as MRP1 inhibitors [24, 25] while 16 anti-cancer hit compounds have not been previously reported to inhibit MRP1 transport activity. The structure of the 18 hit compounds is presented in Fig. 2.3.



Correlation coefficient

Experiment	1	2	3
1	1.00		
2	0.85	1.00	
3	0.82	0.73	1.00

Figure 2.2 Performance of the imaging-based MRP1-mediated doxorubicin accumulation screening assay

Screening of the anti-cancer compound library was performed in three independent experiments at compound concentration of 10 μM. The relative inhibitory activities of each compound were calculated and displayed as a 3D plot. Red dots represent compounds with mean percent inhibition of ≥50%. The table below the plot shows correlation coefficients between any two experiments. 3D scatter plot was generated using SigmaPlot 12.0 and

correlation coefficients were calculated using MS Excel. Correlation and graph exclude 5 compounds identified as autoflourescent.

Table 2.1 Chemotherapeutic targets, % inhibition for calcein/doxorubicin accumulation inhibition for identified MRP1 inhibitors

Compound	Chemotherapeutic targets	% Doxorubicin Inhibition	% Calcein Inhibition
MK-571		100.0 ± 0	100.0 ± 0
Tipifarnib	Transferase	86.3 ± 9.3	199.1 ± 56.8
AZD1208	Pim	71.2 ± 13.8	145.7 ± 25.3
Rapamycin	Autophagy	66.1 ± 10.0	142.5 ± 12.2
Deforolimus	mTOR	74.6 ± 10.9	111.9 ± 19.4
HS-173	PI3K	86.7 ± 7.7	94.0 ± 21.8
YM201636	PI3K	54.3 ± 5.9	81.9 ± 4.9
ESI-09			74.6 ± 11.8
Everolimus	mTOR	69.2 ± 4.6	72.6 ± 7.8
TAK-733	MEK	59.4 ± 8.9	67.4 ± 12.7
CX-6258	Pim		57.1 ± 16.4
Cyclosporin A		61.3 ± 11.6	50.2 ± 2.7
Temsirolimus	mTOR	45.0 ± 4.0	43.3 ± 4.2
GSK2126458	mTOR	94.0 ± 13.5	
Flavopiridol	CDK	68.4 ± 7.1	
Mifepristone		67.5 ± 7.1	
NVP-BSK805	JAK	65.8 ± 0.8	
Saracatinib	Src	65.7 ± 3.4	
Alisertib	Aurora A kinase	64.1 ± 10.3	
OSI-420	EGFR	57.8 ± 2.7	
LY294002	PI3K	56.6 ± 5.3	
Rosiglitazone		56.1 ± 3.6	
Alvespimycin	HSP90	55.9 ± 7.4	
MK-2206	AKT	54.1 ± 16.0	
Celecoxib	COX-2	53.4 ± 6.7	
Amuvatinib	C-Kit	53.3 ± 6.5	
LY2228820	P38 MAPK	53.3 ± 4.1	
GSK461364	PIK1	52.7 ± 1.3	
GW4064	FXR	52.3 ± 1.8	
Afatinib	EGFR	50.9 ± 7.7	
Doramapimod	MAPK	50.8 ± 2.0	

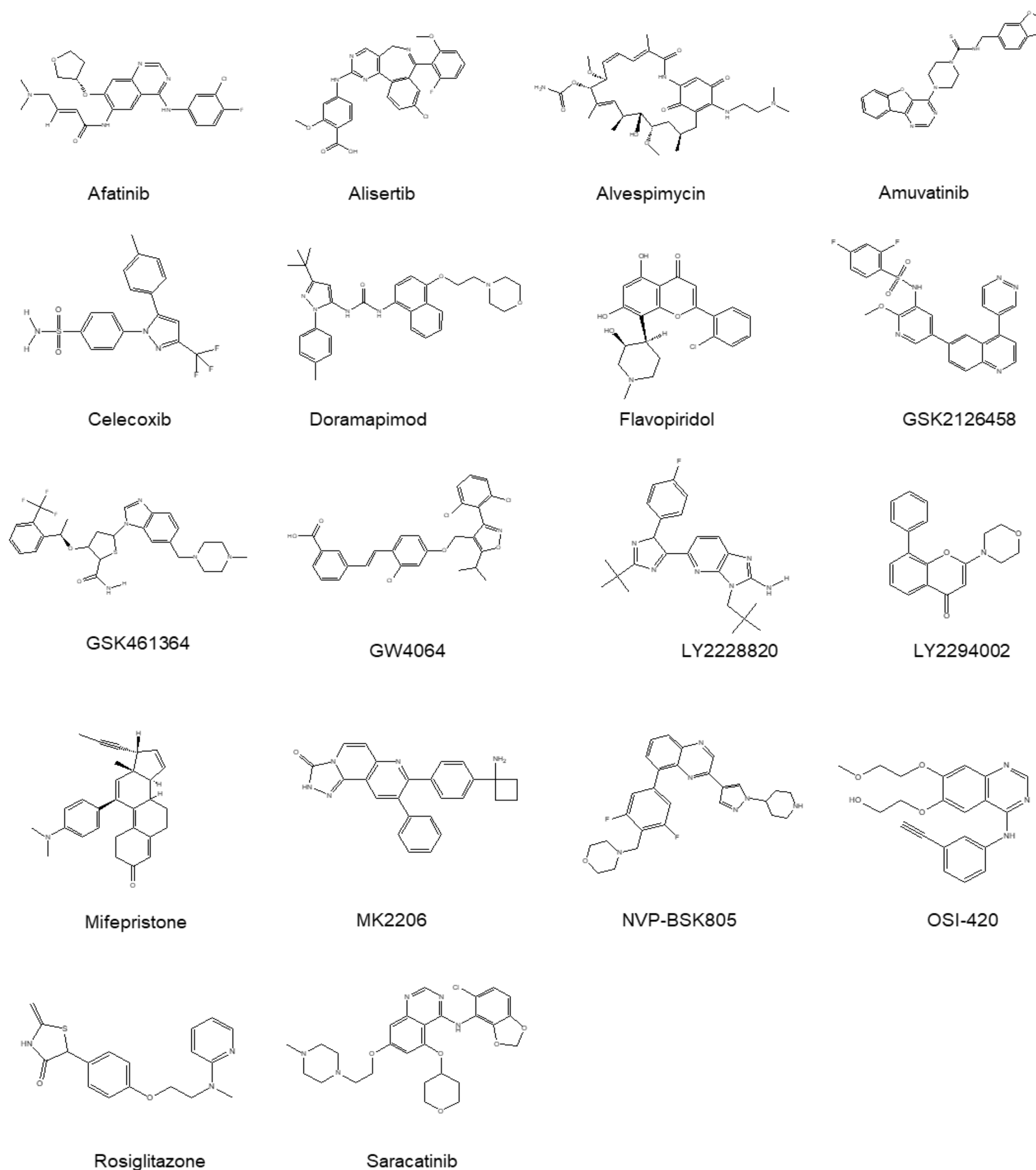


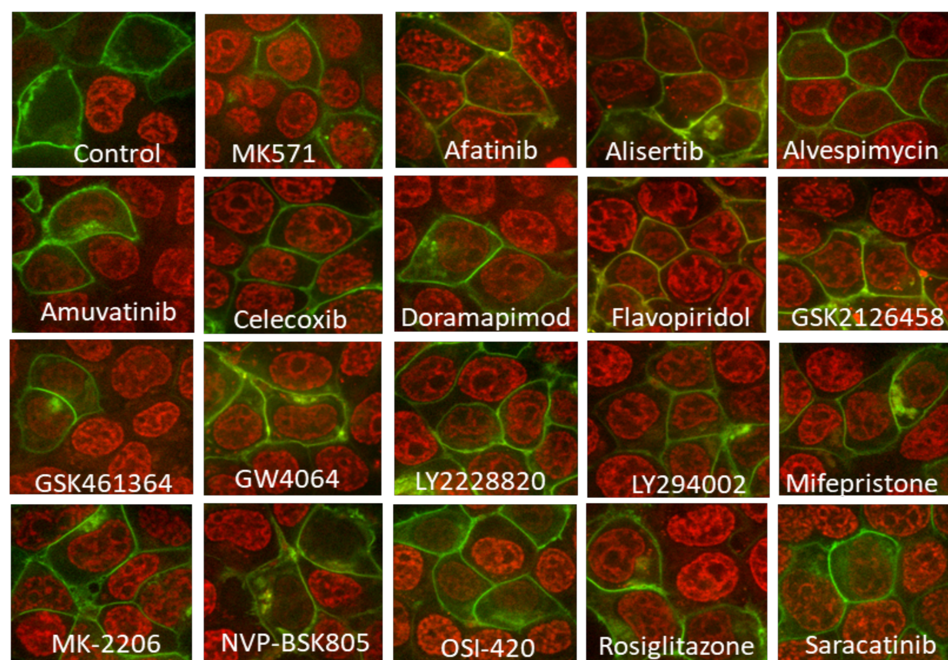
Figure 2.3 Chemical Structures of the selected 18 hit compounds.

3.3 Validation of identified MRP1 inhibitors

To verify the inhibitory activities of the hit compounds obtained from the initial high-content screening and to identify any false hits, we performed doxorubicin accumulation assay using two different traditional and well-established methods. First, we used confocal microscopy to visualize the inhibitory effects of the hit compounds on MRP1-mediated doxorubicin efflux. For this assay, HEK293T cells were transiently transfected with a vector encoding recombinant MRP1-GFP protein. The beauty of this experiment is that transient transfection allows mixed population of cells with and without MRP1-GFP and creates negative control within the system to easily visualize MRP1-dependent doxorubicin efflux side by side in the same image. As shown in Fig. 2.4A, no drug treatment control cells showed high doxorubicin accumulation in the nuclei of non-transfected cells, while doxorubicin fluorescence was very low or undetectable in cells expressing MRP1-GFP. MRP1-mediated efflux of doxorubicin was blocked by MK-571 (50 μM), which was used as the positive control. All the test compound (10 μM) treatments increased doxorubicin accumulation in MRP1-GFP expressing cells to a certain extent reflecting inhibition of doxorubicin efflux by MRP1. Treatment with NVP-BSK805 showed the weakest response. Next, we also performed more quantitative doxorubicin accumulation assay using flow cytometry method. In this assay, MRP1-overexpressing H69AR stable cells were pre-treated with the test compounds for 1 hour before treatment with doxorubicin for another 2 hours. As shown in Figure 4B, MK-571 at 50 μM enhanced doxorubicin accumulation in H69AR cells by 2-fold compared to no treatment control. All the tested hit compounds (10 μM) exhibited increased doxorubicin accumulation in H69AR cells to varying levels (~1.5 to 2.7-fold). Based on both confocal and flow cytometry assays, hit compounds

GSK2126458, doramapimod, MK2206, mifepristone and celecoxib showed strong inhibition of doxorubicin efflux by MRP1 while the remaining compounds showed moderate to weak inhibition.

A



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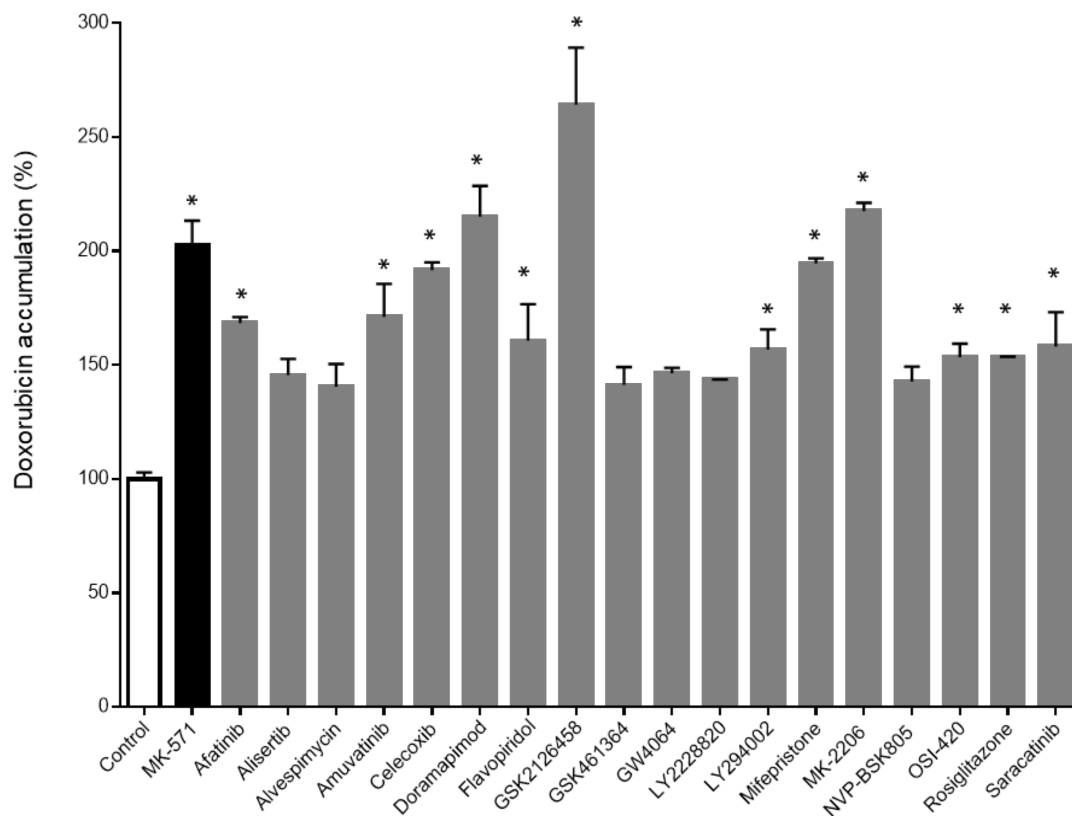

Figure 2.4 Validation of MRP1-inhibitory activity of identified hit compounds

A. HEK293T cells transiently transfected with MRP1-GFP (green) were pre-treated with 10 μM test compounds for 30 min, before treatment with doxorubicin (red) at 37 °C for 1 h. Images were acquired using confocal microscopy. GFP and doxorubicin were excited at 488 nm, and detected at 475/42 and 605/64 nm, respectively. **B**. H69AR cells were pre-treated with 50 μM MK571 or 10 μM test compounds for 1 h before treatment with 10 μM doxorubicin at 37 °C for 2 h. Fluorescence intensities of intracellular doxorubicin were detected using flow cytometry, with excitation and emission wavelengths of 488 and 610/20 nm, respectively. Data are combined from two experiments and presented as mean ± SEM. *, p value lower than 0.05 compared with control.

3.4 Membrane Transport assay for MRP1 inhibitors

Since MRP1 plays a very important physiological role in detoxification and tissue defense. We were interested in evaluating the effects of the hit compounds on MRP1-mediated transport of endogenous organic anions. For this purpose, we employed membrane-based vesicular uptake assay for $E_2 17\beta G$, a glucuronide conjugate and a prototypical physiological substrate of MRP1. HEK293/pcDNA 3.1 and HEK293/MRP1 cells were used to prepare membrane vesicles. MRP1 transport activity was measured using $[^3H]E_2 17\beta G$ as substrate and hit compound treatments were done at 10 μM. As shown in Fig. 2.5, ATP-dependent uptake of $[^3H]E_2 17\beta G$ into HEK293/pcDNA3.1 (vector control) membrane vesicles was less than 1% of uptake by HEK293/MRP1 membrane vesicles. Uptake of $[^3H]E_2 17\beta G$ into HEK293/MRP1 membrane vesicles was reduced by ~95% with 10 μM MK571. Among the hit compounds, alisertib, GSK461364 and GW4064 exhibited

strong MRP1 inhibition and reduced the uptake of $[^{3}H]E_{2}17\beta G$ by 66%, 67% and 70% respectively. In contrast, afatinib, celecoxib, doramapimod, flavopiridol, LY294002, MK-2206, OSI-420, rosiglitazone and saracatinib showed no inhibition or modest inhibition of $[^{3}H]E_{2}17\beta G$ uptake into HEK293/MRP1 membrane vesicles.

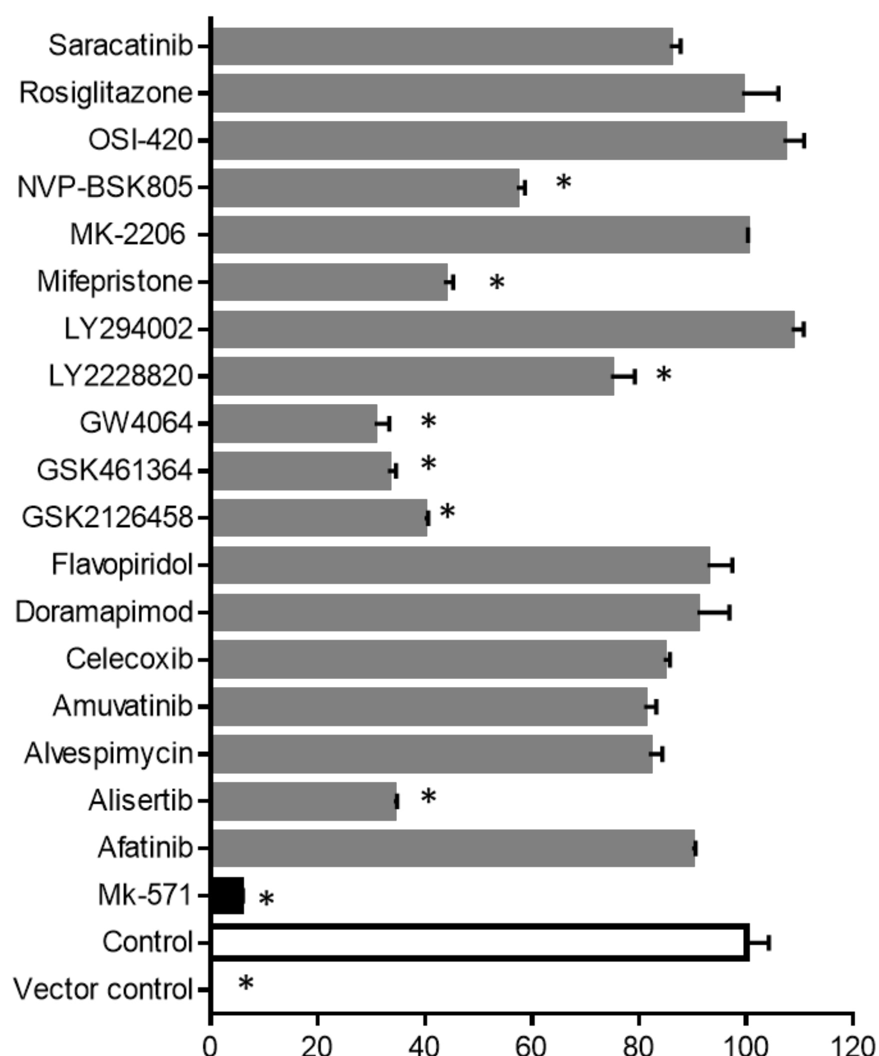

Figure 2.5 Effects of test compounds on $[^3H]E_2 17\beta G$ uptake into MRP1 expressing membrane vesicles.

Membrane vesicles were prepared from stable HEK293/pcDNA3.1 and HEK293/MRP1 cells. Membrane vesicles (2 μg protein) were incubated with 10 μM test compounds. Reactions were performed using 400 nM/20 nCi $[^3H]$ $E_2 17\beta G$ at 37 °C for 1 min. Radioactivity retained on collected membrane vesicles was quantified using liquid scintillation counting. Data are combined from two experiments and presented as mean ± SEM, * p value lower than 0.05 compared with control.

3.5 Resistance reversal by selected MRP1 inhibitors

Development of MDR is the biggest hurdle in the success of chemotherapy. We were interested to determine if the identified anti-cancer hit compounds can reverse drug resistance in a MRP1-overexpressing MDR cell line. MDR reversal ability of a drug may be valuable in combinatorial therapies of malignancies where MRP1 is overexpressed. In addition, drugs which show substrate selectivity and can inhibit the efflux of cancer drugs without significantly interfering with the efflux of physiological organic anions by MRP1 may have a better chance of success in chemotherapy. We selected 6 compounds (afatinib, celecoxib, doramapimod, mifepristone, MK-2206 and rosiglitazone) from the total 18 hit compounds to conduct resistance reversal experiments based on the available data. The abilities of the selected compounds were evaluated to reverse resistance of MRP1-overexpressing H69AR lung cancer cells against vincristine, doxorubicin and etoposide. These cytotoxic anti-cancer agents are very commonly used in chemotherapy and are well-established substrates of MRP1. First, concentration response was conducted for selected drug hits to evaluate the cytotoxicity profile of these compounds in H69AR (data not shown). Selected hit compounds at non-cytotoxic concentrations were then administered in combination with increasing concentrations of vincristine, doxorubicin or etoposide to determine the resistance level of H69AR cells. As shown in Fig. 6, MRP1-overexpressing H69AR cells (solid red) showed MDR and as expected exhibited much lower sensitivity towards each of the three cytotoxic cancer drugs than H69 cells (dotted red). IC_{50} values and fold resistance of the parental H69 and the MRP1-overexpressing derivative H69AR cells treated with vincristine, doxorubicin or etoposide with or without the selected compounds are presented in Table 2.2.

In case of vincristine, H69AR cells were 35-fold more resistant than the parental H69 cells and MRP1 inhibitor MK571 (10 μM) reduced the fold resistance of H69AR cells to 14-fold. Among the selected 6 hit compounds, mifepristone and doramapimod were most effective in sensitizing H69AR cells against vincristine and reduced the fold resistance to 3 and 6-fold respectively. The remaining hit compounds showed comparable reversal as standard MRP1 inhibitor MK571. H69AR cells were 23-fold more resistant towards doxorubicin compared with H69 cells. Mifepristone and rosiglitazone proved most effective in sensitizing H69AR cells against doxorubicin and reduced the fold resistance to 2.6 and 3.1-fold respectively. Afatinib, alisertib, celecoxib and doramapimod showed similar resistance reversal as MK571. In the absence of any inhibitor, H69AR cells also exhibited 5.6-fold more resistance towards etoposide compared with H69 cells. Doramapimod, celecoxib and alisertib were able to completely reverse resistance of H69AR cells against etoposide. In contrast, presence of afatinib failed to show any reduction in the resistance level against etoposide. Hit compounds mifepristone, MK-2206 and rosiglitazone showed only moderate levels of reduction in resistance of H69AR cells against etoposide.

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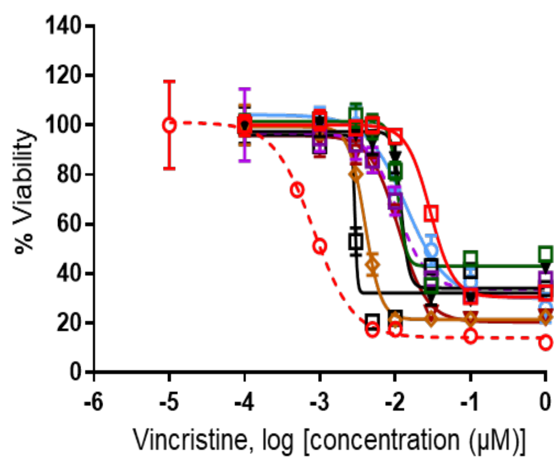

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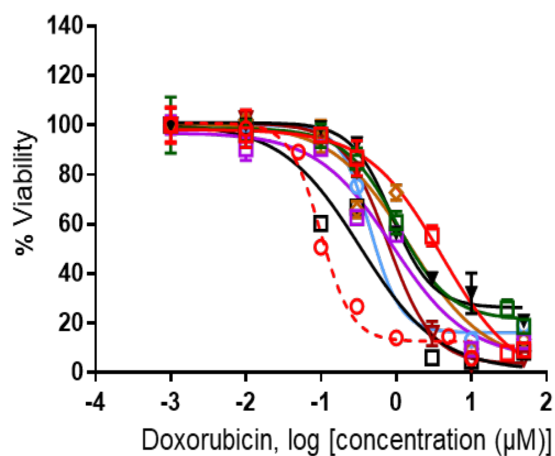

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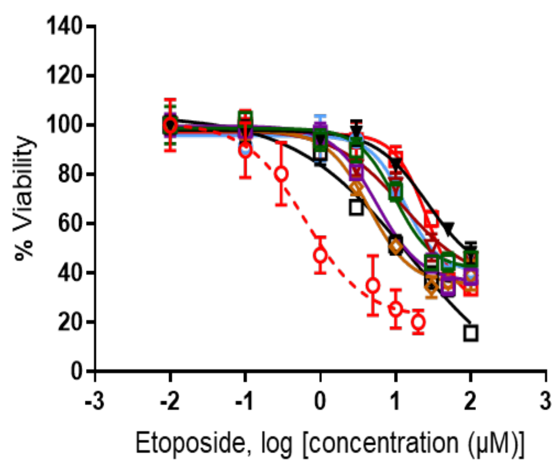

H69
H69AR
H69AR + MK571
H69AR + Afatinib
H69AR + Celecoxib
H69AR + Doramapimod
H69AR + Mifepristone
H69AR + MK-2206
H69AR + Rosiglitazone

Figure 2.6 Reversal of drug resistance towards vincristine, doxorubicin and etoposide in H69AR cells by selected MRP1 inhibitors.

A. H69 and H69AR cells were treated with vincristine at increasing concentrations in the absence and/or presence of selected MRP1 inhibitors. B. H69 and H69AR cells were treated with doxorubicin at increasing concentrations in the absence and/or presence of selected MRP1bitors C. H69 and H69AR cells were treated with etoposide at increasing concentrations in the absence and/or presence of selected MRP1 inhibitors. A, B and C. MK571, doramapimod, mifepristone, and rosiglitazone were at 10 μM, MK-2206 and celecoxib were at 2 μM, afatinib was at 1 μM. Cell viability was evaluated with MTT after 72 h. Data are representative of three experiments and presented as mean ± SD (n = 3).

Table 2.2 The effects of selected MRP1 inhibitors on the IC_{50} values of vincristine, doxorubicin and etoposide in H69 and H69AR cells

Cell line/Treatment	Vincristine		Doxorubicin		Etoposide	
	IC_{50} [a] (nM)	Fold resistance [b]	IC_{50} [a] (μM)	Fold resistance [b]	IC_{50} [a] (μM)	Fold resistance [b]
H69	0.67 ± 0.08	1.00	0.14 ± 0.05	1.00	4.43 ± 1.97	1.00
H69AR	23.35 ± 0.34	34.69	3.31 ± 0.32	22.93	24.68 ± 2.23	5.57
H69AR + MK571 10 μM	9.33 ± 1.43	13.86	1.05 ± 0.04	7.25	7.97 ± 1.10	1.80
H69AR + Afatinib 1 μM	14.68 ± 1.57	21.80	1.16 ± 0.21	8.05	28.34 ± 1.03	6.40
H69AR + Celecoxib 2 μM	10.54 ± 0.19	15.66	1.19 ± 0.15	8.28	4.78 ± 0.35	1.08
H69AR + Doramapimod 10 μM	4.08 ± 0.06	6.06	1.21 ± 0.25	8.39	4.18 ± 0.52	0.94
H69AR + Mifepristone 10 μM	2.13 ± 0.40	3.16	0.38 ± 0.10	2.65	11.58 ± 1.99	2.62
H69AR + MK-2206 2 μM	15.40 ± 2.5	22.87	2.09 ± 0.05	14.50	14.54 ± 1.89	3.28
H69AR + Rosiglitazone 10 μM	14.85 ± 1.13	22.20	0.44 ± 0.05	3.07	13.14 ± 0.03	2.97

[a]Mean ± SEM of n ≥ 3 independent experiments.

[b]Fold resistance is the ratio between IC_{50} value of each treatment and IC_{50} value of vincristine, doxorubicin and etoposide alone in H69 cells.

3.6 Effects of selected hit compounds on drug sensitivity of HEK293/BCRP and HEK293/P-gp cells

Among ABC drug transporters, P-gp, MRP1 and BCRP are most frequently implicated in MDR. Therefore, we were interested in investigating the potential of selected MRP1 inhibitors to reverse drug resistance of P-gp and BCRP-overexpressing cell lines. HEK293/BCRP and HEK293/P-gp cells were treated with increasing concentrations of mitoxantrone and vincristine, respectively, in the presence and absence of selected hit compounds. Ko143 and verapamil were used as known inhibitors of BCRP and P-gp, respectively. As shown in Fig. 7, BCRP and P-gp-overexpressing cells (solid red) exhibited much lower sensitivity towards mitoxantrone and vincristine, respectively, than the vector control cell line HEK293/pcDNA3.1 (dotted red). Treatment of HEK293/BCRP and HEK293/P-gp cells with Ko143 and verapamil, respectively, reversed the resistance of the cells. IC_{50} values and fold resistance are presented in Table 3. Doramapimod and mifepristone were able to completely reverse the resistance of HEK293/BCRP cells against mitoxantrone while the remaining selected hits showed only modest to moderate reversal. In case of HEK293/P-gp cells, mifepristone treatment completely reversed the resistance against vincristine while doramapimod exhibited modest resistance reversal. In contrast, treatment with afatinib or celecoxib led to increased resistance of HEK293/P-gp cells

against vincristine. Alisertib, MK-2206 and rosiglitazone did not significantly reduce the resistance levels of HEK293/P-gp cells.

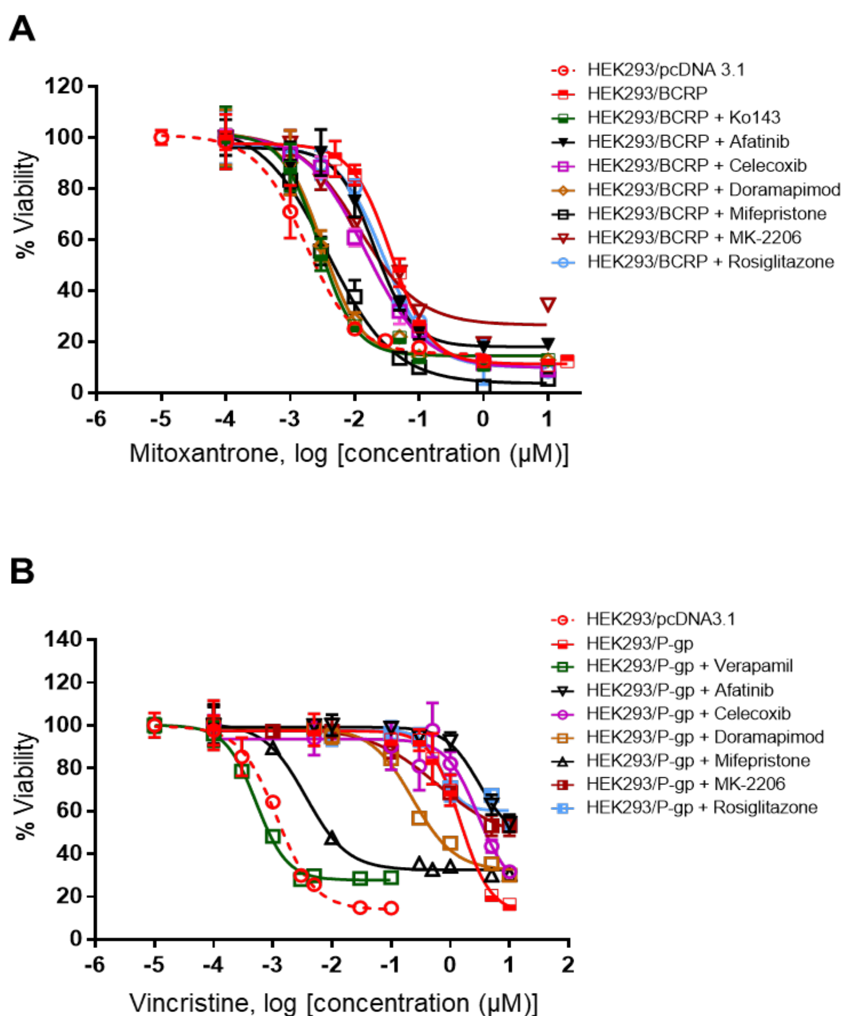

Figure 2.7 Effects of selected MRP1 inhibitors on drug sensitivity of HEK293/BCRP and HEK293/P-gp cells. HEK293/BCRP

(A) and HEK293/P-gp **(B)** were treated with increasing concentrations of mitoxantrone and vincristine, respectively in the absence and /or presence of selected MRP1 inhibitors. Afatinib was at 1μM, celecoxib and MK-2206 were at 2 μM while doramapimod, mifepristone and rosiglitazone were at 10 μM. Ko143 (1 μM) and verapamil (25 μM) were used as positive controls in HEK293/BCRP and HEK293/P-gp cells, respectively.

HEK293/pcDNA cells were included as negative control for drug resistance. Cell viability was evaluated with MTT after 72 h. Data are representative of three experiments and presented as mean ± SD (n = 3).

Table 2.3 Effect of selected MRP1 inhibitors on IC_{50} of mitoxantrone and vincristine and in HEK293/BCRP and, HEK293/P-gp respectively

Cell line/Treatment	**Mitoxantrone**		**Vincristine**	
	IC_{50} [a] (nM)	Fold resistance [b]	IC_{50} [a] (μM)	Fold resistance [b]
HEK293/pcDNA3.1	4.57 ± 1.48	1		
HEK293/BCRP (control)	28.36 ± 4.1	6.21		
HEK293/BCRP + Ko143 1μM	1.33 ± 0.14	0.29		
HEK293/BCRP + Afatinib 1μM	17.37 ± 1.58	3.80		
HEK293/BCRP + Celecoxib 2 μM	19.91 ± 3.68	4.36		
HEK293/BCRP + Doramapimod 10 μM	2.52 ± 0.22	0.55		
HEK293/BCRP + Mifepristone 10 μM	4.40 ± 0.33	0.96		
HEK293/BCRP + MK-2206 2 μM	22.5 ± 1.85	4.93		
HEK293/BCRP + Rosiglitazone 10 μM	20.04 ± 4.68	4.39		
HEK293/pcDNA3.1			0.0037 ± 0.0013	1
HEK293/P-gp (control)			1.40 ± 0.17	376.86
HEK293/ P-gp + Verapamil 25 μM			0.0030 ± 0.0012	0.80
HEK293/ P-gp + Afatinib 1 μM			2.92 ± 0.09	788.73
HEK293/ P-gp + Celecoxib 2 μM			3.83 ± 0.46	1034.51
HEK293/ P-gp + Doramapimod 10 μM			0.12 ± 0.07	31.23
HEK293/ P-gp + Mifepristone 10 μM			0.0032 ± 0.0002	0.88
HEK293/ P-gp + MK-2206 2 μM			0.81 ± 0.46	218.64
HEK293/ P-gp + Rosiglitazone 10 μM			0.58 ± 0.07	157.86

[a] Mean ± SEM of n ≥ 3 independent experiments

[b]Fold resistance is the ratio between IC_{50} value of each treatment and IC_{50} value of mitoxantrone or vincristine alone in the parental cell line HEK293/pcDNA3.1.

4.0 Discussion

MDR is a major problem in the treatment of malignancies as it increases the effective dose of anticancer agents to more lethal levels. The capacity of MRP1 to efflux drugs prevents the effective treatment of cancers when overexpressed. MRP1 although localizes primarily at the plasma membrane of the cells, has also been found in the membranes of sub-cellular organelles such as mitochondria, endoplasmic reticulum and endocytic vesicles [26]. The localization of MRP1 in sub-cellular organelles has been hypothesized to serve as a sequestering mechanism to prevent drugs from reaching their respective intracellular targets [3,27]. MRP1 is known to be overexpressed in cancers such as neuroblastoma [28], acute lymphoblastic leukemia [29], breast [30], tongue [31], brain [32], small-cell lung carcinoma [33] and prostate [34]. It has also been linked with survival of patients with ovarian [35], neuroblastoma [36] and acute leukemia [37]. MRP1-overexpression has been implicated with MDR in lung, breast, prostate cancers and different types of leukemia. Inhibition of MRP1-mediated transport by small molecules to resensitize drug resistant cells provides the possibility of overcoming MDR in malignancies where MRP1 is overexpressed and linked to patient survival.

Chemotherapy is the most common type of treatment for various types of cancers. Conventional cytotoxic anticancer drugs such as vincristine, doxorubicin, cisplatin, paclitaxel, and methotrexate are still commonly used in cancer chemotherapy. Because these agents target essential and fundamental cellular processes, they are very cytotoxic and not cancer specific. Major problems limiting the success of chemotherapy are MDR, tumor cell heterogeneity and toxicity. To address MDR and tumor heterogeneity, combination cancer therapy is frequently used, where drugs from different families

targeting different cellular mechanisms are combined to achieve better outcomes. To address the toxicity issue associated with chemotherapy, the focus in cancer therapeutics is to identify drugs that show high selectivity towards cancer cells and target specific cell signaling pathways. Many new compounds have been discovered that target specific enzymes, kinases, and receptors, and are being tested in clinical trials for better efficacy and reduced toxicity. Given the remarkable ability of MRP1 to significantly alter the bioavailability of a broad spectrum of drugs and the fact that MRP1-overexpression has been implicated in MDR of different types of cancer, it is very important to profile any new anticancer drug candidates for their interaction with this transporter. For successful cancer treatment, especially in cancers where MRP1-overexpression is associated with poor clinical outcome, it will be desirable to either inhibit MRP1 activity or use drugs that are not substrates of MRP1. In this study we investigated the interaction of MRP1 with a unique library of anti-cancer compounds which are being clinically tested as targeted cancer drugs. We are especially interested to identify inhibitors of MRP1 from this library because such compounds can act as dual-edge sword. In addition to working as a targeted cancer drug, it can also reverse MRP1-mediated drug resistance or can be used to improve the efficacy and reduce the toxicity of MRP1 substrate drugs.

In the present study, we have successfully developed and validated a high content imaging-based efflux assay for MRP1 transport activity using doxorubicin as a fluorescent reporter substrate. To the best of our knowledge, doxorubicin has never been successfully used as a substrate in a high content screening or any other high-throughput screening assay to identify inhibitors of MRP1 activity. Screening a unique library of 386 anti-cancer compounds currently under clinical trials targeting 12 types of cancers, we identified a total

of 28 MRP1 inhibitors. The assay exhibited high correlation (0.73–0.85) among the three independent screening experiments. The high quality and performance of this screening assay was demonstrated by Z'-factor of 0.58 across all plates. The high-through screening assay developed in this study has several advantages over assays that measure fluorescence intensity using microplate reader or flow cytometry. This high-content screening method uses automated imaging and offers very advance and sophisticated image analysis tools to remove the non-specific and background fluorescence leading to a very robust and precise assay with high dynamic range between the positive and negative controls. In addition, this live cell-based assay allows simultaneous detection of cell permeable, non-toxic and potent inhibitors. There is convincing biochemical and structural data to suggest that MRP1 has multiple distinct substrate binding sites. However, only calcein-AM as a substrate has been used in cell-based assays for MRP1 inhibition screening. The doxorubicin-based new assay developed was able to identify 10 of 12 hits previously identified using calcein-based screening and in addition discovered many new drugs as inhibitors of MRP1. These findings suggest that doxorubicin binding site overlaps with calcein binding site and may have an additional binding site. Furthermore, the new assay signifies the importance of MRP1 for drug discovery and development.

Among the 28 hit compounds identified as MRP1 inhibitors in the doxorubicin-based screening of anticancer compounds, 10 compounds (tipifarnib, AZD1208, rapamycin, deforolimus, HS-173, YM201636, everolimus, TAK-733, cyclosporin A, and temsirolimus) were recently identified by our group using calcein-based screening assay. Therefore, we focused on the characterization of the remaining 18 MRP1 inhibitors. Two of the novel inhibitors identified in this study, namely alisertib and amuvatinib, are being

actively investigated for their anticancer efficacy and have never been reported to interact with any ABC drug transporter. Alisertib, a second-generation aurora kinase A and B inhibitor with a higher affinity for aurora kinase A is identified for the first time to interact with MRP1. Alisertib showed strong inhibition of MRP1-mediated transport of doxorubicin and $E_2 17\beta G$ (Fig. 3 and 4). P-gp and BCRP expression have been reported to decrease the anti-cancer activity of pan-aurora kinase inhibitor tozasertib but not of the aurora kinase inhibitor alisertib indicating that alisertib is not a substrate of these ABC transporters [38, 39]. Two compounds, celecoxib and LY294002, have been previously shown to inhibit MRP1 efflux function [24, 25] while anti-cancer drugs mifepristone and rosiglitazone although have not been reported to be a substrate or inhibitor of MRP1 directly but have been shown to reverse resistance of cells overexpressing P-gp and MRP1 [40, 41].

Eleven of the 18 hit drugs have been reported previously to be a substrate or inhibitor of P-gp or BCRP but not have been reported to interact with MRP1. GW4064 has been reported to induce the MRP2 mRNA levels [42] while MK2206 was reported to decrease P-gp expression [43] but neither of these drugs has been reported as substrate or inhibitor of P-gp, BCRP or MRP1. In addition to cell-based assays that measured doxorubicin efflux inhibition, we also employed membrane-based vesicular uptake assay to evaluate the effects of hit compounds on MRP1-mediated transport of endogenous organic anion, $E_2 17\beta G$, a prototypical physiological substrate of MRP1. Ten of the 18 compounds (LY294002, OSI-420, rosiglitazone, MK-2206, flavopiridol, doramapimod, afatinib, celecoxib, alvespimycin and amuvatinib) showed no or weak inhibition of MRP1-mediated $E_2 17\beta G$ transport. Substrate selective inhibition of MRP1 may be very critical in the

potential application of these anti-cancer agents in reversing MDR. An anti-cancer agent that blocks the efflux of therapeutic drug without interfering with the efflux of important physiological substrates may exhibit less toxic side effects.

Six of the hit compounds were evaluated to reverse resistance of MRP1-overexpressing H69AR lung cancer cells against vincristine, doxorubicin and etoposide. Mifepristone, doramapimod and celecoxib were most effective in reversal of MRP1-mediated drug resistance. Mifepristone is a progesterone and glucocorticoid hormone antagonist and has been used to treat hypercortisolism in patients with nonpituitary cushing syndrome. Mifepristone has been reported to reverse drug resistance in cells overexpressing P-gp as well as in SGC790/VCR cell line overexpressing both P-gp and MRP1. In this study, mifepristone was able to sensitize MRP1-overexpressing H69AR cells against vincristine (reduced IC_{50} from 23.35 to 2.13 nM), doxorubicin (reduced IC_{50} from 3.3 to 0.38 nM), and etoposide (reduced IC_{50} from 24.68 to 11.58 μM). We report that mifepristone is a MRP1, P-gp and BCRP pan inhibitor since it was able to completely reverse resistance of P-gp and BCRP-overexpressing cells against vincristine and mitoxantrone, respectively. As of June 16^{th,} 2019, there are currently 146 clinical trials registered (clinicaltrials.gov) and many of them are focused on evaluating the anti-cancer potential of mifepristone for various types of cancers. Doramapimod is a p38 MAPK inhibitor and has been reported to enhance cytotoxicity of anticancer drugs in P-gp overexpressing cell lines [44]. We are the first to report its interaction with MRP1. In this study, doramapimod was able to sensitize MRP1-overexpressing H69AR cells against vincristine (reduced IC_{50} from 23.35 to 4.08 nM), doxorubicin (reduced IC_{50} from 3.3 to 1.21 nM), and etoposide (reduced IC_{50} from 24.68 to 4.18 μM). Here we report that doramapimod is a MRP1, BCRP and P-gp pan inhibitor

since it was able to completely reverse resistance of BCRP-overexpressing cells against mitoxantrone and exhibited strong resistance reversal of P-gp overexpressing cells against vincristine. Celecoxib is a selective cyclooxygenase inhibitor. In this study, celecoxib was able to completely reverse the resistance of MRP1-overexpressing H69AR cells against etoposide whereas only modest drug reversal was observed against vincristine and doxorubicin reflecting substrate selective inhibition. In contrast, we observed that celecoxib enhanced the cytotoxicity of vincristine in HEK-P-gp overexpressing cells. In addition, we report that celecoxib was ineffective in reversing resistance of HEK-BCRP overexpressing cells against mitoxantrone. Interestingly, a gene array analysis investigating the expression of all 48 human ABC transporters found *ABCC1* and *ABCC6* to be highly expressed in the MCF7VP breast cancer cell line (etoposide-resistant cell line) compared to the MCF7 (parental sensitive cell line) whereas the expression of other transporters was not appreciably increased [45]. This report is consistent with our findings and highlights the role of MRP1 in resistance against etoposide.

In summary, we demonstrated the effectiveness and value of a novel doxorubicin-based high content imaging-based assay to study compound interaction with MRP1. This study identified 16 anti-cancer agents as MRP1 inhibitors which have not been previously reported as a substrate or inhibitor of MRP1. The anti-cancer drugs which did not exhibit interaction with MRP1 may have a lower risk of being interfered by MRP1-mediated MDR.

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Chapter 3

CRO-9 screening of unique anticancer library identifies novel inhibitors of human MRP1 (ABCC1).

Abstract

Multidrug resistance protein 1 (MRP1/ABCC1) transports a variety of drugs, toxic molecules and important physiological substrates across the plasma membrane and physiological barriers. MRP1 plays an active role in protecting cells by effluxing a vast array of drugs to sub-lethal levels. Additionally, its overexpression has been implicated in multidrug resistance in various cancers. We recently reported development of a high content efflux assay using doxorubicin as fluorescent reporter which identified MRP1 inhibitors missed by calcein-AM in a similar assay. Here, we describe the development of a new high content imaging-based efflux assay using CRO-9 as a fluorescent reporter. Taking advantage of multiple binding sites of MRP1, we screened the same anti-cancer library of 386 compounds using CRO-9. The new assay identified a total of 50 MRP1 inhibitors including 19 inhibitors that have not been previously reported as inhibitors of MRP1. The inhibitory activity of compounds was confirmed using flow cytometry confocal microscopy, and membrane vesicle-based transport assays. Six drugs (GSK650394, KU-0063794, LY2603618, MK0752, NU7441 and ZSTK474) were evaluated for their ability to reverse resistance of MRP1-overexpressing H69AR lung cancer cells against vincristine, and SN38. LY2603618 and ZSTK474 were the most effective in reversing resistance of H69AR cells against vincristine and SN38. NU7441 was more effective in reversing resistance against SN38 as compared to resistance of

H69AR to vincristine. Our findings indicate the importance of MRP1 in drug discovery and demonstrate the advantage of CRO-9 as a fluorescent reporter in a high content screening assay. Anti-cancer agents that exhibit MRP1 inhibition and ability to reverse resistance may be used in combination therapy to improve the efficacy and reduce the toxicity of other cancer chemotherapies.

Keywords: MRP1; ABCC1; ABC transporter; multidrug resistance; MRP1 inhibitors; high content screening; anti-cancer agent; drug-transporter interactions; CRO-9; drug profiling

Chemical compounds studied in this article:

1) AUY922 (PubChem CID: 135539077); 2) BIBR 1532 (PubChem CID: 9927531); 3) Bosutinib (PubChem CID: 5328940); 4) CT99021(9956119); 5) CW069 (PubChem CID: 73427517); 6) Evista (PubChem CID: 59400); 7) Gefitinib (PubChem CID: 123631); 8) GSK1120212 (PubChem CID: 11707110); 9) GSK650394 (PubChem CID: 5022668); 10) Ibrutinib (PubChem CID: 24821094); 11) Ku-0063794 (PubChem CID: 16736978); 12) Ku-55933 (PubChem CID:5278369); 13) Ly2603618 (PubChem CID: 11955855); 14) Mk-0752 (PubChem CID: 9803433); 15) Nu7441(PubChem CID:11327430); 16) Nutlin 3(PubChem CID: 11433190); 17) Nutlin 3a (PubChem CID: 216345); 18) Pf562271(PubChem CID: 11713159); 19) PIK75 (PubChem CID: 9825070); 20) Ponatinib (PubChem CID: 24826799); 21) WIKI4 (PubChem CID: 2984337); 22)Wp1130 (PubChem CID: 11222830); 23) ZSTK474 *(*PubChem CID: 11647372)

1.0 Introduction

One of the challenges to effective cancer treatment is multidrug resistance which decreases long term survival rate of patients despite advances in cancer therapy [1]. Multidrug resistance (MDR) is the phenomenon where cancer cells become resistant and unresponsive to a variety of chemotherapeutic agents. Mechanisms of MDR in cancer includes activation of DNA repair mechanism, interference in apoptotic signaling pathways, activation of drug metabolizing enzymes and overexpression of drug transporters [2]. The overexpression of drug transporters is the most common mechanism of MDR and is widely studied [3]. Underlying this type of MDR is active efflux of variety of structurally and functionally unrelated pharmacological agents from cells. The superfamily of drug transporters called ATP binding cassette (ABC) transporters have been implicated to confer resistance to clinically active drugs especially P-glycoprotein (P-gp/ABCB1), breast cancer resistant protein (BCRP, ABCG2) and multidrug resistant protein 1 (MRP1/ ABCC1)[4, 5]. These drug transporters actively pump out chemotherapeutic drugs reducing the intracellular levels of drugs and consequently drug efficacy. These transporters are also impediments in the treatments of neurological disorders , epilepsy as they are present at the brain blood barrier and extrude xenobiotics from the CNS[6]. The expression of MRP1 in peripheral blood mononuclear cells of children who have intractable epilepsy is increased as compared to children with epilepsy controlled by anti-epileptic drugs (AED) and normal children [7].

The ABC transporters are classified into seven subfamilies (A through G) based on their sequence homology [8]. The mammalian ABC transporters are made up of four domains often encoded as a single polypeptide (e.g. P-gp) but are sometimes two polypeptides that

form homodimers (e.g. ABCG2) or heterodimers (e.g. TAP1/TAP2) [9]. MRP1 and several ABCC homologs have a five-domain structure with two nucleotide binding domains (NBDs) and 17 transmembranes in three membrane spanning domains (MSD0, MSD1, and MDS2) [10].

The NBDs each contain three key motifs i.e. Walker A and B motifs characteristic of P-loop ATPases and an "active transport" signature motif common to all ABC proteins [9]. Transport by MRP1 (and other ABC transporters) is driven by the binding and hydrolysis of ATP, which promotes the required protein conformation changes that aids solute translocation. The two NBDs form a "sandwich" dimer, with two ATP molecules sandwiched in the dimer interface. In the 'sandwich dimer' the two ATP-binding sites comprise of the Walker motifs of one NBD and the active transport signature motif of the other [9]. The ATP-binding sites of MRP1 are functionally nonequivalent. NBD1 has a higher affinity for ATP (but very low ATPase activity) whiles NBD2 has a greater capacity for ATP hydrolysis. MRP1 also has composite substrate-binding sites which permits both cooperativity and competition between various substrates [11].

MRP1 (ABCC1) was first reported by Cole and colleagues to mediate resistance to doxorubicin, etoposide and vincristine [5]. MRP1 is ubiquitously expressed in normal tissues with elevated expression in kidney, lungs, thymus, cardiomyocytes and placenta with lower expression in colon, brain, small intestine and peripheral blood mononuclear cells [11,12]. The expression level of MRP1 can still vary between different cell types in the same tissue. The activity of MRP1 unlike P-gp is not limited to the efflux of xenobiotics but also endobiotics and other physiological substrates. As such it influences physiological processes such as transport of inflammatory mediators (extrusion of leukotriene) and

defense against oxidative stress (transport of glutathione and glutathione conjugates). Additionally, substrates of MRP1 and P-gp differ by their chemical properties. MRP1 mostly transports organic anions, many of which are conjugated with glutathione (GSH, g-Glu-Cys-Gly), glucuronic acid, or sulfate whereas P-gp recognizes unconjugated hydrophobic substrates [13].

Due to its protective roles, MRP1 expression is also elevated in cells with specialized barrier functions including choroid cells and capillary endothelial cells. MRP1 is located on the basolateral membrane of these polarized cells. MRP1 expression is elevated in rapidly proliferative cells such as reactive type II pneumocytes in lungs; and physiological barriers such as brain blood barrier(BBB) [14], blood testes [15], blood placental [16]. The overexpression of MRP1 in cancer cells can affect pharmacokinetic parameters of drug absorption, distribution, metabolism, excretion and toxicity (ADMET) [17]. Elevated levels of MRP1 confers resistance to various natural products and anticancer drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins [4].

Not much is known about modulators of MRP1 at clinical trials. Although P-gp has been studied well, no P-gp modulator has been successful at clinical trials. This is mainly attributed to toxicity related issues and the fact that patients are not selected based on tumor expression of P-pg. To fully understand and determine the impact of ABC transporters on multidrug resistance in clinical trials, subpopulation of patients with ABC transporters as the main mechanism of multidrug resistance should be used. Identification of modulators of MRP1 and understanding fully the physiological and pharmacological consequences of doing so is of great interest due to its potential benefits in the treatment of drug resistant malignancies.

Previously, we developed, optimized and validated a high content imaging-based efflux assay to profile drug interactions with MRP1 [18] using calcein-AM. This assay was more robust and efficient as opposed to more traditional assays such as flow cytometry. This assay identified 12 novel MRP1 inhibitors after screening of a 384 unique anticancer library. Furthermore, the adaptation of this assay using doxorubicin as a fluorescent probe identified 28 inhibitors which included 10 of 12 inhibitors discovered previously with calcein-AM assay as well as 18 MRP1 inhibitors that were missed by the calcein-based screening [19]. The rationale for using doxorubicin was to take advantage of the wide range of the probe and multiple substrate nature of MRP1. Based on the promiscuity of MRP1 substrate binding sites, we tested the hypothesis that using CRO-9 as a fluorescent reporter substrate, which is different in size and shape from doxorubicin and calcein-AM may identify new inhibitors. The screening of a 386 anticancer library with CRO-9 identified a total of 50 MRP1 inhibitors including 19 inhibitors that have not been previously reported as inhibitors. The remaining 31 inhibitors included 10 out of 12 compounds identified with calcein-AM and 17 out of 18 compounds exclusive to doxorubicin- based screening of the same library .We verified the inhibitory activity of the identified compounds exclusive to CRO-9 using flow cytometry and vesicular transport assays. The ability of some selected compounds to reverse the resistance of an MRP1-overexpressing MDR cancer cell line is also demonstrated.

2.0 Materials and methods

2.1 Chemicals

CRO-9, doxorubicin, adenosine monophosphate (AMP), adenosine triphosphate (ATP), estradiol 17-(β-D-Glucuronide) ($E_2 17\beta G$), poly-D-lysine, thiazolyl blue tetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO). MK571 was acquired from Cayman Chemical(Ann Arbor, MI) and [6,7-3H]$E_2 17\beta G$ (49.9 Ci mmol–1) from PerkinElmer (Waltham, MA). Anti-cancer compound library consisting of 386 anti-cancer small molecules under clinical trials for 12 different types of cancers was acquired from Selleck Chemicals (Houston, TX)

2.2 Cell lines and cell culture

H69 and H69AR cells were purchased from ATCC (Manassas, VA). HEK293T cells were provided by Dr. Adam Hoppe (South Dakota State University, Brookings, SD). HEK293 and H69 cell lines were cultured in DMEM (GE Healthcare, Marlborough, MA) and RPMI 1640 (ATCC), respectively, supplemented with 10% fetal bovine serum. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37°C. H69AR cells were exposed to 0.8 μM doxorubicin monthly and cultured drug-free for a week before use.

2.3 Screening for MRP1 inhibition via automated image acquisition and analysis

The assay was developed and optimized using H69 and doxorubicin resistant cell line H69AR. CRO-9 was used as the fluorescent substrate and MK571 as the positive control.

Both cell lines were seeded at 6×10^4 cells per well in 100 μL culture medium in 96-well optical-bottom plates with polymer base (ThermoFisher Scientific, Waltham, MA) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) and allowed to attach overnight. Culture medium was replaced with 80 μL of serum-free medium. Prior to incubation with CRO-9, 10 μL of test compounds (10 μM final concentration), DMSO (0.2% final concentration) as negative control, or MK-571 (50 μM final concentration) as positive control were added and incubated for 30 min. A total of 10 μL of CRO-9 (0.25 μM final concentration) was added and incubated for an additional 30 min. Treatment was removed and 100 μL of PBS containing 10mM HEPES and 4.5% glucose was added post incubation.

Images were obtained using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA) equipped with a 0.70 numerical aperture 60× objective. Eight images (4 bright field and 4 fluorescent) were taken for each well. Fluorescent images were acquired using a texas red filter with excitation and emission wavelengths of 562/40 nm and 624/40 nm, respectively, with an exposure time of 100 ms. Both negative and positive controls were included in every two columns to account for fluorescence decrease across the 96-well plate due to gradual decrease in intracellular accumulation of probe over time. intracellular int fluorescence. Screening was performed in 3 independent experiments. Fluorescent images were analyzed using the MetaXpress software (version 5.10.41, Molecular Devices). Segmentation of fluorescent objects on the texas red channel was achieved using a custom application module based on the 'Find Blobs' module. The custom module enables differentiation of fluorescent accumulation from background and artifacts via segmentation masks based on set parameters for object size.

2.4 Doxorubicin accumulation assay using confocal microscopy

Confocal microscopy was used to confirm inhibition of MRP1 mediated doxorubicin efflux. HEK293T cells were plated on poly-D-lysine-coated cover glass placed in a 6-well plate at a density of 5×10^5 cells/well in 2 mL culture medium. Cells were transiently transfected with an MRP1-GFP expression vector after 24 hours using jetPRIME Transfection Reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's protocol. After 48 hours, cells were pre-treated with 10 μM test compound for 30 min, before incubation with doxorubicin (10 μM) for 1 h. Cells were maintained in buffer (4.5 % glucose, 10mM HEPES, PBS containing Ca^{2+} and Mg^{2+}) as intracellular fluorescence was visualized using a iMIC digital microscope (TILL Photonics GmbH, Gräfelfing, Germany) equipped with a 1.35 numerical aperture 60x oil-immersion objective. Excitation was done at 488 nm for GFP and doxorubicin, with emission bands of 475/42 and 605/64 nm, respectively. Images were processed using ImageJ (NIH, Bethesda, MD).

2.5 Flow cytometry-based CRO-9 accumulation assay

MRP1 mediated efflux of CRO-9was determined in the presence/absence of test compounds using flow cytometry. This was to quantitatively affirm the inhibition activity of drugs identified in the screening assay . H69AR cells were prepared in serum-free culture medium to a density of 7×10^5 cells/mL. One mL of cells was incubated with test compounds (10 μM) at 37 °C for 10 min after which 0.25 μM of CRO-9was added for an additional 30 min. DMSO concentration was maintained at 0.2% (v/v). Efflux activity of

MRP1 was stopped by adding ice-cold PBS buffer. Cells were then collected, washed with cold PBS and resuspended in ice-cold PBS containing 1% formaldehyde. Intracellular fluorescence of CRO-9was detected by BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) equipped with 488nm and > 670nm for excitation and emission respectively. Fluorescence intensity was collected as mean of 10,000 events. Treatments were performed in duplicate and repeated in 3 independent experiments.

2.6 Membrane vesicle preparation

Membrane vesicles were prepared as described in Loe et al., [20] with modifications. Frozen cell pellets of HEK293/pcDNA 3.1 and HEK293/MRP1 were thawed and suspended containing 50 mM Tris.HCl, pH 7.4, 250 mM sucrose, 0.25 mM $CaCl_2$, and 1x complete protease inhibitors (Santa Cruz Biotechnology, Dallas, TX). Cell disruption was achieved *via* nitrogen cavitation at 450 psi for 5 min. The resultant lysates were supplemented with 1mM EDTA and centrifuged at 500 × *g* at 4 °C for 10 min. The supernatant was collected twice by resuspending cell pellets and repeating centrifugation. Pooled supernatant was layered over 35% (w/w) sucrose containing 10 mM Tris.HCl, pH7.4, and 1 mM EDTA and centrifuged at 25,000 rpm at 4 °C for 1 h in a SW28 rotor (Beckman Coulter, Brea, CA). The opaque membrane interface formed was collected and washed twice by ultracentrifugation. The membrane pellet obtained was further resuspended in transport buffer (50 mM Tris.HCl, pH 7.4, and 250 mM sucrose) and passed 20 times through a 27-gauge needle for vesicle formation. Protein concentration was determined using Quick Start Bradford Protein Assay (BioRad, Hercules, CA).

2.7 Membrane vesicular transport assay

A rapid filtration technique was used to measure the ATP-dependent transport of [3H]$E_217\beta G$ into MRP1 enriched inside-out membrane vesicles [20]. The vesicles (2 μg protein) were incubated with 400 nM/20 nCi [3H]$E_217\beta G$ for a minute at 37°C in a 30-μL reaction mixture containing 4 mM AMP or ATP, 10 mM $MgCl_2$, and test compound in transport buffer (250 mM sucrose and 50 mM Tris-HCl, pH 7.4). Total DMSO concentration was kept at 0.3%. Reaction was stopped by the addition of ice-cold buffer and resulting mixture transferred to a 96-well MultiScreenHTS-FB plate (EMD Millipore, Billerica, MA). Filter membranes were washed 4X with 200 μL ice-cold suspension buffer under vacuum aspiration. Radioactivity retained on the membranes was measured with Tri-Carb 4810TR liquid scintillation counter (PerkinElmer, Waltham, MA). ATP-dependent uptake was calculated by subtracting the uptake in the presence of AMP from the uptake in the presence of ATP. Treatments were performed in triplicates.

2.8 Drug sensitivity Assay

The sensitivity of H69 and H69AR cells towards multiple chemotherapeutic drugs (vincristine and SN-38) and the ability of test compounds to reverse the resistance of H69AR cells against these drugs were analyzed using the MTT colorimetric assay. H69 and H69AR cells were seeded in 96-well plates (CellBIND®, Corning) at 2.5×10^4 cells per well in 100 μL culture medium and allowed to attach overnight. The cells were pretreated with 50 μL of test compounds in culture medium and incubated for an hour. Subsequently, 50 μL of cytotoxic drugs (vincristine and SN38) at varying concentrations

was added to the cells. Final DMSO concentration was maintained at 0.2%. Cells were further incubated for 96 h. At the end of the incubation period, 100 μL of culture medium was carefully removed and cells were treated with MTT (0.5 mg/mL) for 4 h. The formazan crystals were dissolved by the addition of 100 μL of 15% SDS containing 10 mM HCl and absorbance at 570 nm were recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicates and repeated three times.

2.9 High content screening data analysis

The MetaXpress software was used for analysis. The mean fluorescent intensity of each well was obtained from averaging the fluorescent intensities of the segmentation mask of 4 captured images. The use of segmentation mask prevents the inclusion of background fluorescence as cells are defined. The relative inhibition of each test compound on CRO-9efflux was determined for each well using the following equation:

$$\% \, inhibition = \; X \;\; T \;\; - X \;\; CRO-9 \qquad X \;\; MK\text{-}571 \;\; - X \;\; CRO-9 \qquad \times 100 \,,$$

where X represents the average fluorescent intensities and T represents the test compound. Both positive and negative controls were placed in every two columns and used for the determination of the percent inhibition for compounds within the same columns. The Z'-factor, a parameter commonly used to infer the versatility and variation of an assay [21] was determined with the following equation:

$$Z \; ' \; \text{-}factor = 1 - \; 3 \;\; \sigma \;\; MK\text{-}571 \;\; + \sigma \;\; CRO-9 \qquad \mu \;\; MK\text{-}571 \;\; - \mu \;\; CRO- \; 9 \qquad ,$$

where σ and μ represent the standard deviations and means, respectively.

2.10 Statistical analysis

Statistical analyses were performed using GraphPad prism (GraphPad Software, San Diego, CA). The differences between mean values were analyzed using linear mixed model analysis. Sidak correction was applied for multiple comparisons. For all analyses, differences were considered significant at *P* value lower than 0.05.

3.0 Results

3.1 Assay development and optimization

We recently reported the development of an imaging-based high content assay for screening for MRP1 inhibitors. This system is more robust and efficient as compared to a fluorescent microplate reader or flow cytometer as it eliminates background fluorescence or artifacts by using the custom module to identify cells. This assay originally developed with calcein-AM [18] had been adapted using doxorubicin [19]. The assay using doxorubicin as a fluorescent reporter identified more inhibitors than calcein-AM after screening the same anti-cancer library. MRP1 as a drug transporter has multiple binding sites thus identification of inhibitors is limited to the fluorescent probe used. In this study, High-Content Screening System, which employs advanced image analysis and quantitation tools, was used to develop an imaging-based high-throughput efflux assay for MRP1using CRO-9 as probe. H69 cell line and its MRP1-overexpressing derivative H69AR cell line were used for the development and optimization of this assay. The parental H69 cells are expected to have higher intracellular accumulation of CRO-9 due to low expression of endogenous MRP1 as compared to H69AR. However, H69AR is expected to have lower intracellular accumulation of CRO-9 due to the efflux activity of MRP1 under similar experimental conditions. For optimization of the assay, both concentration and time dependent accumulation of CRO-9 were studied in both cell lines.

As shown in Figure 3.1A, increased concentrations of CRO-9 yielded an increase in intracellular fluorescence. The intracellular accumulation of CRO-9 was remarkably higher in the parental H69 as compared to the H69AR in all tested concentrations. To confirm that the difference in fluorescence accumulation observed in both cell lines were due to MRP1

activity, MK571, an MRP1 inhibitor was introduced. MK571 did not affect CRO-9 accumulation regardless of the incubation duration in H69 cell line (Figure 3.1B). In contrast, MK571 significantly increased intracellular fluorescence in H69AR by approximately 6-fold at 30 minutes of incubation as indicated in Figure 3.1C.

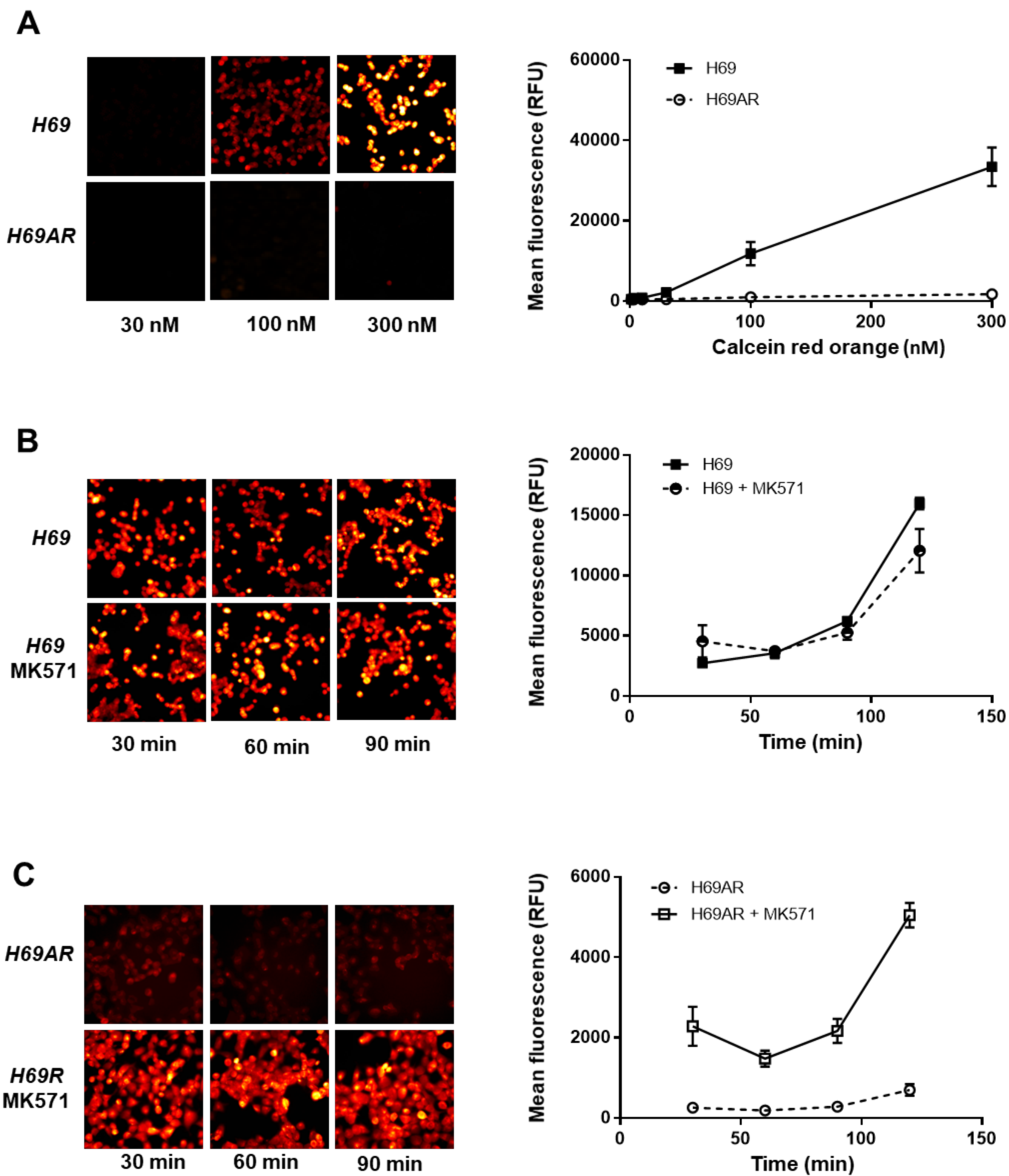

A
H69
H69AR
30 nM
100 nM
300 nM
Mean fluorescence (RFU)
H69
H69AR
Calcein red orange (nM)
B
H69
H69 MK571
30 min
60 min
90 min
H69
H69 + MK571
Time (min)
C
H69AR
H69R MK571
30 min
60 min
90 min
H69AR
H69AR + MK571
Time (min)

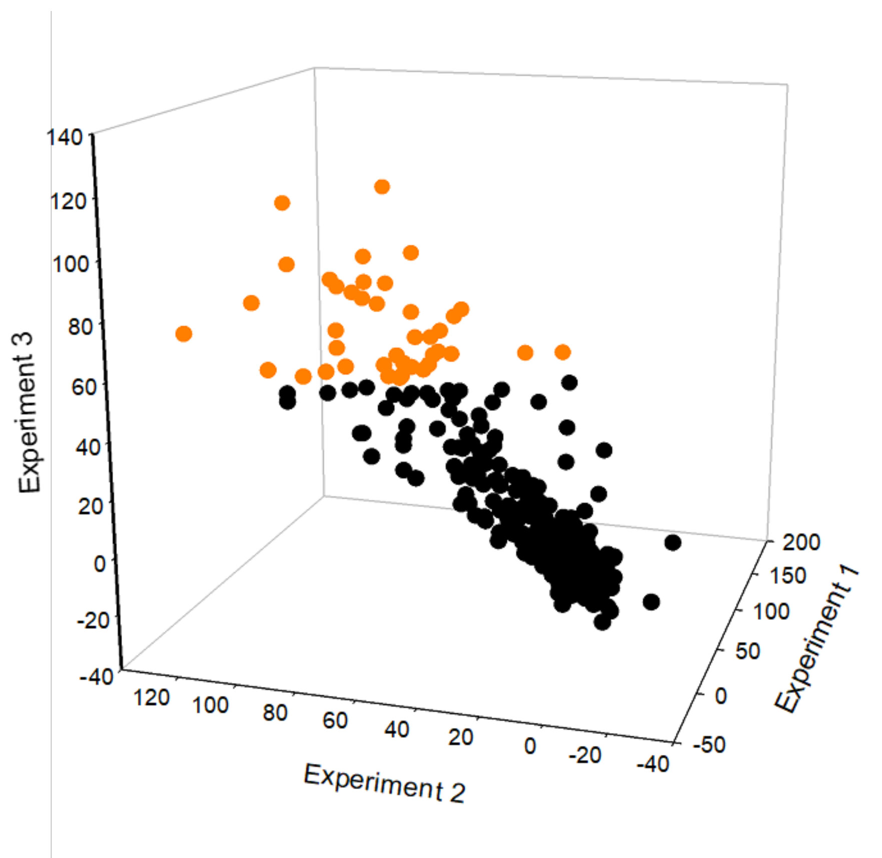
Figure 3.1 Concentration- and time-dependent accumulation of CRO-9 in H69 and H69AR cells**.**

A. H69 and H69AR cells were treated with CRO-9 at various concentrations (10–300 nM) for 1 h. Representative images of CRO-9 treatment at 30, 100, and 300 nM are shown. The average fluorescence intensities derived from the fluorescent images were graphed and shown on the right. **B.** H69 cells were treated with 250 nM CRO-9 in the absence and presence of 50 μM MK571 for 30–90 min. Representative images of CRO-9 treatment at 30, 60, and 90 min are shown. **C.** H69AR cells were treated with 250 nM doxorubicin in the absence and presence of 50 μM MK571 for 30–120 min. Representative images of CRO-9 treatment at 30, 60, and 90 min are shown. Data are representative of two experiments and shown as mean ± SD (n=3).

3.2 Anti-cancer compound library screening for MRP1 inhibitors

After optimization of the high content assay with CRO-9, a 386 anti-cancer library under clinical trials of 12 different cancer types was screened for inhibitors of MRP1. Screening was done in a 96-well format in triplicates. Treatment with 50 μM MK571 (a commonly used MRP1 inhibitor) was considered as 100%MRP1 inhibition for calculating percent inhibition for the test compounds. Three independent screening experiments were done using the 96-well format. The relative MRP1-inhibitory activities of the test compounds from three independent experiments are represented as a 3D scatter plot (Fig. 3.2). The assay had good reproducibility with a correlation range of 0.72-0.78 between any two given experiments. The quality and performance of the assay were also assessed by calculating the Z'-factor from the experiments. The average Z'-factor across all plates was 0.51, indicating a good assay performance. A positive hit was defined as a compound with ≥

50% mean percent inhibition. With this threshold value, we identified 50 hit compounds indicated by orange dots in Figure 3.2. Ten compounds out of the 50 were identified in previous studies in our lab using calcein-AM [18] and doxorubicin [19] as fluorescent reporters. Seventeen out of the remaining forty compounds were exclusive to studies using doxorubicin as fluorescent reporter. The twenty-three compounds that had not been identified in either studies were chosen for further studies.



Correlation coefficient

Experiment	1	2	3
1	1.00		
2	0.74	1.00	
3	0.72	0.78	1.00

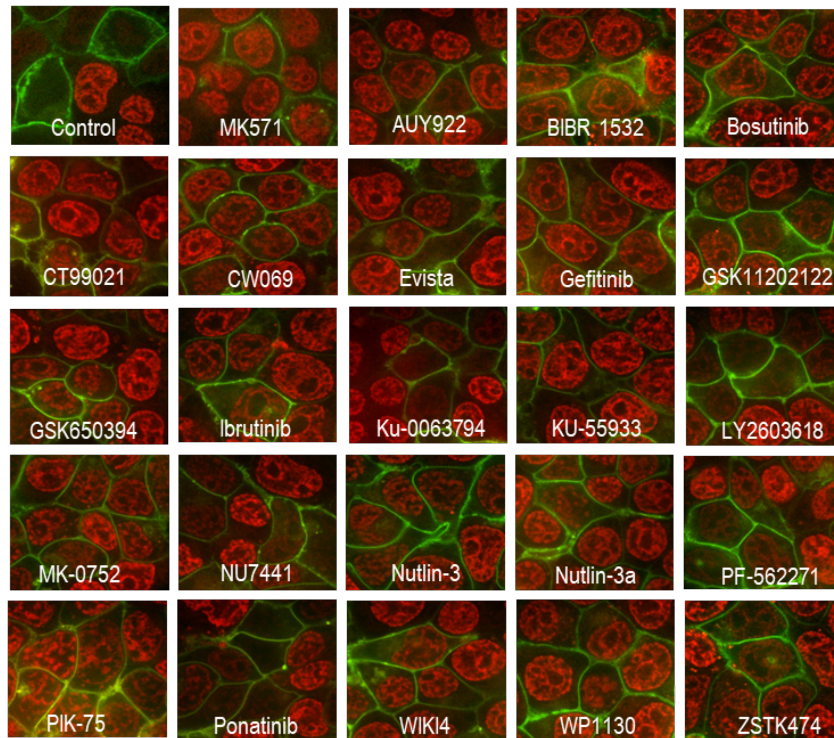
Figure 3.2 Performance of the imaging-based MRP1-mediated CRO-9 accumulation screening assay.

Screening of the anti-cancer compound library was performed in three independent experiments at a compound concentration of 10 μM. The relative inhibitory activities of each compound were calculated and displayed as a 3D plot. Orange dots represent compounds with a mean percent inhibition of ≥50%. The table below the plot shows correlation coefficients between any two experiments. 3D scatter plot was generated using SigmaPlot 12.0 and correlation coefficients were calculated using MS Excel.

3.3 Validation of MRP1 inhibitors

To qualitatively evaluate compound effect on doxorubicin efflux, HEK293T cells were transiently transfected with MRP1-GFP vector and the inhibitory effect of the test compounds on MRP1 activity was visualized using confocal microscopy. As shown in Figure 3.3A, cells treated with DMSO showed high doxorubicin accumulation in the nuclei of non-transfected cells, while cells expressing MRP1-GFP displayed very low doxorubicin accumulation. MRP1-mediated efflux of doxorubicin was reduced by MK571 (50 μM), which was used as the positive control. All the test compounds (10 μM) induced doxorubicin accumulation in MRP1-expressing cells to a certain extent, ranging from strong, moderate to mild. Drugs such as, BIBR 1532, bosutinib, WP1130 and nutlin-3 showed strong inhibition, while GSK11202122, PF562271, ZSTK474, ponatinib and NU7441 showed moderate to mild inhibition. For a more quantitative assessment of hit compounds, flow cytometry was done. To this end H69AR cells were pre-treated with the test compounds for 10 minutes prior to incubation with CRO-9 for 30 minutes. As shown

in Figure 3.3B, MK-571 at 50 μM enhanced doxorubicin accumulation in H69AR cells by 3-fold. All hit compounds (10 μM) except evista, gefitinib, KU-0063794, and NU7441 significantly increased CRO-9 accumulation in H69AR cells by 1.6–2.8-fold.


A
Control
MK571
AUY922
BIBR 1532
Bosutinib
CT99021
CW069
Evista
Gefitinib
GSK11202122
GSK650394
Ibrutinib
Ku-0063794
KU-55933
LY2603618
MK-0752
NU7441
Nutlin-3
Nutlin-3a
PF-562271
PIK-75
Ponatinib
WIKI4
WP1130
ZSTK474

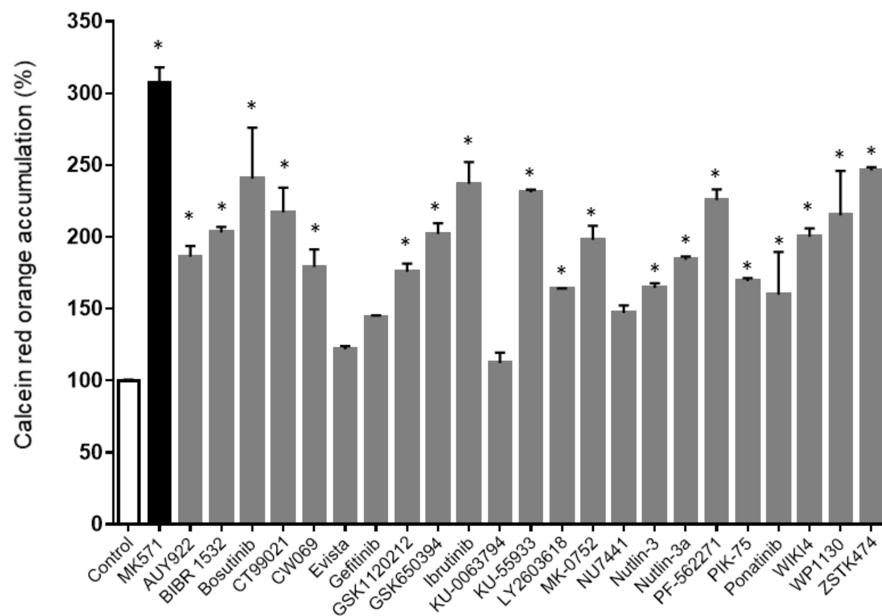

B
Calcein red orange accumulation (%)
350
300
250
200
150
100
50
0
Control
MK571
AUY922
BIBR 1532
Bosutinib
CT99021
CW069
Evista
Gefitinib
GSK1120212
GSK650394
Ibrutinib
KU-0063794
KU-55933
LY2603618
MK-0752
NU7441
Nutlin-3
Nutlin-3a
PF-562271
PIK-75
Ponatinib
WIKI4
WP1130
ZSTK474

Figure 3.3 Validation of MRP1-inhibitory activity of identified hit compounds.

A. HEK293 T cells transiently transfected with MRP1-GFP (green) were pre-treated with 10 μM test com- pounds for 30 min, before treatment with doxorubicin (red) at 37 °C for 1 h. Images were acquired using confocal microscopy. GFP and doxorubicin were excited at 488 nm, and detected at 475/42 and 605/64 nm, respectively. **B.** H69AR cells were pre-treated with 50 μM MK571 or 10 μM test compounds for 10 min before treatment with 250 nM CRO-9 at 37 °C for 30 min Fluorescence intensities of intracellular doxorubicin were detected using flow cytometry, with excitation and emission wavelengths of 488 and 610/20 nm, respectively. Data are combined from two experiments and presented as mean ± SEM. *, p value lower than 0.05 compared with control

3.4 Effects of hit compounds on MRP1 mediated $E_2 17\beta G$ vesicular uptake

Due to the essential physiological role of MRP1 in tissue defense, we were interested in evaluating our hits compounds on MRP1-mediated transport endogenous MRP1 substrates. For this purpose, we employed membrane-based vesicular uptake assay for $E_2 17\beta G$, a glucuronide conjugate and a prototypical physiological substrate of MRP1 Membrane vesicles were prepared from HEK293/pcDNA 3.1 and HEK293/MRP1 cells. The uptake of $[^3H]$ labelled $E_2 17\beta G$ was measured to evaluate the effect of potential MRP1 modulators on $E_2 17\beta G$ efflux. As shown in Figure 3.4, ATP-dependent uptake of $[^3H]E_2 17\beta G$ into HEK293/pcDNA3.1 (vector control) membrane vesicles was less than 1% of uptake by HEK293/MRP1 membrane vesicles. Transport of $[^3H]$ $E_2 17\beta G$ into HEK293/MRP1 membrane vesicles was reduced by 94% with 10 μM MK571. Among the test compounds, evista, GSK1120212, MK-0752 and WIKI4 inhibited $[^3H]E_2 17\beta G$ uptake by more than

50%, while CW069, gefitinib, KU-0063794, KU-55933, and LY2603618 showed little to no inhibition of [^{3}H]E$_2$17βG transport uptake into HEK293/MRP1 membrane vesicles.

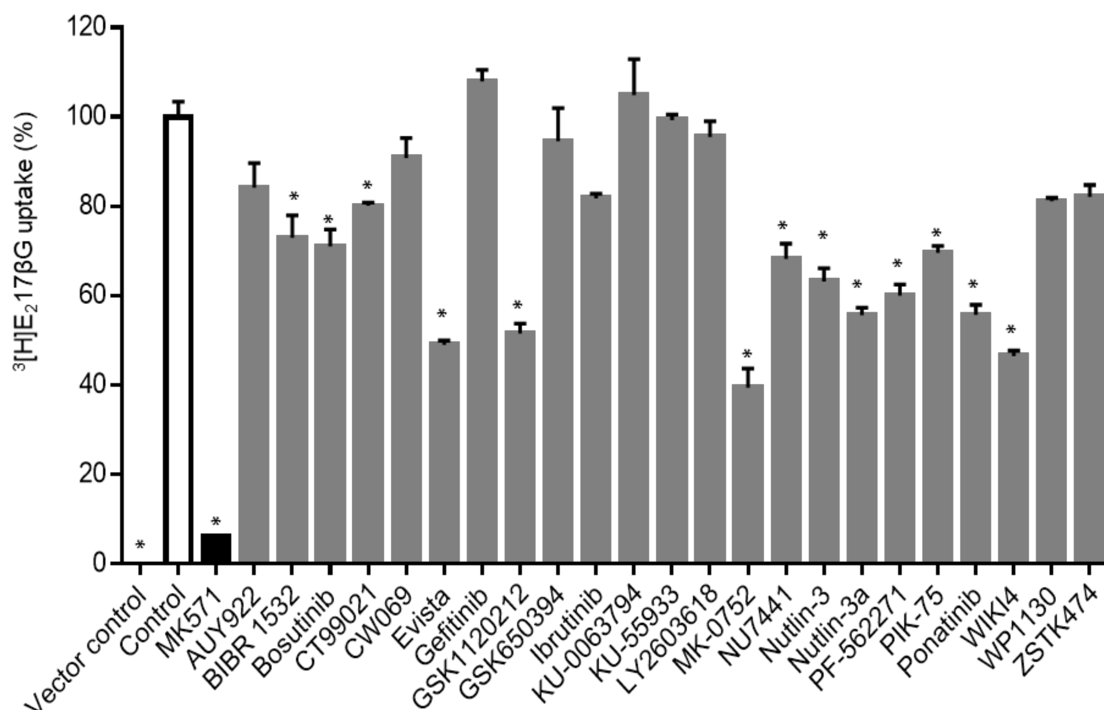


Figure 3.4 Effects of test compounds on [3H]E$_2$17βG uptake into MRP1 expressing membrane vesicles.

Membrane vesicles were prepared from stable HEK293/ pcDNA3.1 and HEK293/MRP1 cells. Membrane vesicles (2 μg protein) were incubated with 10 μM test compounds. Reactions were performed using 400 nM/20 nCi [3H]E$_2$17βG at 37 °C for 1 min. Radioactivity retained on membrane vesicles was quantified using liquid scintillation counting. Data were combined from two experiments and presented as mean ± SEM, * p value lower than 0.05 compared with control.

3.5. Resistance reversal by selected MRP1 inhibitors

Multidrug resistance is a major barrier to the success of chemotherapy. We therefore wanted to verify if the identified anticancer hit compounds had the ability to reverse resistance mediated by MRP1. Drugs that had this ability could be useful in clinical combinatorial therapies. Six potential drug hits (GSK650394, KU-0063794, LY2603618, MK0752, NU7441, ZSTK474) were selected for reversal studies based on available data. The ability of test compounds to reverse MRP1 mediated resistance in H69AR lung cancer cells against vincristine and SN38 was evaluated. These cytotoxic agents are known substrates of MRP1 with different chemical classifications and mode of actions.

First, concentration-response was conducted for selected drug hits to evaluate the cytotoxicity profile of these compounds in H69AR (data not shown). Selected hit compounds at non-cytotoxic concentrations were then administered in combination with increasing concentrations of vincristine or SN38 to determine the ability to reverse resistance in H69AR cells. As shown in Figure 3.5 MRP1-over- expressing H69AR cells (solid red) showed MDR and as expected exhibited much lower sensitivity towards each of the cytotoxic cancer drugs than H69 cells (dotted red). IC_{50} values and fold resistance of the parental H69 and the MRP1-overexpressing derivative H69AR cells treated with vincristine or SN38 with or without the selected compounds are presented in Table 3.1.

Table 3.1 The effects of selected MRP1 inhibitors on the IC_{50} values of vincristine and SN38

Cell line/Treatment	Vincristine		SN38	
	IC_{50} [a] (nM)	Fold resistance [b]	IC_{50} [a] (μM)	Fold resistance [b]
H69	0.861 ± 0.003	1.00	0.0030 ± 0.0004	1.00
H69AR	23.35 ± 3.4	27.11	0.71 ± 0.04	236.90
H69AR + MK571 10 μM	9.33 ± 1.43	10.83	0.28 ± 0.04	94.89
H69AR + GSK650394 10 μM	14.96 ± 0.77	17.37	0.45 ± 0.05	151.57
H69AR + KU-0063794 0.1 μM	12.55 ± 1.11	14.58	0.46 ± 0.04	153.83
H69AR + LY2603618 10 μM	11.12 ± 0.30	12.92	0.0055 ± 0.0006	1.84
H69AR + MK-0752 10 μM	11.98 ± 0.23	13.92	0.52 ± 0.12	171.96
H69AR + NU7441 10μM	13.04 ± 0.52	15.14	0.389 ± 0.001	129.66
H69AR + ZSTK474 2 μM	11.76 ± 0.82	13.66	0.03 ± 0.01	9.74

[a]Mean ± SEM of n ≥ 3 independent experiments.

[b]Fold resistance is the ratio between IC_{50} value of each treatment and IC_{50} value of vincristine and SN38 alone in H69 cells.

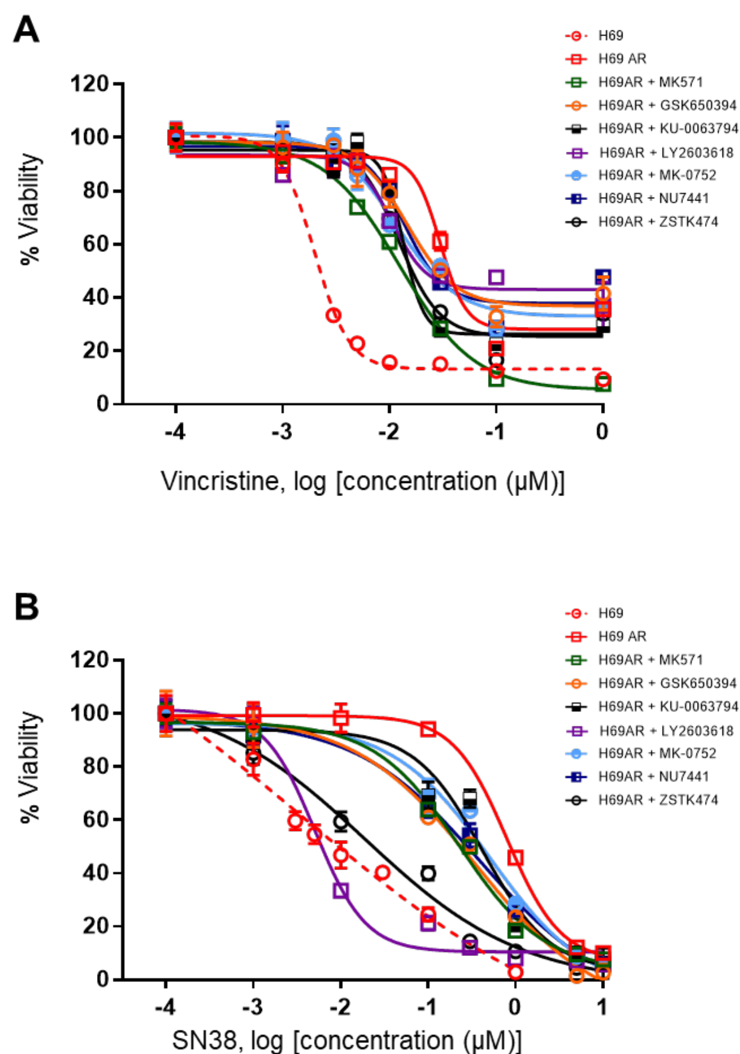


Figure 3.5 Reversal of drug resistance towards vincristine and SN38 in H69AR cells by selected MRP1 inhibitors.

A. H69 and H69AR cells were treated with vincristine at increasing concentrations in the absence and/or presence of selected MRP1 inhibitors. B. H69 and H69AR cells were treated with SN38 at increasing concentrations in the absence and/or presence of selected MRP1 inhibitors. A and B. MK571, GSK650394, LY2603618, MK-0752, NU7441, were at 10 μM, KU-0063794and ZSTK474were at 0.1 μM and 2 μM respectively. Cell viability was evaluated with MTT after 72 h. Data are representative of three experiments and presented as mean ± SD (n = 3).

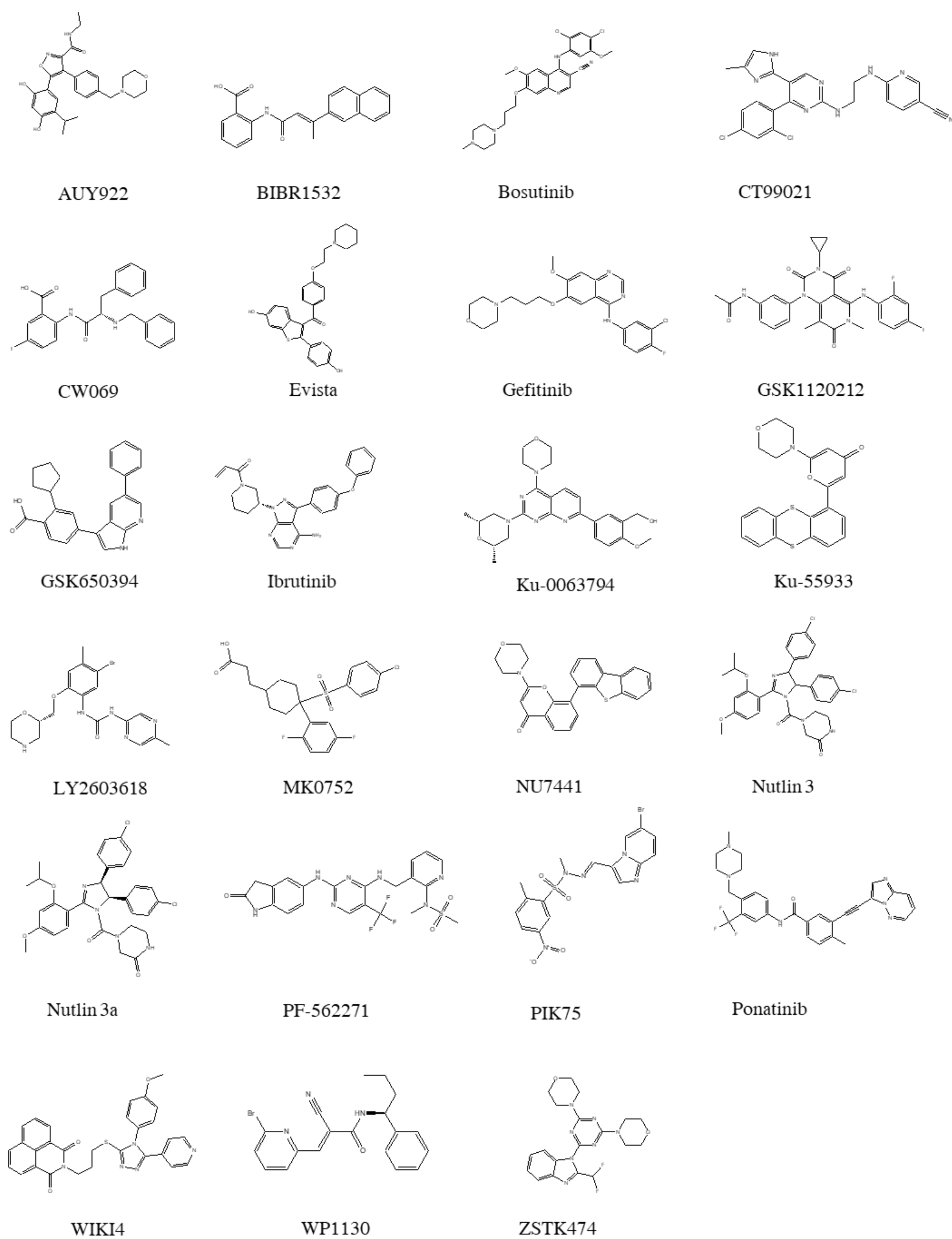


Figure 3.6 Chemical Structures of the selected 23 hit compounds

4.0 Discussion

Chemotherapy is the prevalent form of treatment for advanced stages of most types of cancer. Factors affecting the success of chemotherapy include multidrug resistance (MDR) and toxicity. MDR is known to be a major cause of relapse in chemotherapy. Energy dependent efflux of anticancer drugs by mainly P-gp, MRP1 and BCRP have been reported to be a significant contributing factor to the development of MDR due to their key roles in drug disposition of anticancer therapeutics [3, 22]. Conventional anticancer drugs such as doxorubicin, vincristine, etoposide, methotrexate and carboplatin target basic and essential cellular processes in their mechanism of action. Thus, conventional chemotherapeutics are not specific and results in increased toxicity in patients. Current and emerging chemotherapeutics are drugs that show specificity in cancer cell type and often target specific signaling pathways associated with specific cancers. However, with the ability of drug transporters such as MRP1 to interact and affect the bioavailability of a broad spectrum of drugs, it is essential to profile interactions of new and emerging chemotherapeutics with drug transporters. In cancers such as childhood neuroblastoma, ovarian cancer [23], acute lymphoblastic leukemia [24], the overexpression of MRP1 has been linked to the poor survival rates of patients. Extensive literature on MRP1 also supports the potential use of MRP1 as a biomarker in non-small cell lung cancer (NSCLC) [25].

Current strategies to overcome ABC transporter mediated MDR include the co-administration of transporter inhibitor with chemotherapeutics (combination therapy) or the use of anticancer drugs that are not substrates of MRP1. Several modulators have been developed and investigated over time for their inhibitory activity against MRP1-mediated

transport. Drugs such as indomethacin, probenecid, MK571, and ONO-1078 have been shown to inhibit MRP1 *in vitro* but the clinical significance has been uncertain due to toxicity and specificity related issues [26]. No potent and safe MRP1 modulator has been developed yet. In this study we investigated the interaction of MRP1 with a unique library of anti-cancer compounds currently under clinical trials targeting 12 types of cancers. MRP1 inhibitors from this library has the potential of being used to reverse MRP1 mediated drug resistance in cancers where MRP1 is significantly overexpressed. They can also be used to improve efficacy of other known anticancer agents which are substrates of MRP1.

For this study, we successfully developed and validated a high content imaging-based uptake assay for MRP1 transport activity using CRO-9 as a fluorescent reporter substrate. We are the first to discover CRO-9 as a substrate of MRP1 and develop a novel assay using this dye. Screening of a 386 unique anticancer library with CRO-9, we identified 23 MRP1 inhibitors which were missed by a similar assay using calcein-AM or doxorubicin. The assay exhibited a high correlation (0.72-0.78) between any two experiments among the three independent screening experiments. The high quality, performance and range of this screening assay were demonstrated by a Z'-factor of 0.51 across all plates. High content imaging used in this study offers an advanced image analysis tools which enables the elimination of non-specific and background fluorescence. The assay also allows the simultaneous detection of cell-permeable, non-toxic and potent inhibitors. Majority of inhibitors identified previously from the 386-unique anticancer library using either calcein-AM or doxorubicin as fluorescent reporter were also identified using CRO-9. With extensive biochemical and structural literature suggesting that MRP1 has multiple distinct

substrate binding sites [27, 28], our data implies that CRO-9 may have overlapping binding sites with calcein-AM and doxorubicin and an additional exclusive biding site as well. These differences observed can be due to structural differences between CRO-9, doxorubicin and calcein-AM.

Among the 50 hit compounds identified as MRP1 inhibitors in this study, 10 compounds were common with studies using calcein-AM based screening assay and doxorubicin. An additional 17 compounds including alisertib and amutavinib which were reported for the first time to interact with an ABC transporter using doxorubicin were also identified using CRO-9. Thus, we focused on characterization of the 23 hit compounds exclusive to CRO-9. Among the hit compounds exclusive to CRO-9, Nutlin 3, ZSTK474, ibrutinib and evista has been reported to inhibit the activity of MRP1 transport [29, 30, 4, 31]. Out of the 23 inhibitors identified, 19 have not been previously reported to inhibit the transport by MRP1. Nutlin 3a an enantiomer of nutlin 3 has been reported to affect the P-gp and BCRP mediated transport but not MRP1 [29, 32]. In validating our hit compounds as MRP1 inhibitors, both cell-based assays and vesicular transport assays were employed. Nine drugs (AUY922, CW069, gefitinib, GSK650394, ibrutinib, KU55933, LY2603618, WP1130 and ZSTK474) significantly affected MRP1 mediated transport in cell-based assays measuring CRO-9 efflux but did not affect MRP1 mediated uptake of $E_2 17\beta G$ in enriched membrane vesicles. This demonstrated the substrate selective inhibition ability of some compounds identified as inhibitors in this study. Compounds that show substrate selective inhibition of MRP1 may be useful in specialized treatment of MDR tumors.

Six of the inhibitors identified in this study (GSK650394, KU-0063794, LY2603618, MK0752, NU7441 and ZSTK474) were evaluated for their ability to reverse MRP1

mediated resistance of MRP1-overexpressing lung cancer cell line (H69AR) against vincristine and SN38. We also sought to examine whether reversal of resistance by selected drugs were substrate specific. Vincristine and SN38 are MRP1 substrates with different chemical class/family and cellular mechanism. Among the 6 drugs evaluated, LY2603618 and ZSTK474 were the most effective in reversing MRP1- mediated resistance by reducing fold resistance to vincristine from 27.11 to 12.92 and 13.66 respectively. For MRP1-mediated resistance of H6AR to SN38, LY2603618 and ZSTK474 reduced the fold resistance from 236.90 to 1.84 and 9.74 respectively. LY2603618, a novel MRP1 inhibitor identified in this study is a Chk (Checkpoint kinase) inhibitor which has been used in 7 clinical trials as of September 20th, 2019 (clinicaltrials.gov) for studying the treatment of solid tumors, advanced cancer, pancreatic neoplasms [33], and non-small cell lung cancer (NSCLC) [34]. LY2603618 has been shown to increased cisplatin sensitivity in osteosarcoma, however the interaction of the drug with ABC transporters including MRP1 has never been reported. LY2603618 in our study successfully reduced fold resistance of H69R to vincristine by half (reduced from 27-fold resistance to approximately 13-fold). It also reversed resistance to SN38 almost completely by decreasing the fold resistance from 237-fold difference to 1.8-fold which is similar to the non-resistant parental cell line (H69). With MRP1 being overexpressed in cancers such as non-small lung cancer [25], LY2603618 holds great promise in the treatment of subpopulation of NSCLC patients who have upregulated MRP1 levels.

ZSTK474, a potent phosphoinositide 3-kinase (PI3K) inhibitor identified in this study has been reported to inhibit P-gp, MRP1 and BCRP [35]. It has been reported to have synergistic effect when combined with imatinib in multidrug resistant K562/A02 cells [36].

ZSTK474 has also been shown to reverse MDR resistance in HL60/HL60ADR cells [30] which is consistent with our findings. In our study, ZSTK474 was much more efficient in reversing resistance to SN38 (reduced approximately 237-fold resistance to approximately 10- fold resistance) as compared to vincristine (27-fold resistance to approximately 14-fold resistance).

GSK650394, a serum-regulated kinase 1 (SGK1 inhibitor) which has been investigated for its potential as therapeutic treatment of prostate cancer [37] and colorectal cancer [38] is reported for the first time to inhibit and reverse MRP1 mediated resistance of H69AR to vincristine and SN38. KU-0063794, an mtor inhibitor was able to sensitize H69AR to vincristine (IC_{50} reduced from 23.4 to 12.55 nM) and SN38 (reduced IC_{50} from 0.71 to 0.46 μM). Interactions of KU-0063794 with ABC transporters has also not been reported. NU7441 is a potent DNA-PK inhibitor has been reported to inhibit P-gp mediated efflux of doxorubicin [39] but has not been reported for its inhibitory activity against MRP1.

Another novel inhibitor identified in this study (MK0752) has been in nine phase I /phase II clinical trial for combination therapies for various cancers (clinicaltrials.gov). For advanced metastatic breast cancer, the drug is combined with docetaxel whiles combination with gemcitabine is being done for patients with pancreatic ductal adenocarcinoma. Although reversal of MRP1 resistance has not been demonstrated, MK0752 is a known gamma secretase inhibitor that targets the notch signaling pathway [40]. Some studies have shown that chemotherapy drugs such as doxorubicin induces a Notch 1 dependent upregulation of MRP1 [41, 42]. In this study, MK0752 sensitized MRP1-overexpressing H69AR cells to vincristine (reduced IC_{50} from 23.4 to 11.98 nM)

and SN38 (reduced IC_{50} from 0.71 to 0.52 μM). The effect on MRP1 mediated resistance observed may therefore be due to the effect of the drug on the notch signaling pathway.

In summary, we demonstrated the efficacy of using CRO-9 as a fluorescent reporter in a high content imaging-based assay to profiling interactions with MRP1. Anti-cancer agents which were identified as inhibitors have the potential of being used in combinational therapy to increase the efficacy of other chemotherapeutic drugs. They can also be explored in treatment of subpopulation of patients with multidrug resistance cancers where MRP1 is prominently expressed.

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Chapter 4

RELEVANT CONTRIBUTIONS

1.0 Scope

This chapter entails relevant contributions to other studies in our research group. The research in this chapter is categorized into two projects. The first project was titled "Calcitriol and Calcipotriol Modulate Transport Activity of ABC Transporters and Exhibit Selective Cytotoxicity in MRP1-overexpressing Cells" and published in Drug Metabolism and Disposition [1]. In this project, the focus of the paper was showing the selective cytotoxicity of calcitriol and calcipotriol toward MRP1-overexpressing cells which could be eliminated with MRP1 inhibitor MK571. Data obtained indicated a potential role of calcitriol and its analogs in targeting malignancies in which MRP1 overexpression is prominent and contributes to MDR.

The second project, titled "Development of Novel Intramolecular FRET-Based ABC Transporter Biosensors to Identify New Substrates and Modulators" published in Pharmaceutics [2]. The objective of the research was to develop a functional recombinant MRP1 biosensor protein which could determine compound interactions with MRP1. The clone MRP1-GR-881 was used in screening 40 novel anti-cancer drugs and identified 10 hits that potentially directly interact with MRP1 and could be substrates or modulators. This provides a valuable tool in profiling of drug libraries for interaction with MRP1. Knowledge of drug-MRP1 interactions can provide useful information to improve the efficacy and reduce the toxicity of various therapies.

Calcitriol and Calcipotriol Modulate Transport Activity of ABC Transporters and Exhibit Selective Cytotoxicity in MRP1-overexpressing Cells

1.1 Introduction

Collateral sensitivity is a phenomenon in which the development of resistance toward a cytotoxic agent in the cells simultaneously confers a greater sensitivity to an alternate agent [3]. The possibility of exploiting collateral sensitivity in cancers where P-gp, MRP1, and BCRP is overexpressed is garnering heightened interest [3]. The underlying mechanisms for collateral sensitivity are yet to be delineated but several putative mechanisms have been proposed, including the generation of reactive oxygen species, change in cellular energy levels, extrusion of essential endogenous substrate, and membrane perturbation in the resistant cells [4]. Calcitriol (1, 25-dihydroxyvitamin D_3), the active metabolite of vitamin D_3, is a potent hormone which regulates numerous physiologic processes in human body. Calcitriol is conventionally recognized for its role in bone development via the absorption of calcium and phosphorous [5]. Accumulating data recently shows other non-skeletal functions in conditions such chronic kidney diseases [5]. In cancer, multiple evidence from epidemiologic and preclinical studies suggest that calcitriol reduces cancer risk and progression. However, evidence from randomized clinical trials has been lacking or inconclusive [6,7]. Nonetheless, the interest in the use calcitriol for the prevention and improvement of cancer and other diseases remains high and several large-scale clinical trials are underway to determine the effects of calcitriol on these major diseases [7]. Calcipotriol, a synthetic derivative of calcitriol is conventionally used in the treatment of psoriasis. However, it was also recently shown to be effective immunotherapy against early skin cancer when combined with 5 fluorouracil [8].

In this paper, the focus of my research and contribution was to confirm that the hypersensitivity observed in MRP1 over expressive cell lines (H69AR and HEK/MRP1) after exposure to calcitriol and calcipotriol was due to the transport activity of MRP1.

1.2 Materials and methods

1.2.1 Chemicals

Calcitriol, calcipotriol, MK571, and vincristine were purchased from Cayman Chemical (Ann Arbor, MI). Mitoxantrone, poly-D-lysine, thiazolyl blue tetrazolium bromide (MTT), and verapamil were obtained from Sigma-Aldrich (St. Louis, MO) whiles Ko143 were purchased from Tocris Bioscience (Avonmouth, Bristol, UK).

1.2.2 Cell Lines and Cell Culture

H69 and H69AR cells were purchased from ATCC (Manassas, VA). HEK293/pcDNA3.1, HEK293/MRP1, HEK293/P-gp, and HEK293/BCRP were kindly gifted by Dr. Suresh V. Ambudkar (NIH, Bethesda, MD). HEK293 and H69 cell lines were cultured in DMEM (GE Healthcare, Marlborough, MA) and RPMI 1640 (ATCC), respectively, supplemented with 10% fetal bovine serum. H69AR cells were monthly exposed to 0.8 μM doxorubicin and cultured without drug for 1 week before use in experiments. Cells were cultured at 37°C in a humidified incubator set at 5% CO2.

1.2.3 Western Blot Analysis

Cell lysates were prepared in RIPA buffer (Thermo-Fisher Scientific, Waltham, MA) supplemented with 1× Halt Protease Inhibitor Cocktail (ThermoFisher Scientific). Protein concentration was determined using Pierce BCA Protein Assay (ThermoFisher Scientific). Cell lysates (20 μg protein) were electrophoresed on 7.5% Mini-PROTEAN TGX gels

(BioRad, Hercules, CA) and transferred onto Immobilon PVDF membranes (EMD Millipore, Burlington, MA). Membranes were blocked for 1 hour at room temperature and incubated overnight at 4°C with monoclonal anti-MRP1 antibody (QCRL; Sigma) or anti-α-tubulin antibody (Sigma-Aldrich) at 1:500 and 1:10000 dilutions, respectively. Secondary antibody incubation was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (ThermoFisher Scientific) for 1 hour at room temperature. Target proteins were detected using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer) and imaged using a C-DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE). For protein expression comparison, protein band density was analyzed using the Image Studio Lite (LI-COR Biotechnology, Lincoln, NE) software and corrected for uneven sample loading and transfer using α-tubulin as the loading control.

1.2.4 Cytotoxicity Assay

Cell sensitivity to test compounds was analyzed using the MTT colorimetric assay. HEK293 was plated in 96-well plates (CellBIND; Corning) at 5×10^3 cells per well in 100 μl culture medium and allowed to attach overnight. For treatment 100 μl of test compound of varying concentrations prepared in culture medium was added to the cells. DMSO concentration was maintained at 0.5%. For the effect of inhibitor on collateral sensitivity, 50 μl of MK571 was added and incubated for 1 hour before the addition of varying concentration of calcitriol or Calcipotriol. For drug combination treatments in HEK293/P-gp and HEK293/BCRP cells, 50 μl of calcitriol, calcipotriol, or control compounds were

added and incubated for 1 hour before 50 μl of cytotoxic drugs (vincristine or mitoxantrone) were added. Cells were incubated for 72 hours. At the end of the incubation period, 100 μl of culture medium was carefully removed, and cells were treated with MTT (0.45 mg/ml) for 4 hours. The formazan crystals were dissolved by the addition of 100 μl 15% SDS containing 10 mM HCl and absorbance at 570 nm were recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland)

1.2.5 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). The differences between mean values were analyzed using linear mixed model analysis. Sidak correction was applied for multiple comparisons. For all analyses, differences were considered significant at *P* value lower than 0.05.

1.3 Results

Based on the strong modulatory activity of vitamin D analogues (calcitriol and calcipotriol) in cell and membrane-based assays, their interaction with MRP1 was further studied. Preliminary studies showed a hypersensitivity of MRP1-overexpressing cells toward calcitriol and calcipotriol. Data from the cytotoxic studies revealed a concentration-dependent cytotoxic effect of calcitriol and calcipotriol, calcipotriol, with IC_{50} values of 11.0 ± 0.7 and 8.9 ± 1.3 μM, respectively, in MRP1 over-expressing H69AR cells (Fig. 4.1A; Table 4.1). However, the IC_{50} values for calcitriol and calcipotriol in the parental H69 cells were threefold higher than the resistant cell line. In HEK293/MRP1 cells, calcitriol and calcipotriol also exhibited a concentration-dependent cytotoxic effect, with IC_{50} values of 8.9 ± 1.3 and 6.0 ± 1.8 μM, respectively, which were lower than IC_{50} values in parental HEK293/pcDNA3.1 by four-fold. (Fig. 4.1B; Table 4.1).

Table 4.1 IC_{50} values derived from cytotoxicity assays

Assay	IC_{50} (μM)[a]	
	Calcitriol	Calcipotriol
Cytotoxicity		
H69	29.0 ± 1.2 *	23.9 ± 0.5 *
H69AR (Control)	11.0 ± 0.7	8.5 ± 1.0
H69AR + MK571 (10 μM)	16.4 ± 1.9	15.0 ± 1.1
H69AR + MK571 (25 μM)	35.3 ± 2.2 *	36.3 ± 2.3 **
H69AR + MK571 (50 μM)	61.6 ± 1.0 **	42.4 ± 1.0 **
HEK293/pcDNA3.1	33.4 ± 2.5 **	23.3 ± 3.7 **
HEK293/MRP1	8.9 ± 1.3	6.0 ± 1.8
HEK293/MRP1 + MK571 (10 μM)	11.7 ± 1.1	16.5 ± 0.1
HEK293/MRP1 + MK571 (25 μM)	16.1 ± 0.5 *	23.6 ± 0.6 *
HEK293/MRP1 + MK571 (50 μM)	35.3 ± 4.0 **	27.5 ± 0.2 *

a Mean ± SEM of n = 3 independent experiments

* $p < 0.05$, ** $p < 0.01$ significantly different between indicated groups, calculated using linear mixed model with Sidak post hoc test.

To test whether the observed collateral sensitivity is dependent on MRP1 activity, we measured the cytotoxicity of calcitriol and calcipotriol treatments in MRP1 over-expressing H69AR and HEK293/MRP1 cells in the presence of 10 and 50 μM MK571 (MRP1 inhibitor). Our results showed that collateral sensitivity of MRP1 over-expressing cell lines toward calcitriol and calcipotriol can be eliminated by MRP1 inhibitor MK571 (Fig. 4.1; Table 4.1). By using inhibitor at 10 μM (partial MRP1 inhibition) and 50 μM (complete MRP1 inhibition) the data indicated that collateral sensitivity is proportional to the amount of active MRP1.

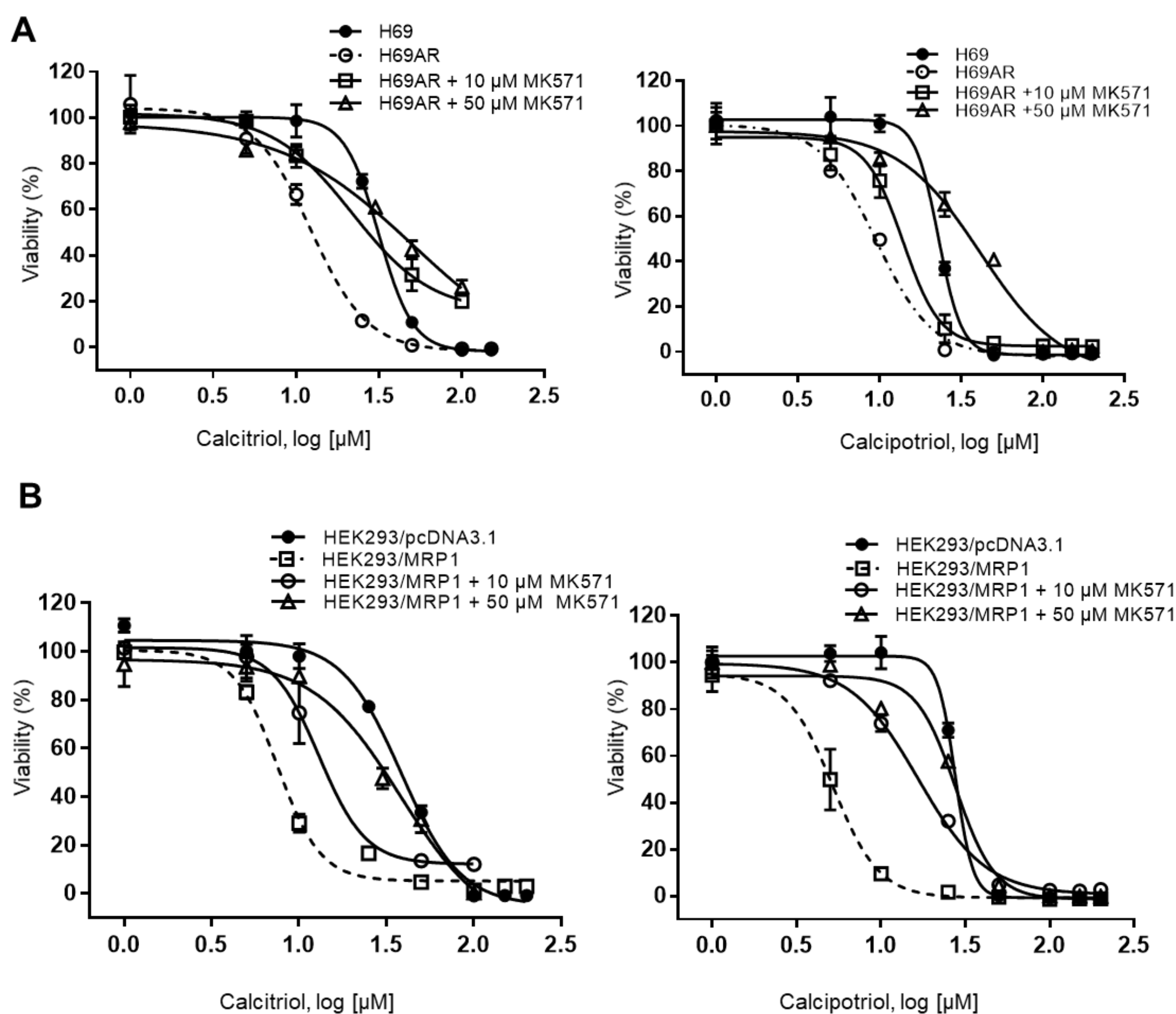

Figure 4.1 Collateral sensitivity of MRP1-overexpressing cells toward calcitriol and calcipotriol.

H69 and H69AR cells were treated with increasing concentrations of calcitriol or calcipotriol in the absence or presence of MK571 for 96 hours. (A). Similar experiments were performed using calcitriol or calcipotriol in HEK293/pcDNA3.1 and HEK293/MRP1 cells in the absence or presence of MK571 for 72 hour (B). Cell viability was evaluated with MTT assay. Data are representative of three experiments and expressed as mean 6 S.D. (n = 3).

1.3.1 Effects of calcitriol and calcipotriol on MRP1 protein expression levels.

To investigate the effect of calcitriol and calcipotriol on the protein expression of MRP1, western blot was performed on HEK293/MRP1 and H69AR after treatment with 1 μM and 10 μM of both compounds for 48 h. The protein expression levels of MRP1 in both cell lines were not significantly altered by 1 μM calcitriol and calcipotriol (data not shown). However, at an increased concentration of 10 μM, calcitriol lowered the protein expression in H69AR cells but not HEK293/MRP1 as shown in Figure 4.2. However, calcipotriol treatment at 10 μM concentration did not significantly change MRP1 expression levels in both cell lines.

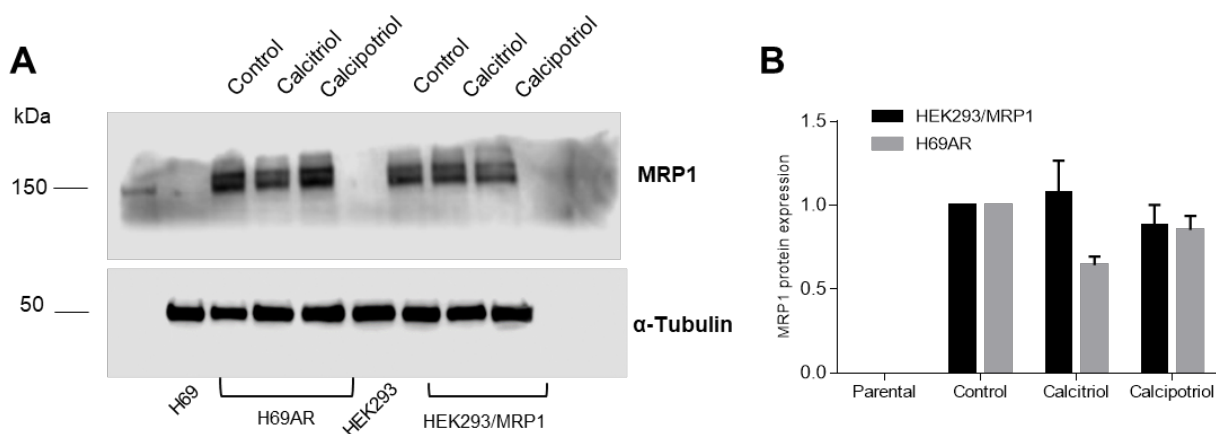

Figure 4.2 Effects of calcitriol and calcipotriol on protein expression of MRP1

Cells were treated with DMSO, calcitriol, or calcipotriol (10 μM) for 72 hours. (A) Immunoblot analysis of whole cell lysates prepared from indicated cell lines with indicated treatments was performed as described in the Materials and Methods section. Data are representative of three experiments. Mean MRP1 protein expression (S.E.M., n = 3) shown as a fold change of the DMSO-treated control is shown in (B). Protein band density was analyzed using the Image Studio Lite (LI-COR Biotechnology) software and corrected for uneven sample loading and transfer using a-tubulin as the loading control.

1.3.2 Effects of Calcitriol and Calcipotriol on Drug Sensitivity of HEK293/P-gp and HEK293/BCRP Cells

Previous results had shown that both calcitriol and calcipotriol did not cause hypersensitivity in both HEK293/P-gp and HEK293/BCRP cells. Thus, we intended to find out if calcitriol or calcipotriol had the ability to reverse drug resistance observed in HEK293/P-gp and HEK293/BCRP against cytotoxic substrates of these cell lines.

HEK293/P-gp and HEK293/BCRP cells were treated with anticancer drugs vincristine and mitoxantrone, respectively, in the presence and absence of 10 μM calcitriol and calcipotriol. Verapamil (25 μM) and Ko143 (1 μM) were used as positive controls for P-gp and BCRP, respectively. As shown in Figure 4.3, HEK293/P-gp and HEK293/BCRP cells exhibited significant drug resistance as compared with the vector control cell line HEK293/pcDNA3.1. Treatment with verapamil and Ko143 in HEK293/P-gp and HEK293/BCRP cells, respectively, reversed the resistance of the cells toward the anticancer agents. In both cell lines, co-treatment with 10 μM calcitriol significantly reversed the resistance of the cells to a certain extent. However, no significant reversal was observed in cells co-treated with 10 μM calcipotriol.

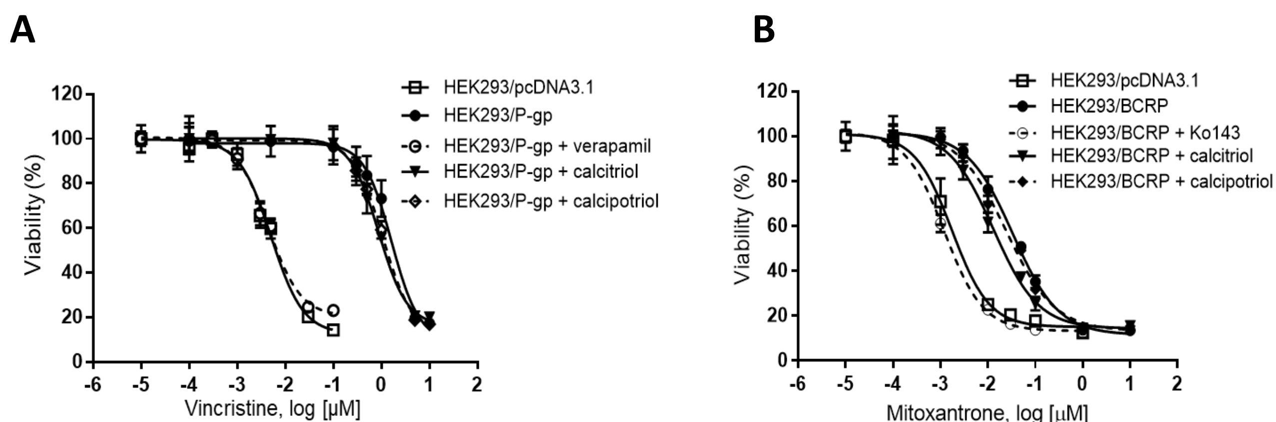

Figure 4.3 Effects of calcitriol and calcipotriol on drug sensitivity of HEK293/P-gp and HEK293/BCRP cells. HEK293/P-gp.

(A) and HEK293/BCRP (B) cells were treated with increasing concentrations of vincristine and mitoxantrone, respectively, in the absence and presence of calcitriol or calcipotriol (10 μM) for 72 hours. Verapamil (25 μM) and Ko143 (1 μM) were used as positive controls in HEK293/P-gp and HEK293/BCRP cells, respectively. HEK293/pcDNA cells were included as negative control for drug resistance. Cell viability was evaluated with MTT assay. Data are representative of three experiments and expressed as mean 6 S.D. (n =3).

1.4 Discussion

This study reported for the first time that the active metabolite of vitamin D3, calcitriol, and its analog calcipotriol, cause selective cytotoxicity in MRP1-overexpressing, but not P-gp- and BCRP-overexpressing cell lines. Calcitriol was also capable of significantly reversing resistance of P-gp and BCRP over-expressive cell lines to vincristine and mitoxantrone respectively. Albeit, calcipotriol did reverse resistance but not significantly in both cell lines. The addition of MRP1 inhibitor MK571 abolished the hypersensitivity previously observed in H69AR and HEK/MRP1 cells. This confirmed that the collateral sensitivity in the cell lines was dependent on the transport activity of MRP1.

Combinatorial approach in cancer therapy which involves combination treatment of modulators and cytotoxic anticancer agents has been the main approach in targeting ABC transporter related MDR. Currently, majority of clinical trials using this method has been conducted using P-gp modulators as P-gp is the most important ABC transporter involved in MDR. However, these modulators have yielded unsatisfactory results leading to skepticism about the feasibility and efficacy of modulators/inhibitors in reversing ABC transporter mediated MDR. [9, 10]. This has garnered sufficient interest in exploiting collateral sensitivity as a substitute of the use of modulators in clinical trials. Collateral sensitivity is a phenomenon where drug resistant cells exhibit hypersensitivity to alternate drugs in comparison with non-drug resistant cells [3, 11]. MRP1-overexpressing H69AR and HEK293/MRP1 cells exhibited collateral sensitivity toward calcitriol and calcipotriol. This phenomenon was specific to MRP1-overexpressing cells as we did not observe any collateral sensitivity in HEK293/P-gp or HEK293/BCRP cells. The selective cytotoxic activity of calcitriol and calcipotriol to MRP1 over-expressing cells could be attributed to

a variety of reasons including stimulation of GSH efflux, up-regulation of apoptosis in a MRP1-dependant manner [12, 13, 14]. In addition, calcipotriol treatment at 1 and 10 μM did not affect the MRP1 protein levels in HEK293/MRP1 and H69AR cells, however calcitriol treatment at 10 μM reduced the protein expression level of MRP1 significantly in H69AR but not HEK293/MRP1. These differences could be due to the physiological differences in the cell lines as they were derived differently. H69AR cells were derived from doxorubicin selection of H69 cells and HEK293/MRP1 were obtained through stable transfection of HEK293 cells.

In conclusion, the main focus of this study was to provide a new perspective to the application of calcitriol and its analogs in cancer treatment. Specifically, our work suggests the potential use of calcitriol and its analogs in selectively targeting tumors with the MDR phenotype conferred by MRP1-overexpression and provides the rational for sequential use of calcitriol and other anti-cancer agents to circumvent the development of MRP1-mediated MDR in clinical chemotherapy. Future work should be focused on designing calcitriol analogs with improved potency and validating the feasibility of applying the MRP1-selective effect of calcitriol in vivo.

Development of Novel Intramolecular FRET-Based ABC Transporter Biosensors to Identify New Substrates and Modulators

1.0 Introduction

Multidrug resistance protein 1 (MRP1) can efflux a wide variety of molecules including toxic chemicals, drugs, and their derivatives out of cells. Drug substrates of MRP1 include anti-cancer agents, antibiotics, anti-viral, and anti-human immunodeficiency virus (HIV) [15]. Efflux of these drugs can interfere with their sub cellular concentration thereby reducing drug efficacy. Although drug development involves many stages, time consuming and costly, a significant number of drugs still fail in clinical trials due to toxicity and inefficacy related issues [2]. Profiling of drugs with relevant drug transporters can help eliminate undesirable drugs at early stages of drug development to reduce economic burden. Profiling drug interactions with MRP1 can identify drugs at risk losing their efficacy as a result of MRP1 overexpression or useful novel inhibitors useful for clinical chemotherapy, especially in malignancies where MRP1 is overexpressed.

Previously, we had engineered a two-color MRP1 construct which quantified intramolecular fluorescence resonance energy transfer (FRET) changes as an index of NBD conformational changes [16]. The underlying principle is that substrate binding results in conformational changes in the NDB. To identify novel substrates and modulators of MRP1 by exploiting intramolecular fluorescence resonance energy transfer (FRET), we genetically engineered six different two-color MRP1 proteins by changing green fluorescent protein (GFP) insertion sites, while keeping the red fluorescent protein (RFP) at the C-terminal of MRP1.

1.2 Materials and methods

1.2.1 Cell Lines and Cell Culture

HEK293T (human embryonic kidney) cell line was donated by Dr. Adam Hoppe (South Dakota State University, Brookings, SD, USA). Cells were cultured in DMEM (GE Healthcare, Marlborough, MA) and supplemented with 10% fetal bovine serum. Cells were cultured at 37°C in a humidified incubator set at 5% CO2.

1.2.2 Detection of MRP1 Localization

HEK293T cells were plated on poly-D-lysine-coated cover glass placed in a 6-well plate at a density of 5×10^5 cells/well in 2 mL culture medium. Cells were transiently transfected with the six two-color MRP1 plasmids after 24 hours using jetPRIME Transfection Reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's protocol. After 48 hours, cells were imaged an iMIC digital microscope (TILL Photonics GmbH, Gräfelfing, Germany) equipped with a 1.35 numerical aperture 60x oil-immersion objective whiles maintained in buffer (4.5 % glucose, 10mM HEPES, PBS containing Ca^{2+} and Mg^{2+}). GFP and RFP were excited at 470 nm and 561 nm, respectively. Emissions of GFP and RFP were correspondingly achieved at 496–530 nm and 573–637 nm. All images were processed with the ImageJ software (NIH).

1.2.3 Doxorubicin Accumulation Assay

HEK293T cells were plated at 5×10^5 cells/well in 2 mL of complete medium on a poly D-Lysine coated coverslip in a six-well plate. After 24 h, the HEK293T cells were transiently transfected with the six different two-color MRP1 constructs using jetPRIME transfection reagent according to manufacturer's protocol. After 48 hours, cells were treated with 10 μM of doxorubicin and incubated for an hour. Cells were then maintained in buffer (4.5 % glucose, 10 mM HEPES, PBS containing Ca^{2+} and Mg^{2+}) and imaged using an iMIC digital microscope (TILL Photonics GmbH, Gräfelfing, Germany) equipped with a 1.35 numerical aperture 60x oil-immersion objective. GFP and doxorubicin were excited at 470 nm wavelength, with emission bands of 480–530 nm for GFP and 570–605 nm for doxorubicin. RFP was excited at 561 nm with an emission band of 573–637 nm. Images were processed with ImageJ (NIH).

1.3 Results and Discussion

1.3.1 Localization and Transport Activity of Two-Color MRP1 Proteins in Live Cells

Fluorescent protein tags like GFP or RFP are usually fused at the amino-terminal or carboxyl-terminal, and do not often cause protein folding and trafficking issues. However, insertion of GFP within the coding sequence of MRP1 could potentially cause problems with the correct folding and trafficking of MRP1. Although all the two-color MRP1 recombinant proteins showed the expected size, we wanted to verify that both fluorophores (GFP and RFP) matured properly and that the recombinant MRP1 proteins were folded, trafficked, and localized properly at the plasma membrane of cells. HEK293T cells were transfected with cDNA expression vectors encoding different two-color MRP1 proteins, and confocal microscopy was used to visualize the localization and expression of recombinant MRP1 proteins. Confocal images in Figure 4.4 indicated that all two-color MRP1 recombinant proteins except GR-648 localized properly at the plasma membrane. This construct was not included in further studies. The GR-859 recombinant protein showed partial mislocalization and intracellular retention, but the recombinant protein was predominantly localized at the plasma membrane

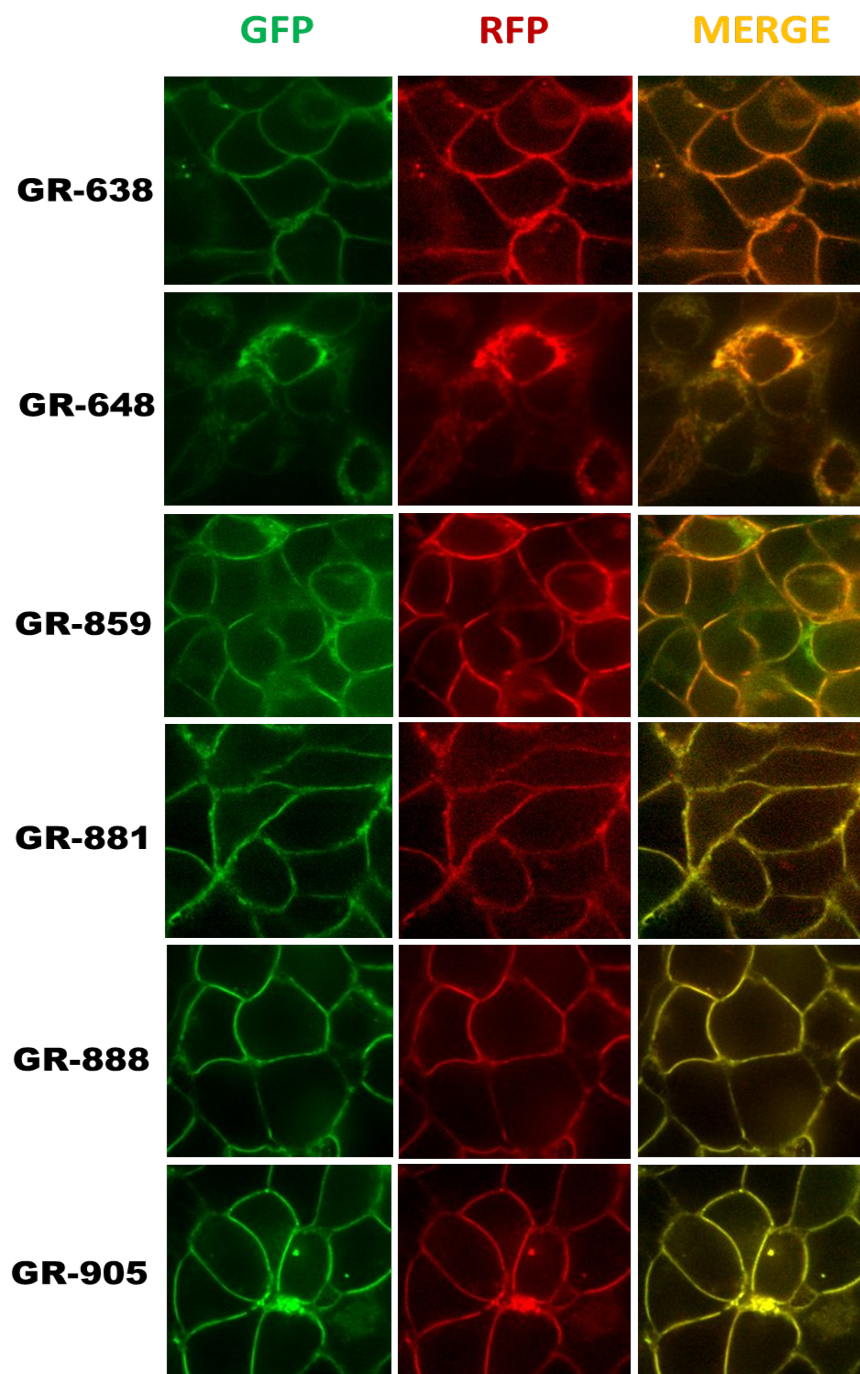
GFP
RFP
MERGE
GR-638
GR-648
GR-859
GR-881
GR-888
GR-905

Figure 4.4 Localization and expression of two-color MRP1 proteins

HEK-293 (human embryonic kidney) cells were plated on glass-bottom chambered coverslips.

Fluorescent images were taken using a confocal microscope equipped with a 63× oil-immersion objective. GFP and TagRFP were excited at 470 nm and 561 nm, respectively. Emission was collected at 480–530 nm for GFP and 580–669 nm for RFP.

To determine if the two-color MRP1 recombinant proteins were functional, their transport activities were evaluated in live cells by measuring accumulation of the fluorescent anti-cancer drug, doxorubicin, a well-known substrate of MRP1 [17]. HEK293T cells were transfected with cDNA expression vectors encoding different two-color MRP1 proteins, and confocal microscopy was used to visualize the accumulation of doxorubicin (Dox) inside the cells. Transiently transfected cells are expected to have a mixed population of cells, transfected and untransfected cells. Transfected cells will express the two-color MRP1 at the plasma membrane of cells whiles untransfected cells will not. Doxorubicin, a substrate of MRP1 executes its cytotoxic action in the nucleus where intercalates into DNA. Thus, cells with a functional MRP1 clone will exhibit a lower intracellular accumulation of doxorubicin in the nuclei as compared with cells with a non-functional MRP1. This disparity is due to the efflux of doxorubicin by a functional MRP1. Doxorubicin fluorescence was very low in cells expressing either GR-638, GR-881, GR-888, or GR-905 (Figure 4.5). These results demonstrate that recombinant MRP1 proteins GR-638, GR-881, GR-888, and GR-905 were functionally active. In contrast, cells transfected with GR-859 showed high doxorubicin accumulation in the nucleus, indicating

that this recombinant protein was not functional, despite having the expected size and proper localization at the plasma membrane.

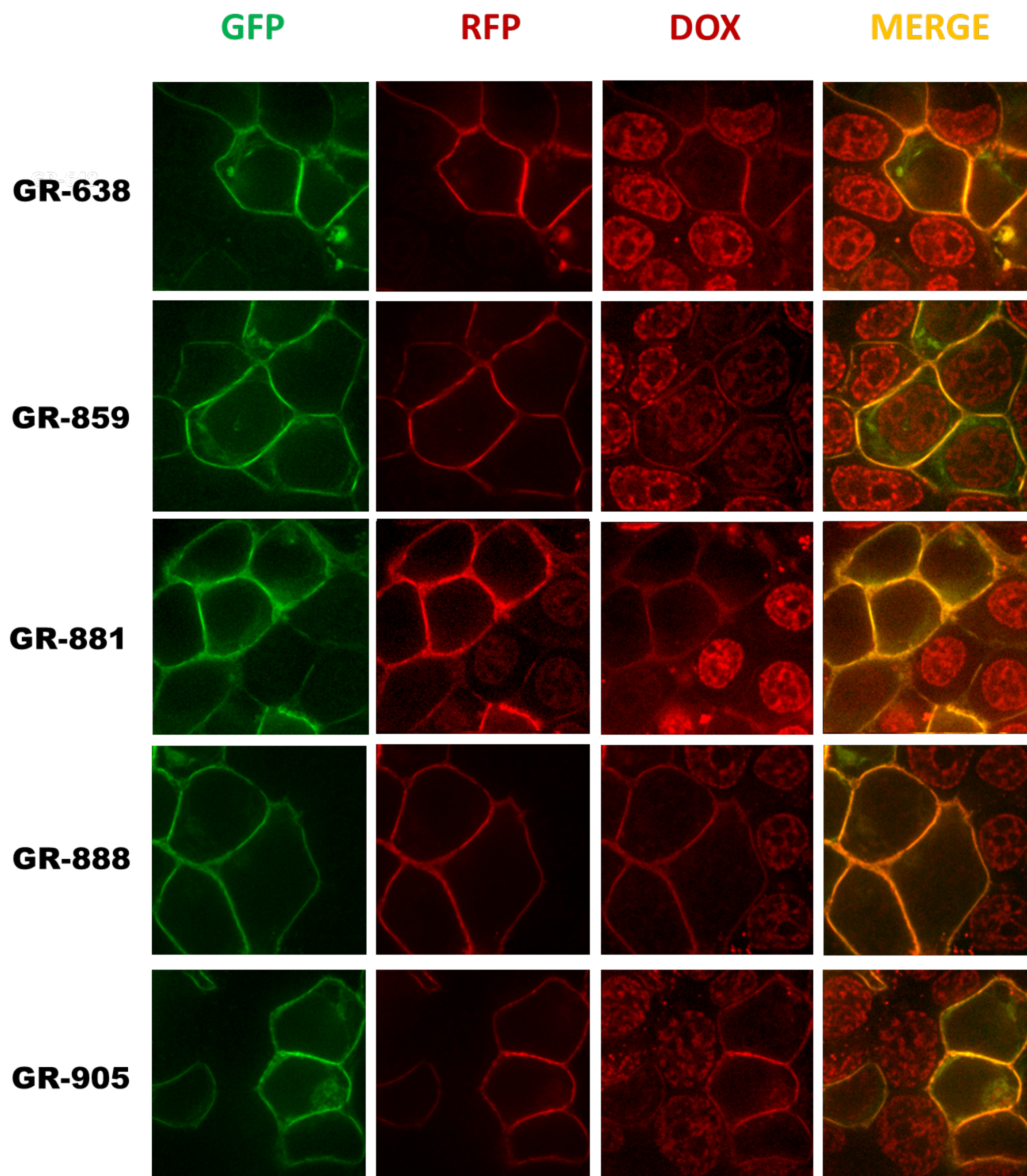
GFP
RFP
DOX
MERGE
GR-638
GR-859
GR-881
GR-888
GR-905

Figure 4.5 Doxorubicin (Dox) accumulation assay

HEK293T cells were transiently transfected with six different two-color MRP1 constructs and incubated for 48 h, after which doxorubicin treatment was done. Images were taken using a confocal microscope equipped with a 63× objective. GFP and Dox were excited at 470 nm wavelength using an Ar laser, with emission bands of 480–530 nm for GFP and 573–637 nm for Dox. RFP was excited at 561 nm, and its emission was collected at 580–669 nm. Data were collected from all three channels—GFP, RFP and Dox. RFP and Dox have significant emission wavelength overlap, explaining the membranous signal seen for the two-color MRP1 recombinant proteins in the Dox channel.

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Chapter 5

FINAL DISCUSSION AND GENERAL CONCLUSIONS

Few ABC transporters are recognized as the key drug transporters to be assessed for their interaction with new drugs in the drug discovery process. They play a pivotal role in governing the transit of a broad spectrum of endogenous substrates and drugs across major organs and physiological barriers [1]. MRP1 is one of the most important ABC transporters which affects drug distribution. It mediates tissue defense in organs such as lung, skin heart, kidneys, small intestines and pharmacological barriers like blood brain, blood, testes and blood placental barrier. In addition to limiting absorption and bioavailability of drugs, MRP1 also regulates drug elimination into urine and feces [2]. MRP1 is overexpressed in a diverse range of cancers and has been implicated in poor survival rates in cancers like acute lymphoblastic leukemia [3], and ovarian cancer [4]. MRP1 may have the widest spectrum of substrates among all clinically significant ABC transporters (P-gp and BCRP). Cells that overexpress MRP1 are resistant to a variety of drugs including anthracyclines, camptothecins, antimetabolites, epipodophyllotoxins and vinca alkaloids [5]. Unlike P-gp, MRP1 does not transport taxanes [5]. Due to the variability in its clinical significance and wide substrate range, finding modulators/inhibitors for MRP1 has been more challenging than P-gp. This may be due to MRP1's preference of interacting with anionic compounds [6]. Anionic compounds do not efficiently enter cells due to the hydrophobic nature of cell walls. As a result, higher doses may be required to attain enough intracellular concentration for effective inhibition. This increases the likelihood of toxicity *in vivo*. Organic anion inhibitors such as probenecid, sulfinpyrazone and indomethacin have been used *in vitro* to

inhibit MRP1. However, since their inhibition activity is not specific to MRP1 (includes all organic anion importers and exporters) they cannot be used clinically as an MRP1 inhibitor [7]. MK571 was initially thought to be a specific inhibitor of ABC (MRP) sub family of ABC transporters [8]. It was later discovered to inhibit P-gp and BCRP and issues regarding toxicity has prevented its use in clinical trials [9]. Considerable efforts have been made to develop inhibitors in order to improve efficacy of anticancer drugs and reduce transporter mediated MDR. To date, no potent and safe MRP1 modulator has been developed and tested in clinical trials and efforts to find the 'ideal' drug candidate is ongoing. In this study we investigated the interaction of MRP1 with a unique library of anti-cancer compounds which is are undergoing clinical trials targeting 12 types of cancers using a high content imaging-based efflux assay.

Over the years, one of the most widely used cell-based assays for assessment of compound interaction with ABC transporters has been the fluorescent substrate accumulation assay. This assay measures the intracellular accumulation of a fluorescent substrate in a transporter-overexpressing cell line in the presence or absence of test compounds. Assays using fluorescent probes to evaluate transporter activity are termed as dye extrusion assay or uptake assays [10]. The accumulation assay has been utilized to study the interactions of ABC transporters. The general principle of accumulation assays is the fluorometric determination of intracellular accumulation of a specific probe such as calcein-AM. This assay has been extensively used to study drug interactions with MRP1 [11, 12] and P-gp [13]. Calcein-AM easily gets into cells by passive diffusion where it is cleaved by endogenous cellular esterases to form a hydrophilic non permeable fluorescent compound [10]. Since calcein-AM is a good substrate of MRP1 and P-gp, it has been used for high

throughput screening of inhibitors of ABC transporters. Some anticancer drugs such as doxorubicin and mitoxantrone are fluorescent and thus drug accumulation can be used to study efflux activity of ABC transporters. Detection and quantification of intracellular accumulation of fluorescent probe is typically determined by flow cytometry. One of the major disadvantages using flow cytometry or microplate readout is the inability to exclude non-specific and background fluorescence which may result in false positives. Also, the effect of cytotoxic drugs on cell viability cannot be simultaneously detected with inhibitory activity of the drug.

In recent years, imaging-based high content screening (HCS) using automated microscope platform has gained increasing popularity in drug discovery field of research. HCS enables high-throughput imaging of single or multiple biological activities measured as intensity or spatial localization of fluorescent dyes or proteins in cells. HCS platforms offer unique advantages over traditional high throughput platforms. The key advantage is its ability to offer multiple readouts. The readout is typically a fixed endpoint based on object segmentation. Object segmentation allows definition of cells which minimizes background noise and facilitates automated analysis. Additionally, this imaging-based uptake assay is more sensitive than assays based on fluorescence plate reader, which is more suited for assays done in a homogenous condition. Moreover, cells can be imaged *in situ* without cell suspension preparations and multiple washing steps, which increase the processing time and limit the assay throughput. Furthermore, cell viability and density before and after treatment can be visually inspected to evaluate compound cytotoxicity, which may interfere with the assay.

Our research lab has previously screened a unique anticancer library for MRP1 inhibitors using a high content imaging-based assay which used calcein-AM as a fluorescent reporter. This assay identified 12 inhibitors of MRP1 after screening a library of 386 anticancer agents. Since MRP1 has distinct multiple binding sites, we decided to use other fluorescent reporters to screen the same library. In this study, our main objective was to develop, and validate a high content imaging-based efflux assay using doxorubicin and CRO-9 as fluorescent reporter substrates. We are confident that we are the first research group to use doxorubicin and CRO-9 as a reporter substrate since there is no available literature supporting that it has been previously used as a substrate in a high content screening or any other high-throughput screening assay to identify inhibitors of MRP1. The screening process in the doxorubicin assay identified a total of 28 inhibitors, which included 10 of 12 inhibitors discovered previously with calcein-AM assay as well as 18 MRP1 inhibitors that were missed by the calcein AM-based screening. Eleven out of the 18 drugs were reported for the first time to inhibit MRP1 mediated transport. Notable among the inhibitors identified using doxorubicin were afatinib, celecoxib, doramapimod, mifepristone, MK-2206 and rosiglitazone which showed the ability to reverse MRP1 mediated resistance of H69AR to vincristine, doxorubicin and etoposide. The drugs showed varied extent of resistance reversal in all three substrates used. Mifepristone and doramapimod were most effective in reversal of resistance against vincristine while mifepristone and rosiglitazone had greater effect on re-sensitizing H69AR cells to doxorubicin. Celecoxib exhibited selective MRP1 inhibition by completely reversing resistance of H69AR to etoposide but not having similar effect with vincristine and doxorubicin.

The assay utilizing CRO-9 further identified inhibitors that were missed by both calcein-AM and doxorubicin. A total of 50 MRP1 inhibitors including 19 inhibitors that have not been previously reported as inhibitors of MRP1 were identified. Though there were some inhibitors exclusive to the assay with doxorubicin as fluorescent reporter, 23 potential hits identified by CRO-9 were missed by doxorubicin. Selected drugs (GSK650394, KU-0063794, LY2603618, MK0752, NU7441 and ZSTK474) were able to decrease the fold resistance of H69AR to vincristine and SN38. LY2603618 and ZSTK474 were the most effective in reversing the resistance of H69AR cells against vincristine and SN38. NU7441 was much more effective in reversing resistance against SN38 as compared to resistance of H69AR to vincristine. Drugs identified in this study are being studied for the treatment of various cancers. From our findings some of these drugs hold great potential in the treatment of subpopulation of patients with malignancies where MRP1 is prominently expressed.

With new test compounds and pharmacological libraries being developed against a wide range of diseases, it is important to develop screening methods with high specificity and reliability for identifying modulators of ABC transporters especially for transporters that are implicated in multidrug resistance phenotype such as MRP1. Consequently, the use of just one traditional *in vitro* screening method may prove problematic as the possibility of false negative results is increased due to relatively low sensitivity of these methods and selective inhibitory activity of some compounds.

Taken together, we conclude that this high content imaging-based assay is effective in identifying novel modulators of MRP1. Based on the International Transporter Consortium (ITC) recommendation and increased literature supporting the role of MRP1 in the

development of MDR in cancer, we anticipate that our high content imaging-based assay will be a great alternative to traditional *in vitro* methods for pharmaceutical industries interested in profiling drug interactions with MRP1. In our opinion, there is a lot of potential in utilizing this high-content imaged assay developed in our laboratory as doxorubicin and CRO-9 seem to be good fluorescent reporters in screening for MRP1 modulators. This assay also has the potential to aid in the investigation of compound effects on ABC transporter expression, trafficking, and function. For example, the expression and trafficking of ABC transporters, and how small molecules affect these cellular events, can be probed using antibodies in conjunction with reporter substrates described in this paper. This strategy can be applied on clinically relevant mutants of ABC transporters to screen for small molecules that can correct anomaly in their expression, trafficking, and degradation.

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APPENDIX A

Fluorescent substrates of ABC transporters and compounds shown to inhibit efflux

	P-gp	MRP1	BCRP
BODIPY FL EDA	Lasalocid, Loxapine, Mometasone, Pimozide, Nicardipine, Verapamil	Nicardipine, Lasalocid, Pimozide, Mometasone, Verapamil	Lasalocid
BODIPY FL Forskolin	Loxapine, Mometasone, Pimozide, Nicardipine	Nicardipine	Nicardipine
BODIPY FL Histamine	Mometasone, Nicardipine	Pimozide, Mometasone, Verapamil, Nicardipine	Mometasone, Nicardipine, Pimozide
BODIPY FL Prazosin	Pimozide, Mometasone, Verapamil, Nicardipine, Loxapine	Loxapine, Nicardipine, Mometasone, Verapamil	Mometasone, Nicardipine
BODIPY FL Thapsigargin	Mometasone, Loxapine, Nicardipine, Verapamil	Loxapine, Nicardipine, Mometasone	
BODIPY FL Verapamil, HCl	Loxapine, Pimozide, Mometasone, Verapamil, Nicardipine	Loxapine, Pimozide, Mometasone, Verapamil, Nicardipine	
BODIPY FL Vinblastine	Mometasone, Loxapine, Nicardipine, Verapamil	Mometasone, Verapamil, Nicardipine	
Calcein AM	Mometasone, XR9576, Nicardipine, Pimozide	MK-571, Nicardipine, Mometasone, Pimozide	
CBIC2(3) (JC-1)	Mometasone, Pimozide, Nicardipine	Mometasone, Pimozide, Nicardipine	Mometasone, Pimozide, Nicardipine
CellTracker Orange CMTMR	Mometasone, Pimozide, Nicardipine, Verapamil	Nicardipine, Verapamil	
DiIC1(5)	Mometasone, Pimozide, Nicardipine	Mometasone, Pimozide	
DiNOC1(3) (JC-9)	Mometasone, Pimozide, Nicardipine, Verapamil, Loxapine	Mometasone, Pimozide, Nicardipine, Verapamil, Loxapine	
DiOC2(3)	Mometasone, Pimozide, Nicardipine, Verapamil, Loxapine	Mometasone, Pimozide, Nicardipine, Verapamil, Loxapine	
DiOC5(3)	Mometasone, Pimozide, Nicardipine	Mometasone, Verapamil, Pimozide	
DiOC6(3)	Mometasone, Pimozide, Nicardipine, Verapamil	Pimozide, Verapamil	
DiSC3(5)	Mometasone, Pimozide, Nicardipine, Verapamil, Loxapine	Mometasone	
Doxorubicin	Substrate	Substrate	Substrate

eFluxx-ID Gold	Substrate	Substrate	Substrate
eFluxx-ID Green	Substrate	Substrate	Substrate
ER-Tracker Green	Mometasone Nicardipine	Nicardipine Pimozide	
LDS 751	Mometasone Pimozide Nicardipine Verapamil Loxapine		
MitoTracker Green FM	Mometasone Pimozide Nicardipine Verapamil Loxapine	Mometasone Nicardipine Verapamil	
MitoTracker Orange CMTMRos	Mometasone Pimozide Nicardipine		
Mitoxantrone	Substrate	Substrate	Ko143 GF120918 FTC
Pheophorbide A			Substrate
Rhodamine 123	PSC833		
Rhodamine 6G chloride	Mometasone Nicardipine		
Rhodamine B, hexyl ester, perchlorate (R6)	Mometasone Pimozide Nicardipine	Mometasone Pimozide	
SYTO 13	Mometasone Pimozide Nicardipine Verapamil Loxapine	Mometasone Pimozide Nicardipine Verapamil Loxapine	
SYTO 16	Mometasone Pimozide Nicardipine	Pimozide	
SYTO 9	Mometasone Pimozide Nicardipine	Mometasone Verapamil Nicardipine	
Tetramethylrosamine Chloride	Mometasone Pimozide Nicardipine	Mometasone Verapamil Pimozide	
TMRE	Mometasone Nicardipine		

APPENDIX B

Radioactive substrates of ABC Transporters

P-gp	**MRP1**	**MRP2**	**MRP3**	**MRP4**	**BCRP**
[ring C, *methoxy*-^{3}H]-Colchicine	[^{14}C]-1-Chloro-2,4-Dinitrobenzene ([^{14}C]-CDNB)	[^{14}C]-1-Chloro-2,4-Dinitrobenzene ([^{14}C]-CDNB)	[6,7-^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)	[6,7-^{3}H] Estradiol 17-(β -d-Glucuronide) ([^{3}H]-$E_2$17βG)	[^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)
[^{3}H]-Daunorubicin	[^{3}H]-Daunorubicin	[^{3}H]-Docetaxel		[3',5',7'-^{3}H(n)]-Methotrexate	[^{3}H]-Methotrexate
[G-^{3}H]-Digoxin	[6,7-^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)	[^{3}H]-Etoposide			[^{3}H]-Mitoxantrone
[^{3}H]-Paclitaxel	[^{3}H]-Leukotriene C4 ([^{3}H]-LTC4)	[6,7-^{3}H] Estradiol 17-(β-d-Glucuronide) ([^{3}H]-$E_2$17βG)			[^{14}C]-2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine ([^{14}C]-PhIP)
[7-*methoxy*-^{3}H]-Prazosin		[3',5',7'-^{3}H(n)]-Methotrexate			
[^{3}H]-Vinblastine		[^{3}H]-Paclitaxel			
[G-^{3}H]-Vinblastine Sulfate		[^{3}H]-Vinblastine			