2021

Characterization of Cucurbitacin-Inspired Estrone Analogues as Novel Inhibitors of Human ATP- Binding Cassette Proteins (ABCB1 and ABCC1)

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CHARACTERIZATION OF CUCURBITACIN -INSPIRED ESTRONE ANALOGUES AS NOVEL INHIBITORS OF HUMAN ATP-BINDING CASSETTE PROTEINS (ABCB1 AND ABCC1)

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biochemistry

South Dakota State University

2021

DISSERTATION ACCEPTANCE PAGE

Jennifer Kyerematen

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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This dissertation is dedicated to the Almighty God for establishing my existence and giving me the strength to achieve this feat in spite of my weakness. I am indebted to Your unconditional love in my life.

And to the loving memory of my dearest father, Edmond Kyerematen, who taught me that I can always achieve my dreams with determination, hard work, perseverance and patience. I am the individual I find myself today because of your constant reassurance. I wish you were here and I definitely know you will be proud of this achievement.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic advisor, Dr. Surtaj Iram for his guidance, mentorship and kind words of encouragement that pushed me farther beyond the limit I thought I could go. I am indebted to my advisory committee members, Prof. Fathi Halaweish, Dr. Rachel Willand Charnley, Prof. Alireza Salehnia and Dr. Severine Van Slambrouck (former committee member) for their periodic assessment of my research, timely support, guidance and being instrumental in helping this dissertation into completion. I am especially thankful to the Halaweish Lab group (Prof. Fathi Halaweish, Dr. Mater Mahnashi) for providing the compounds that enabled me to execute this study. I am also thankful to my lab colleagues for their constant support and assistance during my research work. I am grateful to the Department of Chemistry and Biochemistry and South Dakota State University for the unwavering financial support throughout my graduate studies.

A huge gratitude goes to my fiancé, Dr. Aubrey Kusi-Appiah for his love, understanding and support and for not allowing me to give up when the going was tough. I am extremely grateful to my family for their love, prayers and sacrifices they have made in my life. To Aunt Doris and my awesome siblings, Christabel, Gloria, Kojo, Nana Boakye and Junior, thank you for understanding and tolerating my silence the past five years.

Finally, I will like to thank my friends, my church family, Holy Life Tabernacle and the Brookings community for creating an amazing positive environment during my studies.

CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
ADMET	absorption, distribution, metabolism, excretion and toxicity
ADP	adenosine diphosphate
AML	acute myeloid leukemia
ATP	adenosine triphosphate
BCRP	breast cancer resistant protein
CFTR	cystic fibrosis transmembrane regulator
CIEA	cucurbitacins-inspired estrone analogues
cMOAT	canalicular multispecific organic anion transporter
CYP3A4	Cytochrome P450 3A4
ECF	Energy-Coupling Factor transporters
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FC	flow cytometry
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GSH	reduced glutathione
HEK 293	human embryonic kidney 293
HCS	high content screening
HTS	high throughput screening
IC_{50}	concentration required to obtain 50% inhibition
JAK/STAT	Janus kinases/signal transducer and activator of transcription proteins

LTC_4	cysteinyl leukotriene
MAPK	mitogen-activated protein kinase
MRP1	multidrug resistance protein 1
MSD	membrane spanning domain
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBD	nucleotide binding domain
NCEs	new chemical entities
NSLC	non-small lung cancer
OATP1B3	organic anion transporting polypeptide
PFIC	progressive familial intrahepatic cholestasis
P-gp	permeability glycoprotein
PXE	pseudoxanthoma elasticum
RFP	red fluorescent protein
ROS	reactive oxidative species
SBP	substrate binding protein
SLCO1B3	solute carrier organic anion transporters
SUR	sulfonylurea receptor
THC	tetrahydrocurcumin
TKIs	tyrosine kinase inhibitors
TMD	transmembrane domain
VLCFA	very long chain fatty acids

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ABSTRACT

CHARACTERIZATION OF CUCURBITACIN -INSPIRED ESTRONE ANALOGUES AS NOVEL INHIBITORS OF HUMAN ATP-BINDING CASSETTE PROTEINS (ABCB1 AND ABCC1)

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2021

ATP-binding cassette (ABC) transporters are a large class of integral membrane proteins that contribute to key physiological functions in all organisms by utilizing ATP binding and hydrolysis to transport diverse substrates across membrane barriers. P-glycoprotein (P-gp/ ABCB1) and Multidrug Resistance protein 1 (MRP1/ABCC1) are widely reported ABC transporters associated with multidrug resistance in cancer. Multidrug resistance (MDR) mediated by P-gp and MRP1 is responsible for treatment failures of many metastatic cancers as a result of reduced accumulation, bioavailability and diminished potency of anticancer drugs. Currently, known P-gp and MRP1 inhibitors are limited due to toxicity, lack of selectivity and low therapeutic benefit which necessitates the investigation of novel compounds with improved potency and specificity. In this study, we investigated the inhibition potential of newly synthesized cucurbitacin-inspired estrone analogs (CIEAs) on P-gp and MRP1 mediated MDR. Utilizing a high content fluorescence-based transport activity assay initially developed in our lab, a library of 81 CIEAs were screened to identify inhibitors for P-gp and MRP1

proteins. The screening assay identified eight P-gp inhibitors using calcein-AM as a fluorescent reporter. A total of six MRP1 inhibitors were identified using doxorubicin and calcein red orange as fluorescent reporters. The inhibitory effect of the identified compounds on P-gp and MRP1 activity were confirmed using established cell-based functional assays. The identified inhibitors significantly increased the accumulation of P-gp substrate, calcein-AM and MRP1 substrates, doxorubicin and calcein red orange. Interestingly, the identified inhibitors demonstrated selective sensitization of P-gp and MRP1 overexpressing cancer cell lines towards different anticancer drugs. On the other hand, the identified inhibitors did not alter protein levels of both P-gp and MRP1. Furthermore, our in silico and FRET-based spectroscopic studies showed that the inhibitor compounds directly interact with P-gp and MRP1 proteins. Together, our findings demonstrated that CIEAs are promising drug candidates for further development into therapeutic agents against P-gp and MRP1-mediated multidrug resistance in cancer.

Chapter 1

Introduction and Background

1.1 Cell Transport and Membrane Transport Proteins

Cell transport is the movement of molecules across biological membranes. Biological membranes serve as the boundary that separates the cell and other intracellular organelles from their external environment. These membranes are essential for intracellular transport by controlling the selective movement of molecules in and out of cells. Generally, transport of molecules is mediated by specific membrane-associated proteins, though some transport processes are unassisted by proteins. These membrane-associated proteins are called membrane transport proteins. Membrane transport proteins play a critical role in regulating several biochemical pathways. Notably, they regulate the entry of essential substances such as nutrients and ions necessary for metabolism into organelles thus maintaining cellular homeostasis while also driving the removal of endogenous waste, toxic substances, metabolites and drugs out of the cell. Consistent with their crucial role, transporters or transporter related proteins are encoded by approximately 2000 genes in the human genome [1]. In addition, their importance may be appreciated from the fact that cells that have defective transport systems have been linked to several pathological conditions such as metabolic disorders [2, 3] and cancer [4]. Also, it is not surprising that membrane transporters constitute over 50% of all known drug targets [5]. Therefore, an extensive knowledge in membrane transport proteins is central to understanding cell metabolism and function. Membrane transport proteins or membrane transporters (sometimes used interchangeably) are grouped into four types:

aquaporins; ion channels; transporters; and ATP-powered pumps [6]. Aquaporins are water channel proteins that utilize osmotic gradients across membranes to transport uncharged molecules (water) into and out of cells [7]. Ion channels on the other hand, are the cell's electric potential regulator proteins that facilitate the transport of charged species or ions down their electrochemical gradient across the plasma membrane. While transporters drive the movement of specific molecules along or against their concentration gradients by undergoing conformational changes, ATP-powered pumps transport molecules against their concentration gradients across the plasma membrane using energy derived from ATP hydrolysis [6]. Membrane transporters can also be classified as passive or active based on their reliability on cellular energy [6]. Passive transporters also known as uniporters or facilitative transporters, mediate the energy-independent transport of substrates across the plasma membrane down their concentration gradient with the aid of special carrier proteins. Active transporters instead, translocate substrates against or down their concentration gradient in an energy-dependent manner. However, active transporters that directly depend on energy derived from ATP hydrolysis to mediate transport are known as primary active transporters. Secondary active transporters indirectly utilize energy from coupling reactions of the transported molecule with another molecule along their concentration gradient sometimes driven by H^+ ions (proton) or sodium motive forces. The transporter is termed as symporter when the transported and co-transported molecules are moved in the same direction or antiporter when both molecules are transported in opposite directions. A diagrammatic presentation of various mechanisms of transport in biological membranes is shown in Figure 1.1.

ATP-binding cassette (ABC) transporters, the transporters of interest in this study are examples of ATP-powered pumps or transport ATPases that include F-, V- and P-type ATPases and also fall under primary active transporters [8]. An overview of the ATP transporter family is discussed in the next sections.

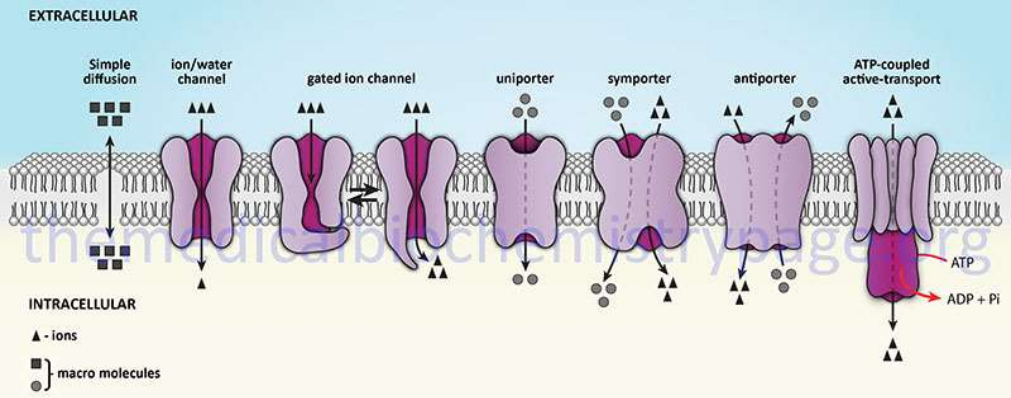

Figure 1.1 Membrane transport processes. Diagrammatic representation of the various mechanisms for the passage/transport of ions and molecules across biological membranes [9].

1.2 The ABC Transporter Family

ABC transporters constitute a large family of integral transmembrane proteins that contribute to key physiological processes in all organisms. They are involved in transporting diverse substrates across membrane barriers using energy derived from ATP hydrolysis. Substrates of ABC transporters may include small molecules such as ions, amino acids, polysaccharides, vitamins, lipids, antibiotics, and drugs to even large molecules like proteins [10]. This transport function leads to physiological processes such

as cell growth and development, nutrients translocation, cellular detoxification, lipid homeostasis, signal transduction and cellular defense [11].

The fundamental protein architecture of a typical ABC transporter consists of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) [12]. The TMDs are also called membrane spanning domains (MSD) in some literature. The NBDs, also known as ATP-binding cassette (ABC) from which this family of transporters derive their name are highly conserved and serve as the motor for energy generation from ATP binding and hydrolysis. Unlike the NBDs, TMDs are more diverse, and responsible for substrate recognition and translocation [12]. During transport, substrates may be recruited either from the inner leaflet (cis-side) or the outer leaflet (trans-side) of the cell membrane. Subsequently, there is ATP binding and hydrolysis at the cytoplasmic side of the membrane which then initiates the opening and closing of the TMDs for possible substrate transport [13]. Based on this cis or trans direction of transport, a given ABC transporter may function as an importer or exporter. Importers direct substrates from the trans-side to the cis-side of the membrane. In contrast, ABC exporters translocate substrates from the cytoplasm to the trans-side, or from the hydrophobic portion of the lipid bilayer to the trans-side or across the inner and outer parts of the bilayer [13, 14]. In general, exporters are found in all kingdoms while importers are limited to bacteria and plants [15, 16]. In bacteria, ABC importers mediate the influx of essential nutrients while exporters typically efflux drug substrates across the membrane [17]. An interesting bacterial ABC exporter is LmrA, found in *Lactobacillus lactis*. LmrA was the first ABC-type transporter discovered to mediate antibiotic resistance in bacteria [18].

Importers and exporters also differ by the structural organization of their NBD and TMD domains. The NBD and TMDs in exporters can be combined in various ways creating a full transporter with varying order of domains. However, for importers, the TMD and NBD domains are distinct chains and can associate to form a homo- or hetero-dimeric domains to create a half transporter [14, 17]. Despite these differences, it is suggested that both transporter types bind ATP and substrates in a similar fashion. In effect, exporters and importers utilize the same ATP binding and hydrolysis mechanism to alternate between inward-facing and outward-facing conformations of the transporter [19, 20]. However, for bacteria importers, substrate translocation requires a high affinity substrate binding protein (SBP) for delivery to the TMDs [21]. With ongoing studies on characterization of ABC transporters, more sub-classifications based on structural and functional diversity have been identified [13, 14, 17]. For instance the Type I and II sub-groups have been reported for both ABC exporters and importers. A more recent group, the Energy-Coupling Factor transporters (ECF) or Type III ABC importers have also been reported to play specific functions in prokaryotes [22, 23].

1.3 Human ABC Transporter Genes and Associated Diseases

The mammalian species have a total of 56 ABC transporter genes of which 48 genes have been identified in the human genome [10]. The 48 human ABC transporter genes can be grouped into seven unique subfamilies designated ABCA - ABCG according to their domain organization and sequence homology. Human ABC transporters are well known to play pivotal roles in many cellular processes such that abnormalities in their genes have consequent genetic conditions. At present, 14 ABC

transporter genes are related to various genetic disorders. A brief description of the subfamilies and their associated genetic diseases are discussed in this section.

The ABCA subfamily consists of twelve full transporters and are subdivided into two groups based on phylogenetic relationships [24]. They are mainly responsible for lipid transport and homeostasis, regulation of membrane trafficking [25] and retinal transport [26]. Mutations in the ABCA1 transporter gene results in Tangier disease, a disorder associated with lipoprotein metabolism [27]; Sjögren syndrome, an autoimmune disease is due to defects in ABCA7 gene [28] while defects in ABCA4 genes have been associated with retinal diseases [29].

The ABCB transporters are the only subfamily made up of both half and full transporters [30]. They are involved in mitochondrial transport, oxidative stress defense [31], intracellular peptide translocation, antigen processing , DNA repair and chromosome recombination [32]. Defects in some of these transporter genes (ABCB4 and ABCB11) have been linked to a group of liver diseases known as progressive familial intrahepatic cholestasis (PFIC) [33]. They are also remarkable for their affinity to multiple substrates including drugs. The most prominent of this class is ABCB1(Permeability-glycoprotein) which plays a significant role in multidrug resistance associated with many tumor cells.

The ABCC subfamily constitute 12 full transporters which are commonly drug transporters and multidrug resistance proteins (MRPs). This group also contains ‘non typical’ ABC transporters like the cystic fibrosis transmembrane regulator (CFTR/ABCC7) which is particularly a cAMP-regulated chloride ion channel [34] and sulfonylurea receptor (SUR/ABCC8/9), a potassium channel [35, 36]. Aside drug

transport, the ABCC transporters are also engaged in lipid transport [37], nucleotide and nucleoside analog transport [38]; glutathione and anion conjugate transport [30]; signal transduction and chemotaxis; inflammatory responses; cell migration and detoxification [39]. Some of the known genetic disorders associated with this subfamily are cystic fibrosis (CF) and Dubin-Johnson syndrome. CF is an autosomal recessive disorder caused by mutations in CFTR/ABCC7 gene which results in the malfunction of various body systems such as the respiratory, digestive and endocrine systems [40]. Although several CFTR gene mutations have been identified, deletion of three bases coding for phenylalanine at position 508 (F508del) is the most common mutation associated with CF [40]. The mutation leads to protein misfolding with defective protein trafficking and post translational modifications. Dubin-Johnson syndrome results from mutations in ABCC2/MRP2 or canalicular multispecific organic anion transporter (cMOAT) gene. Such mutations include deletions of Arg1392 and Met1393, causing abnormalities in protein maturation and trafficking [41]. ABCC2 is an organic anion transporter localized mostly to canalicular membranes of hepatocytes where it is actively involved in biliary transport of endogenous and exogenous compounds hence the disease is characterized by a bile-associated liver disorder [41][42]. Defects in ABCC6 genes have also been reported to cause pseudoxanthoma elasticum (PXE), a connective tissue disorder [43]. Consistent with their drug transport function, members of this subfamily (ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC10 and ABCC11) are multidrug resistance proteins and are highly expressed in a variety of cancers where they contribute to chemoresistance and disease progression [44]. The most well studied among this group is ABCC1/ MRP1.

The ABCD subfamily genes consist of four half transporters mainly involved in peroxisomal transport of very long chain fatty acids (VLCFA) [45]. An exception to this function is the ABCD4 transporter, which is localized in lysosomes and endoplasmic reticulum (ER) and involved in intracellular transport of Vitamin B_{12} [46]. The effects of ABCD gene mutations are peroxisomal disorders such as X-linked adrenoleukodystrophy (ABCD1), which is caused by toxic concentrations of VLCFA. Mutations in ABCD3 have also been linked to spleen disorders (hepatosplenomegaly) while defects in ABCD4 gene are associated with abnormal Vitamin B_{12} metabolic disorders [46, 47]. The ABCE and ABCF subfamilies though called ABC transporters, do not act as transporters due to the absence of TMD in their structure. They are rather thought to play significant roles in cell division and regulation of translation initiation factors [48-50].

The last group of the ABC transporters, the ABCG subfamily members are lipid and cholesterol transporters. Mutations in their genes are associated with toxic intracellular amounts of cholesterol in liver and intestines [51]. ABCG2 or breast cancer resistant protein (BCRP) is the most-studied member of this subfamily and function as a drug efflux pump that confers drug resistance in many cancer cells. It is also known to play a vital role in renal uric acid transport. Therefore mutations in ABCG2 gene result in gout, a renal disease characterized by excess plasma accumulation of urate crystals [52].

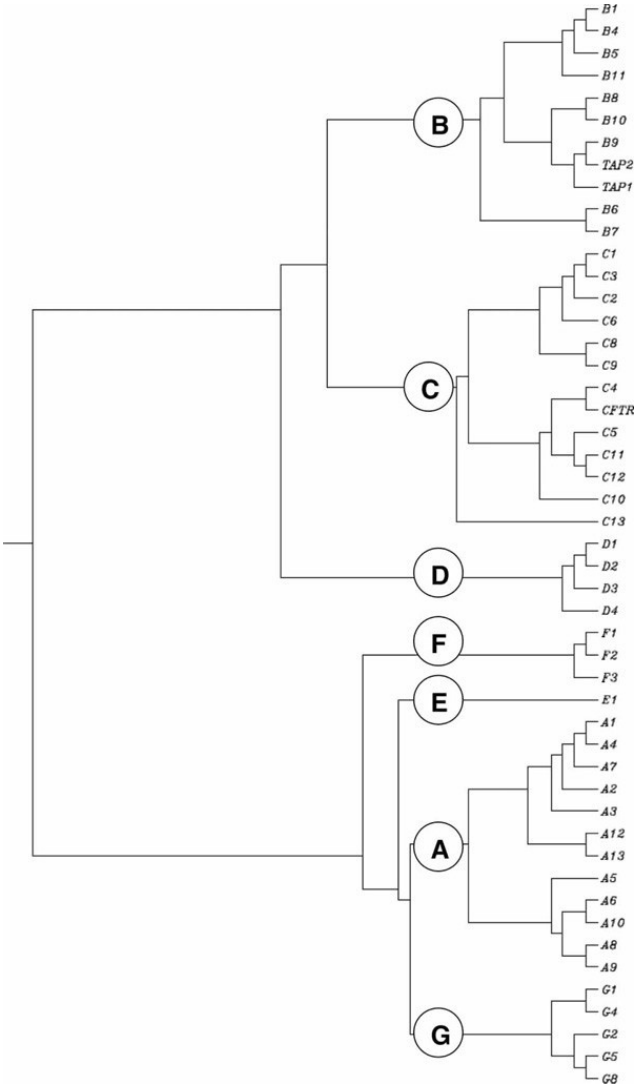

Figure 1.2 Phylogenetic tree showing human ABC transporter gene family. The root 'ABC' is omitted from the figure to simplify it. Thus, the correct gene name for 'B1' is ABCB1, for 'B4' is ABCB4, and so forth [6].

1.3 Structure, Organization and Mechanistic function of ABC Transporters

A typical eukaryotic ABC transporter consists of a single polypeptide organized into four functional units or domains that can be found associated in the membrane. The functional units consisting of two nucleotide binding domains (NBDs) and two

transmembrane domains (TMDs) make up the fundamental architecture of the transporter. The NBDs belong to a group of nucleotide binding proteins (NTP) known as P-loop NTPases that require the catalytic activity of magnesium ions to function [53]. The NBDs are also hydrophilic in nature and located at the cytoplasmic side of the membrane [54]. Each NBD contains two subdomains (Figure 1.3B): a catalytic or RecA-like domain and a helical domain.

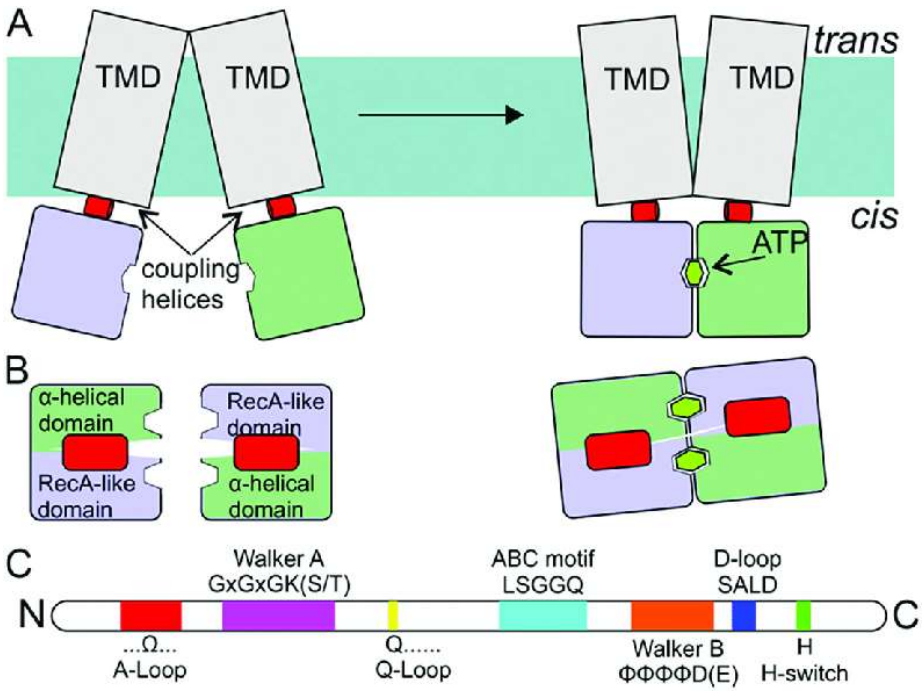

Figure 1.3 Schematic representation of NBDs and coupling helices. (A) Side view (from the membrane plane) of an ABC transporter. NBDs (blue and green) are attached to the TMDs (gray) via so-called coupling helices (red) present in loops of the TMDs. ATP binding and hydrolysis cause rearrangements in the NBDs, which are propagated to the TMDs via the coupling helices. (B) Top view (along an axis perpendicular to the

membrane) of the NBDs and the coupling helices from the TMDs. (C) The relative positions of sequence motifs in NBDs [13]

The domains are evolutionary highly conserved with various sequence motifs performing specific functions for ATP catalysis during substrate transport [13, 14, 54, 55]. The RecA-like domain starts from the N-terminal with the A-loop motif (Figure 1.3C). The A-loop has a conserved aromatic residue (mainly tyrosine) responsible for assembling the adenine ring of ATP for better anchorage during ATP binding. Next, the Walker A (P-loop) motif (GXXGXGK(S/T)) which contains highly conserved lysine residues is the part of the NBD that interacts with the phosphate groups of ATP. The Q-loop follows the Walker A motif. This has highly conserved glutamine residues and serves as the active site for ATP hydrolysis and the main point of connection with the TMDs. The C or ABC motif begins the helical domain. The consensus sequence of this motif (LSGGQ) is the signature feature of all ABC transporters and it functions to position the helix for interaction with the phosphate groups of ATP. The Walker B (φφφφDE, φ = hydrophobic residue) motif with its conserved glutamate residue acts as a polarizing base for ATP against attack by water molecules. The D-loop motif (SALD) comes next to the Walker B and helps to create the platform for ATP hydrolysis. The H-loop or H-switch ends the NBD at the C terminus. It has a highly conserved histidine residue responsible for essential interaction with other motifs at the catalytic site.

Unlike the NBDs, the TMDs are highly hydrophobic and depending on the transporter subfamily, each TMD may contain 6-11 (usually 6 for eukaryotic ABC exporters) transmembrane spanning segments [56]. Thus, for a full human ABC

transporter, the TMD may contain a total of 12 transmembrane spanning α-helix segments. Largely, the TMDs show no substantial sequence similarity and are thus structurally heterogenous though they may share similar network within the same transporter subfamily. The structural dissimilarity thereof for TMDs is suggested to be responsible for the broad specificity of ABC transporters to a variety of substrates. This is especially evident for the multidrug transporter P-glycoprotein (P-gp) which has been termed to exhibit 'polyspecificity' to a number of substrates [57]. In addition, experiments done on human P-gp and multidrug exporter Sav1866 from Staphylococcus aureus revealed that the TMD helical segments are arranged in a manner that creates a substrate translocation pathway extending from the cytoplasm towards the membrane exterior [14, 58].

Under normal cell function, ABC transporters transverse substrates across the membrane against a concentration gradient using energy derived from ATP binding and hydrolysis. For exporters, this transport process is done in a unidirectional manner. This means that the transporter functional domains must operate in a complementary manner for the transport to occur. The transduction of conformational changes between the NBDs and TMDs is said to be crucial for this mechanistic transport process. Despite the NBD-TMD dissimilarities, their domain interactions are made possible through coupling helices identified in the TMDs [19]. The coupling helix describes a brief α-helical motif present at one of the cytoplasmic projections of the TMD that fits into a pocket of a NDB. The coupling interaction between the TMDs and NBDs in this manner is necessary for transmitting conformational changes from the catalytic site of the NBDs to the TMDs. The pocket of the NBD where this coupling interaction occurs is the border between the

helical and RecA-like subdomains where the Q-loop motif is located. The interface of the two subdomains plus the conserved glutamine in the Q-loop motif facilitates this interaction. Moreover, crystallography studies on some ABC transporters also suggest that the dimerization of NBDs in a 'sandwich' fashion is central to the conformational changes in TMDs [59]. Consequently, more studies have proposed several mechanistic transport models such as the ATP- switch [60], alternating catalytic site [61], constant contact [62] and the reciprocating twin-channel [63].

The ATP- switch model proposed by Higgins and Linton is the most commonly cited mechanistic model for ABC proteins [60]. This model describes the switching of two conformations of the NBDs alongside ATP binding and hydrolysis and the resulting conformational changes induction in the TMDs. With respect to substrate translocation in ABC exporters, the transport cycle is triggered by binding of the substrate to the TMDs. The presumable binding of substrate to the high-affinity TMDs site promotes the transformation of the open NBD dimer (low ATP affinity) conformation to the closed dimer (high ATP affinity) conformation. In essence, the binding of substrate favors the closed dimer conformation by lowering the activation energy of the process and also increasing the NBDs affinity for ATP. Binding of two ATP molecules at the interface of the NBDs and the closed dimer formation induce non-covalent conformational changes in the TMDs. The changes in the conformation of the TMDs reduce the binding affinity of the substrate allowing it to be released. ATP is subsequently hydrolyzed with release of ADP, and inorganic phosphate. The NBDs are dissociated and finally reset to their physiological ground state or non-dimeric form to reinitiate another transport cycle. The

substrate-induced conformational changes consistent with the ATP-switch model have been reported in transport studies for P-gp [64, 65] and MRP 1 [66].

1.5 Human ABC Transporters and Multidrug Resistance in Cancer

The World Health Organization (WHO; www.who.int) reports cancer as the second leading cause of mortality worldwide after heart disease, accounting for ~ 9.6 million deaths or 1 in every 6 deaths in 2018. The number of new cancer cases reported in 2018 was ~ 18.1 million and by 2030, this number is expected to increase by more than 80% annually [67]. Current cancer treatment modalities include chemotherapy, radiotherapy, immunotherapy and surgery though chemotherapy remains the traditional approach for managing cancer [68]. Notwithstanding the substantial progress made in chemotherapy, survival rates in many cancer types still remain significantly low unfortunately due to multidrug resistance (MDR) mechanisms. MDR is believed to be responsible for treatment failure in more than 90% of patients with metastatic cancers [69]. The multidrug resistance term describes the ability of cancer cells to develop cross resistance to a number of structurally and pharmacologically unrelated chemotherapeutic agents [70] . The underlying mechanisms (Figure 1.4) identified to cause MDR include increased drug efflux, reduced drug influx, drug target alteration, deactivation of drug metabolic and apoptotic mechanisms as well as mutations among others. The most frequent and the widely studied mechanism is increased drug efflux mediated by ABC transporters [71]. Amongst the ABC efflux pumps identified as drug transporters, P-glycoprotein/ABCB1, MRP1/ABCC1 and BCRP/ABCG2 have gained more attention in

MDR related cancers. This work focuses on multidrug resistance mediated by P-glycoprotein and MRP1.

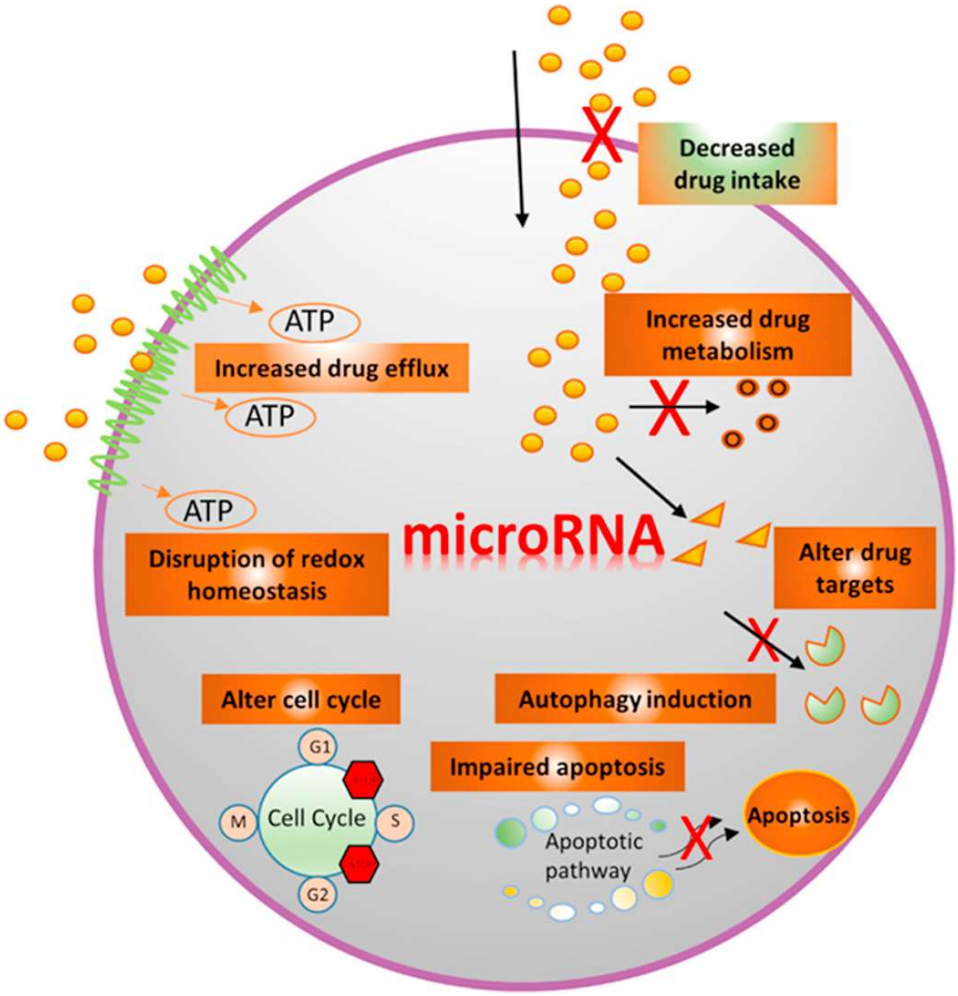

Figure 1.4. Mechanisms of multidrug resistance in cancer [72].

1.6 Permeability Glycoprotein (P-gp/ABCB1)

P-gp is the first to be discovered in the human ABC transporter family and the most extensively characterized ABC protein. P-gp protein was originally identified overexpressed in tumor cells of Chinese hamster selected for multidrug resistance and was later cloned in mouse and humans [73]. The fundamental core of P-gp consists of two NBDs and two TMDs as shown in Figure 1.5. The NBD and TMD domains organization for substrate translocation using energy derived from ATP binding and hydrolysis depicted by P-gp is characteristically the same for the family of ABC efflux transporters. The P-gp gene (mdr1) is encoded with 1280 amino acids giving a 170 kDa protein separated into two homologous halves located at the N- and C- terminals [74, 75]. Each homologous half contains a NBD accompanied by a TMD with six transmembrane spanning helices. A flexible linker peptide or region of approximately 75 amino acids connects the two halves. The linker region is responsible for proper interaction of the two halves and also couple ATPase activity for drug binding and efficient P-gp transport function [74, 75].

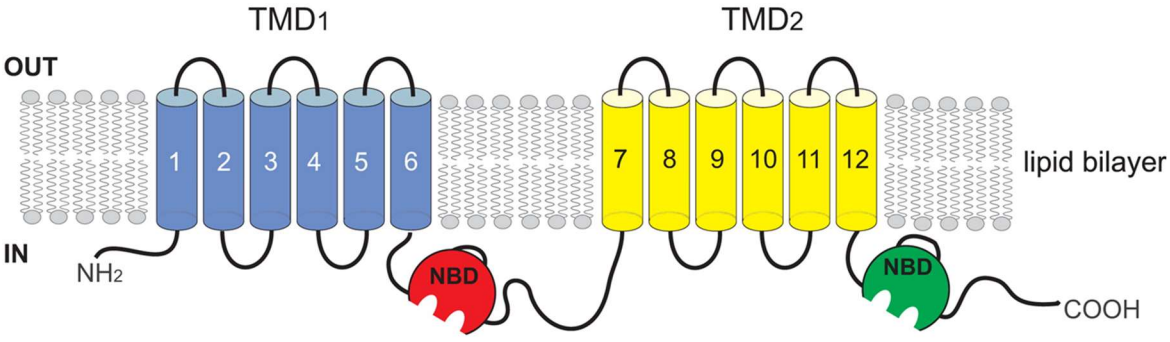

Figure 1.5 Typical structural architecture of P-gp transporter. Adapted from [76]

The diversity of substrates transported by P-gp may include neutral or positively charged compounds but mainly amphipathic or hydrophobic compounds that can appreciably penetrate the plasma membrane into tissues [77]. Many of the substrates include various anticancer drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins and clinically important therapeutics like HIV protease inhibitors, cardiac glycosides, immunosuppressive agents, antibiotics and corticoids [77]. Considering the unique ability of P-gp to transport a variety of chemically diverse compounds, the molecular basis behind its broad substrate and polyspecificity is still not clear. Nonetheless, critical studies of P-gp structure, the nature of its TMDs and the drug binding sites have shed light onto its unique properties. High-resolution crystal structures of mouse P-gp (87% sequence similarity to human P-gp) by Aller et al revealed that P-gp can differentiate between stereoisomeric compounds and consequently generate distinct binding spots with varied orientational chemistries for multiple substrates [57]. Furthermore, an inward facing conformation of the TMDs facilitates the formation of an internal cavity (of volume ~6,000 $Å^3$) which is large enough to hold at least two different substrates concurrently [78]. Additionally, the TMDs conformations promote different topologies of the drug binding pocket which is characterized by highly conserved residues [79]. The flexibility of the drug binding pocket topologies in addition to the highly conserved residues in the pocket may account for the substrate polyspecificity nature of P-gp. The ability of P-gp to transport diverse molecules especially hydrophobic substrates can be explained by the abundance of hydrophobic and aromatic residues in the drug binding pocket that permit the recruitment and partitioning of hydrophobic substrates into the lipid bilayer of the membrane. During its transport

activity, P-gp is able to identify substrates from the bilayer or inner leaflet of the membrane and ‘flip’ or extrude them into the outer leaflet of the membrane or extracellular environment. Mutational and photolabeling experiments of P-gp have also confirmed the contribution of the TMDs to substrate specificity [74, 80]. In short, the TMDs, the large internal cavity, the drug binding pockets and conformational changes allow P-gp to scan, accommodate and bind diverse substrates and subsequently extrude them out of the cell.

The distribution of P-gp in normal physiology is important for major cellular functions like lipid homeostasis, metabolite excretion, among others. P-gp is localized to apical membranes of specialized epithelial cells of the intestines, kidney, liver and pancreas where major metabolites excretion occur [81]. P-gp is also expressed in endothelial cells of luminal blood barriers of sensitive tissues such as the brain, nerves, testis and the placenta and thus plays an important physiological role in tissue defense against xenobiotics [82]. Consistent to the essential role played by P-gp in sensitive tissues, the pathological effects of cytotoxic drugs in the brain, testes and placenta have been demonstrated in numerous experimental models. An important example is the increase in fetal sensitivity to teratogenic substrates among other cytotoxins and subsequent neonatal abnormalities often reported in the placenta of P-gp knockout mice [83, 84]. On the other hand, overexpression of P-gp in brain and nerve tissues in diseased conditions may impede pharmacological behavior of selective therapeutics such as antiepileptics, antiemetics and antidiarrheals targeting the brain or central nervous system (CNS) [85].

Given its crucial physiological functions in normal cells, P-gp overexpression in tumor cells is a crucial index for drug pharmacokinetics such as the absorption, distribution, metabolism and excretion (ADME) of orally administered chemotherapeutic drugs. Reduced intracellular drug bioavailability is a major obstacle to successful chemotherapy. Although not always the case, a viable correlation between elevated levels of P-gp, tumor insensitivity and patient response or survival rates have been established in some hematological malignancies (leukemias), neuroblastoma, colon, adrenal, bladder, breast and endometrial carcinomas [86-89].

1.7 Multidrug Resistance Protein 1 (MRP1/ABCC1)

Multidrug resistance protein 1 (MRP1) is the second known ABC transporter implicated in clinical multidrug resistance. MRP1 was initially identified for its role in conferring resistance to a number of anticancer drugs in a doxorubicin-selected lung cancer cell line (NCI-H69) which was not overexpressing P-gp [90]. The 190 kDa MRP1 (encoded with 1531 amino acids) has an extra membrane spanning domain (MSD0) (Figure 1.6) with five transmembrane helices at the N-terminal region in addition to the typical four core domains (two NBDs and two MSDs) observed in P-gp and other ABC proteins [91]. The twelve transmembrane helices in the MSD1 and MSD2 (as shown in Figure 1.6) form the substrate translocation pore through which solutes are transported, however, the function of the MSD0 in relation to MRP1 transport activity is not well understood [92]. MRP1 transport is governed by ATP binding and hydrolysis with conformational change transduction from the NBDs to the MSDs. Transport is initiated by the binding of substrate and ATP to the two NBDs, which is then accompanied by a

head-to-tail orientation and sandwich dimer formation of the NBDs. ATP-binding and dimerization of the NBDs are facilitated by three characteristic sequence motifs in the NBDs: the Walker A, Walker B and the signature C motifs [63, 92] . Interestingly, ATP binding to the NBDs is asymmetrical, generating two sites namely: the 'degenerate' and 'consensus' sites [63, 93]. Whereas the NBD1 has a low ATPase activity with higher affinity for ATP binding at the degenerate site, the NBD2 on the other hand, has a higher ATP hydrolysis ability at the consensus site [93]. The asymmetric catalytic activity at the NBDs is a characteristic feature of MRP1 and the other members of its subfamily but not observed in P-gp.

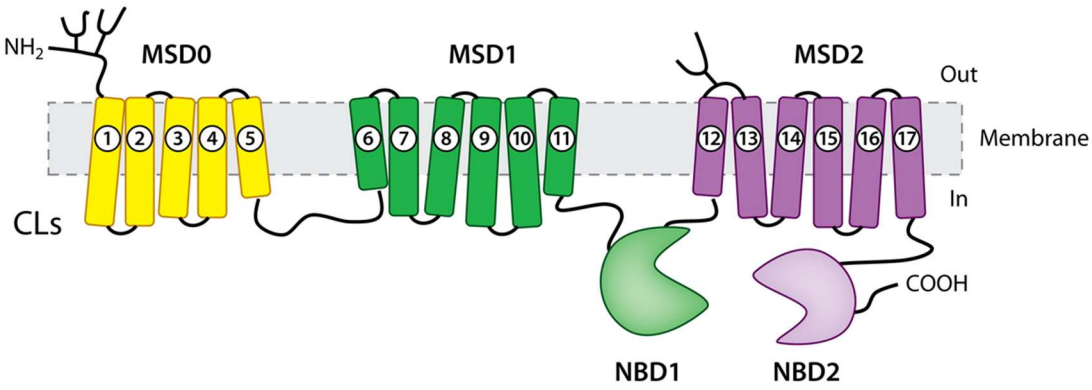

Figure 1.6 Topology and domain structure of MRP1 [94].

The substrates of MRP1 are mostly organic anions from endogenous or exogenous sources several of which are conjugated to glutathione, glucuronic acid or sulfates [95]. Especially, the transmembranes, (TMs) 10, 11, 16 and 17 in MRP1 are

remarkably amphipathic and thus contain predominantly polar amino acids. Conversely, corresponding TMs (5, 6, 11 and 12) of P-gp are less amphipathic accounting for the ability of P-gp to transport mostly hydrophobic substrates but not hydrophilic organic anionic compounds [92]. Furthermore, the TMDs in MRP1 have comparably large number of ionizable amino acids. Evidence suggests that the TMDs residues have hydrogen-bonding abilities that favor intrahelical or interhelical ion pair interactions with hydrophilic organic anions [96]. MRP1 also transports a broad range of antineoplastic agents such as vinca alkaloids, epipodophyllotoxins, methotrexate and camptothecins many of which are also substrates of P-gp. Many clinically important therapeutics e.g. antibiotics, antivirals, antidepressants, statins and metal oxyanions are also substrates of MRP1 [94, 97]. Additionally, MRP1 is the primary transporter of endogenous anionic substrates such as glutathione (GSH), cobalamin, bilirubin, estradiol glucuronide (E217βG) and cysteinyl leukotriene (LTC_4) [98-100]. The ATP-dependent transport of the antioxidant GSH and the pro-inflammatory molecule LTC_4, presents MRP1 as an essential mediator in cellular redox homeostasis, cell differentiation, proliferation and immunological reactions. Therefore, it is not surprising that MRP1is associated with the etiology of many metabolic, cardiovascular, neurological, immunological and tumor related diseases in humans [92].

MRP1 is normally distributed in tissues including the lung, gastrointestinal tract, cardiac, liver, pancreas, adrenal cortex, skin and skeletal muscle but unlike P-gp, MRP1 is localized to basolateral membranes of polarized epithelia [101]. However, like P-gp, MRP1 is also expressed in pharmacological sensitive barriers and therefore exerts chemoprotective functions in these tissues. In fact, a study demonstrated that co-

expression of both MRP1 and P-gp in sensitive tissue compartments is essential for cumulative tissue protection. In mice genetically lacking both P-gp (mdr1a/1b) and MRP1 (mrp1) genes, it was reported that a substantial fold increase in toxicity of chemotherapeutic drugs, vincristine and etoposide was observed in embryonic fibroblasts as compared to drug doses in only P-gp lacking mice [102].

Similarly, MRP1 expression in many drug-resistant malignant and non-malignant diseases is a major factor to successful clinical outcomes. Especially in malignant diseases, MRP1 expression is contributive indicator for drug absorption and disposition. An unfavorable pharmacokinetic response leads to poor clinical outcome in many patients particularly with non-small lung cancer (NSLC), acute myeloid leukemia, (AML), and breast cancer [103-105]. In addition, high MRP1 expression levels have been linked to aggressive and multidrug resistance traits in neuroblastoma [106], and thus MRP1 is a key individual prognostic indicator of childhood neuroblastoma

It can be safe to say that both P-gp and MRP1 are key contributors to clinical multidrug resistance in cancer due to their distribution, expression and the wide diversity of xenobiotic substrates transported by them. To date, several attempts to forestall MDR mediated by these ABC proteins remains a challenge. The prevailing challenges facing MDR circumvention are discussed in the next section.

1.8 Current strategies for P-gp and MRP1 inhibition

The rationale to circumvent MDR is to introduce compounds that will inhibit the activity of ABC drug efflux pumps and thus re-sensitize drug resistant tumors to respond to chemotherapeutic agents. The objective is to enhance the intracellular accumulation of

the chemotherapeutic drug and improve the overall clinical outcome in many cancers. Although several laboratory or preclinical studies geared toward inhibiting these ABC proteins have shown promising outcomes, unfortunately transition and replication in clinical settings have not been favorable. The possible reason(s) accounting for the poor outcomes have been discussed in literature [107, 108], however, a brief summary of selected inhibitor compounds against P-gp and MRP1 and their limitations are discussed here.

1.8.1 First generation P-gp inhibitors

Over the last four decades, an extensive search for compounds against P-gp, have led to three pharmacological generations of P-gp inhibitors [109]. The first generation inhibitors were introduced by taking advantage of the polyspecificity nature of P-gp and included drugs already in use for other therapeutic applications such as verapamil, felodipine, nifedipine, chlorpromazine, quinine and cyclosporin A. Verapamil, a calcium channel blocker showed effective MDR reversing ability, however, its use in clinical trials was terminated as a result of serious cardiotoxicity. [110]. Also, verapamil required high plasma concentrations in order to achieve a significant P-gp blocking activity [111, 112]. Likewise, cyclosporin A, an immunosuppressive agent did not improve treatment outcomes in a randomized phase II clinical trials of patients with relapsing acute myeloid leukemia in combination therapy with etoposide and mitoxantrone due to increased toxicity [113, 114]. The apparent challenge that faced this generation of inhibitors was their low specificity to P-gp. The reason being that they were not specifically developed for P-gp inhibition. Also, many of them emerged as substrates of P-gp and acted

competitively with other chemotherapeutic substrates. Coupled with the low specificity and different pharmacokinetic effects of these inhibitors, high doses were required for their potency hence the undesirable toxic effects observed.

1.8.2 Second generation P-gp inhibitors

Owing to the limitations of the first generation compounds, the second generation inhibitors were developed as derivatives from the first generation compounds through chemical optimizations. The second generation inhibitors lacked the original pharmacological activity of their parent compounds but were still hindered by low specificity, toxicity and pharmacokinetic effects. This generation of inhibitors include dexverapamil, emopamil, and PSC-833 [115]. The prominent among them, PSC-833 (valspodar), is an analogue of cyclosporin A with no immunosuppressive activity. Studies have indicated that PSC-833, though was more specific and less toxic than its parent compound, interfered with the activity of CYP3A4 which belongs to the family of drug metabolizing enzymes, cytochrome P450 3A4, when used in combination with anticancer drugs [116, 117]. Since the metabolism and excretion of most drugs including anticancer drugs depend on CYP3A4 activity, inhibition of CYP3A4 by these inhibitors likely affected anticancer drugs in two ways: i) reduced the therapeutic effect of the drug due to decreased metabolism by CYP3A4 (ii) increased drug exposure or plasma drug concentration with resultant toxic effects due to decreased drug clearance. To overcome these challenges, a low cytotoxic drug dosage was required in order to limit pharmacokinetic interactions and ensure patient safety. The low cytotoxic dosage was

insufficient to produce the desired therapeutic benefit and therefore rendered this generation of inhibitors clinically non-beneficial [117].

1.8.3 Third generation P-gp inhibitors

To solve the problems of non-specificity, low potency and toxicity, the third generation inhibitor compounds were designed using combinatorial chemistry and structural-activity relationship (SAR) approaches. Notable among these compounds that were able to get to the clinical trial stages include tariquidar (XR9576), zosuquidar (LY335979), and laniquidar (R101933) [118]. A Phase I study with tariquidar in combination with anticancer drug vinorelbine or P-gp fluorescent substrate rhodamine showed tariquidar to be a potent P-gp inhibitor with no considerable toxicity or pharmacokinetic effects [119]. However, in another study where tariquidar was combined with docetaxel in patients with lung, ovarian or cervical cancers, tariquidar was observed to have no clinical advantage on patients even though no intolerable pharmacokinetic interactions were recorded [120]. Similarly, in a randomized placebo-controlled double-blind study of patients with newly diagnosed AML or high-risk myelodysplastic syndrome, zosuquidar showed no effect on the overall survival or remission rate of patients [121]. The possible reasons for this roadblock has been attributed to the following: patients selection not based on high P-gp expression, presence of other efflux mechanisms or not accounting for them and possible occurrence of transporter polymorphisms resulting in interpatient variability among others [107].

1.8.4 MRP1 Inhibitors

Compared to P-gp, the search for MRP1 inhibitors is still in early stages since MRP1 was discovered years after P-gp. Additionally, the preference of MRP1 for organic anionic substrates have slowed down the search for MRP1-specific inhibitors. The selected inhibitor should possibly have anionic properties to possess a high affinity towards MRP1 for efficient inhibition. There is also the problem of inadequate intracellular drug accumulation (of the inhibitor) to cause the desired therapeutic effect due to inability of anionic compounds to effectively penetrate into cells. Nonetheless, there has been some progress made with the search for small molecule MRP1 inhibitors such as MK571, probenecid, sulfinpyrazone, benzbromarone, and indomethacin. MK571, the popular MRP1 inhibitor is an LTC_4 analogue primarily developed as a leukotriene D_4 receptor antagonist for the treatment of asthma [94]. It was identified as a competitive inhibitor of LTC_4 (and other organic anionic substrates of MRP1) and also reversed drug (particularly vincristine) resistance in MRP1 overexpressing cells [122]. However, MK571 lacks specificity as it also inhibits other organic anion uptake systems like organic anion transporting polypeptide or solute carrier organic anion transporters (OATP1B3/SLCO1B3) and other proteins in the ABCC family [94, 123]. Sulfinpyrazone, benzbromarone and probenecid have also been reported as organic anion uptake transporter inhibitors [77].

Some P-gp inhibitors have been identified to modulate the function of MRP1. Among them, cyclosporin A and its analogue PSC 833 are reportedly known to inhibit MRP1 though with poor specificity [98]. Tyrosine kinase inhibitors (TKIs) like imatinib, ibrutinib, and AG1393 have also shown inhibitory activity on MRP1 [124-126]. Zheng at el, in a previous study demonstrated that vandetanib, a commercial orally administered drug inhibiting multiple receptor tyrosine kinases has dual inhibition on both MRP1 and BCRP- mediated drug resistance in a dose-dependent manner [125]. A recent study also found GSK1904529A, a potent inhibitor of insulin-like growth factor-I receptor (IGF-IR) tyrosine kinase, to inhibit the function of MRP1 by increasing the sensitivity of MRP1 overexpressing MDR cells and also enhancing the efficacy of MRP1 anticancer substrates [127]. Novel strategies using nanoparticle drug delivery systems have also shown promise in MRP1 inhibition [128, 129]. Notwithstanding, the data accumulated on current MRP1 inhibitors, their limitations have not positioned them as potential candidates for clinical trials evaluation. Some common P-gp and MRP1 inhibitors are shown in Table1.1

Table 1.1 Some common P-gp and MRP1 inhibitors

P-gp Inhibitors			MRP1 inhibitors
First generation	Second generation	Third generation	
Verapamil	Dexverapamil	Tariquidar (XR9576)	MK571
Reserpine	Gallopamil PSC	KR30031	Probenecid
Progesterone	833 (Valspodar)	Cyclopropyldiben -zosuberane	Indomethacin
Tamoxifen	VX-710 (Biricodar)	Zosuquidar (LY335979)	Imatinib
Felodipine	MS-209 GF	Laniquidar (R101933)	Ibrutinib
Nifedipine	120918 (Elacridar)	Substituted diarylimidazole	AG1393
Erythromycin	Reversin 121	ONT-093	
Flufenazine	Reversin 125		
Diltiazem			
Chlorpromazine			
Quinidine			
Cyclosporin A			

1.9 Natural Products as potential source of drug strategies against multidrug resistance in cancer

Though both P-gp and MRP1 inhibitors have failed to show success in therapeutic and clinical settings to date, utilizing modulators (inhibitors) to block ABC transporter-mediated activity is still the cheapest, simplest and direct way for circumventing multidrug resistance. Therefore, it is prudent that the search for inhibitors is not

abandoned, but rather extended to include a manifold alternative strategies. Current alternative strategies used to target MDR include gene regulation technologies such as antisense oligonucleotides, RNA interference, transcriptional regulation; nanomedicine technologies, or a combination of both technologies, and the application of natural products [130, 131]. Amongst them, natural products application has garnered more attention in the search for MDR inhibitors. Historically, natural products and their drug derivatives are sources of many therapeutic agents for treatment of approximately 87% classified human diseases [132]. Furthermore, drugs derived from natural products are among the top 35 ethical drugs sold globally [133]. A study has shown that more than half of prescription drugs in the United States most of which fall under anti-allergic, analgesics and cardiovascular agents are obtained from natural products [134]. The extensive structural and chemical diversity of natural products make them suitable for semi- and total synthetic modifications. In addition, natural products are highly potent, safe and tolerable to use with minimal inherent toxicity compared to synthetically derived drugs [134]. Therefore, natural products are significant sources of new pharmacological leads in the drug discovery and development process.

In the face of the challenges encountered by current P-gp and MRP1 inhibitors, compounds from natural origin will be more desirable for developing drug strategies to overcome multidrug resistance due to the advantages already mentioned. Indeed, some studies have referred to promising natural products-derived compounds as the fourth generation inhibitors. Natural products such as alkaloids, coumarins, curcumin, flavonoids, and terpenoids have already been reported to have inhibitory and considerable MDR reversing activities in many cancer cell lines with minimal toxicity. Notably

flavonoids, a group of plant-derived phenolic compounds were found to significantly enhance the accumulation of P-gp substrate, daunomycin in vitro and also conferred absent to moderate pharmacokinetic effect on doxorubicin, cyclosporin A and paclitaxel in an in vivo study [135]. In a study screening dietary flavonoids, quercetin and rutin were found to inhibit P-gp function and also reduced paclitaxel resistance in P-gp overexpressing human KB CH^R 8-5 carcinoma multidrug resistance cell lines [136]. Coumarins and their derivatives have also shown to reverse P-gp resistance and increased chemotherapeutic drug bioavailability in cancer cells [137, 138]. Similarly, tetrahydrocurcumin (THC), the main metabolite of curcumin (derived from turmeric) inhibited the function of P-gp, MRP1 and BCRP and also re-sensitized their multidrug resistance cell lines to chemotherapeutic drugs [139].

Cucurbitacins are the main natural product of interest in this work. The origin, structure and potential of cucurbitacins as therapeutic candidates for attenuating multidrug resistance in cancer are discussed in the next section.

1.9.1 Cucurbitacins – origin and structure

Cucurbitacins are a class of diverse compounds made up of highly oxidized tetracyclic triterpenoids and present in different species of the plant family *Cucurbitaceae* [140]. They are predominantly found in plants such as cucumber, honeydew melons, Crenshaw melon, pumpkin, cantaloupe, squash, pumpkin, angled gourd, bottle gourd, watermelon and colocynth. [141]. They are also known to be responsible for the bitter taste in these plants. The chemical structure of cucurbitacin includes a steroidal carbon

skeleton (Figure 1.7) or basic triterpenoid (carbon-30 compound with IUPAC name 19(10–9ß)-abeo-5α-lanostanes) built from six isoprene units with oxygen substitutions at different positions [140, 141].

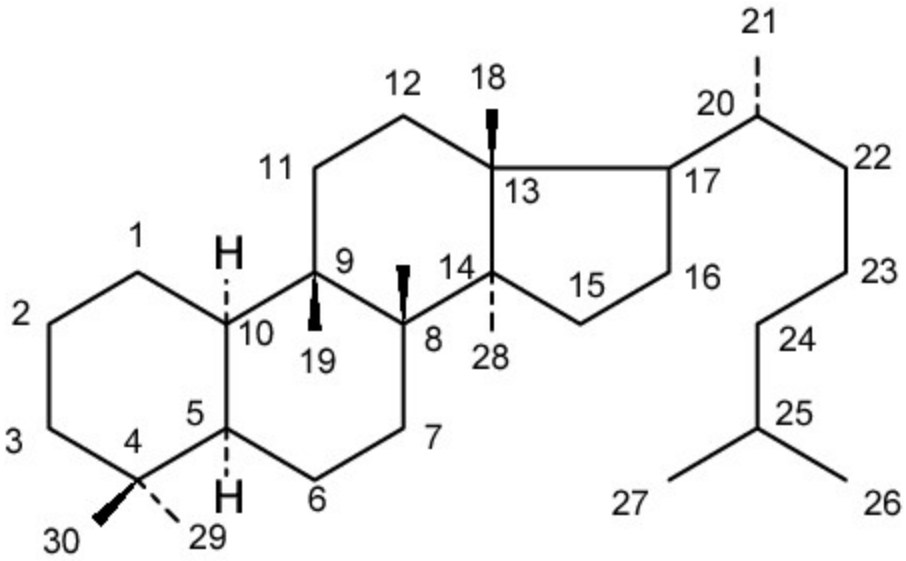

Figure 1.7 Cucurbitacin skeleton [141].

Cucurbitacins are classified into twelve types designated A to T. The diversity of the different cucurbitacin classes is based on the positions of substituent molecules (hydroxylated, unsaturated, keto, glycosyl and acyl groups) attached to them. [142]. As reported by Miro, cucurbitacins A, B,C, D, E, F, I, O and Q contain unsaturated substitutions at carbon position 23 (C-23). On the other hand, cucurbitacins G, H, J and K are typically unsubstituted except for a hydroxyl group on C-24. In general, the basic structure of cucurbitacins is characterized by a gem-dimethyl group at C-4 and an isopropyl group at C-24. There is also presence of methyl groups at positions C-9,C-13,C-14, and C-20. Cucurbitacins basic structure is also defined by unsaturation at C-5

[140]. Cucurbitacins can also be identified based on whether they are naturally occurring or products from enzymatic reactions. [140]. Cucurbitacins B and E are believed to be the naturally occurring cucurbitacins in plants from which the other types are generated through metabolic processes. For instance, cucurbitacins A, C, D, F, G, and H, are products from metabolism of cucurbitacin B while cucurbitacins I, J, K, and L are derived from metabolism of cucurbitacin E [140]. Furthermore, cucurbitacins B and D could be converted to their reduced forms, 23,24-dihydrocucurbitacin B and 23,24-dihydrocucurbitacin D, respectively.

1.9.2 Cucurbitacins Antitumor Activities

Cucurbitacins (Cucs) were earlier used for treatment of liver diseases such as jaundice, cirrhosis and hepatitis in folk medicine due to their natural hepatoprotective properties [142]. Besides this property, cucurbitacins also possess other important pharmacological traits and were thus utilized as purgatives, anti-inflammatory and antimicrobial agents [143]. The antitumor activity of cucurbitacins was discovered more than 50 years ago, however, due to serious toxic effects in humans, their usage subsequently diminished [144]. Fortunately, developments leading to in vitro anticancer drug screening on 60 human tumor cell lines by the National Cancer Institute (NCI-60) [140] restored their potential as prospective source of anticancer compound candidates. To understand the biological mechanism of cucurbitacins, recently, more research have focused on their cytotoxic and antitumor activities.

Cucurbitacins B, D, E and I are amongst the widely explored cucurbitacins in cancer studies [145]. Accumulated data have shown evidence of their remarkable

antitumor activities in various cancer profiles both in vitro and in vivo. Cucurbitacin B particularly, has exhibited potent cytotoxic and antitumor activities in more than 100 tumor panels e.g. breast, colon, leukemia and lung cancers [146, 147]. The antitumor activity of cucurbitacin B has been attributed to its ability to induce various phases of cell cycle arrest and apoptosis by modulating several signaling pathways in tumor cells. For instance, cucurbitacin B induced G2 arrest and caspase-dependent apoptosis in human colon adenocarcinoma SW480 cells in a reactive oxidative species (ROS)-dependent manner while activating caspases and suppressing the expression of cyclin B1 and cdc25C proteins [148]. Cucurbitacin B has also displayed in vitro and in vivo antiproliferative and apoptotic activities through JAK/STAT inhibition mechanism in human pancreatic cancer cells [149]. Treatment of breast cancer cell lines (MCF-7 and MDA-MB-231) with cucurbitacins B and E glucosides extracted from the leaves of *Citrullus colocynthis* inhibited tumor growth through cell cycle arrest and apoptosis [150].

Similarly, a plethora of reports have so far demonstrated cucurbitacin D has inhibitory effect on leukemic, hepatic, colon, lung, breast and central nervous system carcinoma cell lines [140]. In a recent study, cucurbitacin D suppressed metastasis in prostate cancer cells via a glucose regulation mechanism [151]. Cucurbitacin E in a similar study was reported to play a significant role in microtubule skeletal elements (actin and vimentin) disruption, which are responsible for tumor aggression in prostrate carcinoma [145, 152]. In addition, a combination of cucurbitacin E with doxorubicin treatment enhanced the chemotherapeutic and collective antitumorigenic effect both in vitro and in vivo in gastric cancer [153]. The induction of cell cycle arrest and apoptosis

through inhibiting the PI3KAkt signaling pathway in multiple cancer cell lines have been reported for cucurbitacin C [154]. Cucurbitacin I has shown effective tumor growth inhibition in breast and prostate carcinoma cell lines (MDA-MB-231, MDA-MB-468, Panc-1), both in vitro and in vivo [145].

1.9.3 Cucurbitacin-inspired estrone analogs (CIEA)

Notwithstanding the numerous evidence showing the anticancer activities of cucurbitacins, they are still limited as sources of pharmacological candidates due to the following:

a) Cucurbitacins are available at only minute concentrations in their natural sources (plants), therefore isolating them in large concentrations is tedious and usually unsuccessful.
b) The structural complexity and diversity in functional groups or pharmacophores of cucurbitacins makes total synthesis difficult.
c) Cucurbitacins induce toxic adverse effects in mammals even at low doses.

To overcome these challenges and also generate them as suitable drug candidates, the Halaweish group utilized a steroidal scaffold, estrone, as starting skeleton before installing the biologically active pharmacophores of cucurbitacins. The approach was possible due to the similarity between cucurbitacin and estrone skeletons. The estrone chemical structure contains a tetracyclic system that mimics the cucurbitacin skeleton (Figure 1.8). However, the two structures differ by the presence of aliphatic ring and a gem-dimethyl group at C-4 in the cucurbitacin structure, which is absent in the estrone structure. Thus, utilizing estrone main skeleton, the enone or α-βunsaturated ketone side

chain of cucurbitacins which is a significant pharmacophore for their biological activity was assembled to create different estrone derivatives. The intent was to create new estrone derivatives that have superior pharmacological activity but lack their estrogenic activity. Further structural modifications such as double bond installation at C-16/C-17, hydroxyl group substitution at C-3 with methoxy and sulfamoyl groups [155] were also used to generate different derivatives of cucurbitacins-inspired estrone analogues (CIEA).

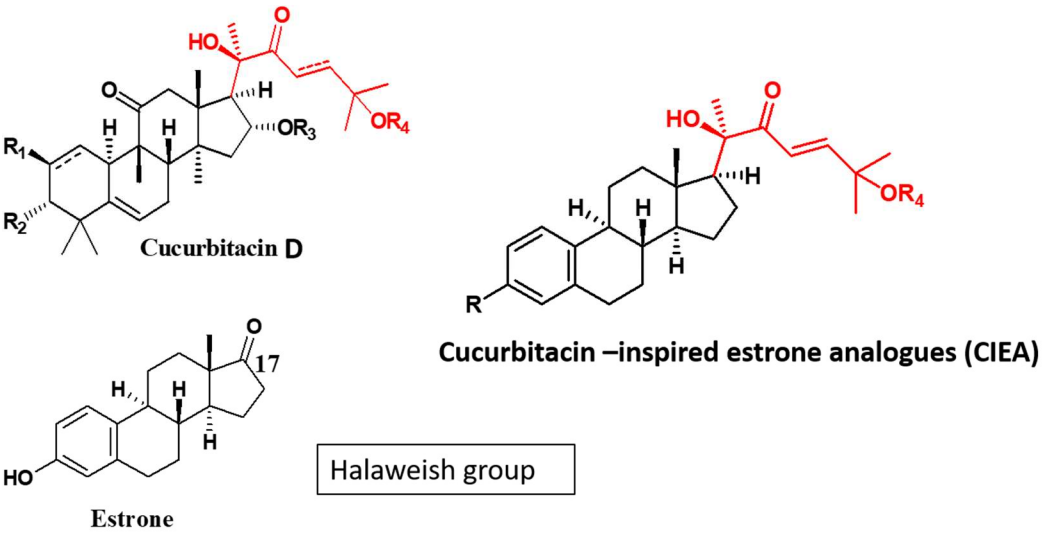

Figure 1.8 Design strategy for cucurbitacin-inspired estrone derivatives developed by the Halaweish group.

1.10 Biochemical Approaches for Studying ABC Transporter Modulators

ABC transporters have the ability to recognize and transport a broad range of molecules that include therapeutically important drugs and thus play an active role in drug pharmacokinetics (absorption, distribution, metabolism, excretion and toxicity (ADMET)). Compounds that interact with and influence the activity of ABC transporters are generally referred to as modulators in some literature [91]. Modulators can behave as

substrates (carried across the plasma membrane by the transporter), inhibitors, (impede the transport of other compounds), inducers (amplify transporter expression) or activators (improve transporter activity) however, one compound can have overlapping mechanisms of action [156]. The mechanisms of action presented by different modulators are important for therapeutic exploitation to overcome MDR in cancer. For compound interactions with ABC transporters, a substrate drug is usually used as 'probe' to investigate potential inhibitors. The mechanism of inhibitors to block transporter mediated efflux of substrates can be one of the following ways: i) by direct inhibition of the transporter binding sites and thus preventing the binding of the substrate, ii) inhibition of ATP binding or hydrolysis; or iii) inhibiting the coupling of ATP hydrolysis to substrate translocation [156]. One of the most studied substrate-inhibitor interaction with ABC transporter systems is the drug-drug interaction involving digoxin and quinidine with P-gp. A study reported that quinidine increased plasma digoxin levels in both P-gp overexpressing cell lines and mouse models by inhibiting P-gp mediated transport of digoxin into the gut and kidneys which enhanced the bioavailability of digoxin [157]. Digoxin is a cardiac glycoside and function as both a substrate and inhibitor of P-gp. Therapeutically, digoxin is said to have a narrow effective window in which case coadministration with P-gp inhibitors may result in toxic drug accumulations. In such instance, inducers or activators can be useful to reduce toxicity to target tissues. Inducers are involved in increasing transporter expression whereas activators stimulate transporter efflux activity. Dexamethasone, doxorubicin, vinblastine and rifampicin are inducers for both P-gp and MRP1 [91]. P-gp inducers are said to be regulated by various nuclear factors while P-gp activators promote transporter activity by: (a) increasing the velocity

of the transport or (b) stimulating the affinity of ATP for NBDs to increase ATP binding; or inducing TMDs conformational changes as a result of substrate-specific binding to the transporter [158, 159]. Inducers and activators have been used to detoxify potentially cytotoxic compounds in many cellular models such as the blood-brain barrier, the cardiovascular system and liver [91].

Thus, for novel drug candidates targeting multidrug resistance in cancer, it is important to profile them with ABC transporters to elucidate their mode of action and toxic effects, which can be used to predict their drug interaction in vivo. In view of the important roles played by ABC transporters in the pharmacokinetics of various drugs, the US Food and Drugs Administration (FDA), has recommended the evaluation of various drug candidates in vitro to determine their interaction with selected ABC transporters [160]. Some established in vitro assays for studying ABC transporter-drug interactions applicable to this research are discussed in the sub sections.

1.10.1 High Content Fluorescence-based Screening

High throughput screening (HTS) is among one of the first techniques used in the drug discovery process especially in the pharmaceutical industry to investigate the interaction of new chemical entities with their biological targets. HTS performed in vitro provides low-cost alternatives to unwarranted animal testing which might be painstaking and time consuming. HTS assays have been employed to investigate interaction of large compound libraries with ABC transporters. Lee et al, using a luminescence-based HTS tested a library of 10,804 compounds on a P-gp overexpressing colchicine-selected subline KB-8-5-11, and identified 55 novel P-gp substrates [161]. In recent years, the

incorporation of instruments with optical sensitivities for imaging-based screening have more significantly improved conventional HTS techniques. Ansbro et al. reported a high content screening (HCS) assay which was used to evaluate the activity of a kinase inhibitor library on P-gp mediated calcein-AM efflux in MDR P-gp overexpressing KB-V1 cell using a fluorescent and phase-contrast live cell imaging system [162]. The phase-contrast system allowed simultaneous identification of cell viability and density before and after treatments, which helped to monitor cytotoxic effects in cells in situ. In comparison, the fluorescent imaging system enabled live cell capture and quantification of fluorescent images against a background fluorescence through segmentation masks to obtain intracellular fluorescence intensities as a measure of the performance of the compounds of interest. The HCS also has the advantage of not requiring multiple washing procedures and therefore tenable for in situ cell-based systems. Recently in our group, a high content imaging-based efflux assay was developed to investigate the interaction of a 386 anti-cancer compound library with MRP1 using calcein-AM and doxorubicin as fluorescent reporters in two independent studies respectively [163, 164]. The assay was effective in identifying novel inhibitors for MRP1. In this research study, the high content imaging-based efflux assay was replicated to screen a novel compound library of CIEA to identify inhibitors of P-gp and MRP1.

1.10.2 Flow Cytometry-based Accumulation or Efflux assays

Flow cytometry (FC) is a one of the common cell-based assays for studying the functional characterization of ABC transporter-drug interactions. FC makes use of fluorescent dyes that may bind to various cellular molecular targets such as DNA or

RNA. Moreover, specific antibodies tagged with fluorescent dyes can be used to target specific cell membrane or intracellular proteins [91]. In the FC principle, fluorescent particles (fluorochromes) present in cells stained with the fluorescent material are able to undergo excitation at higher energies when passed through a light source. Upon returning to their non-excited states, the fluorochromes emit light at specific wavelengths depending on the dye used. The fluorescence emitted is detected by specific filters and counted by a flow cytometer. The technique allows for simultaneous measurements of different cellular properties with several fluorochromes excited by a single laser to yield different emission wavelengths. The activity of efflux transporters may be evaluated on the basis of fluorescent dye accumulation/efflux or both performed on cell suspensions or monolayers [165]. In both assays, the accumulation or efflux of the specific ABC transporter fluorescent substrate over a time period is measured in the presence or absence of their specific inhibitors or activators. In accumulation assays, fluorescent dye accumulation is higher in cells with high transporter expression under conditions where transporter is inhibited compared to the control (absence of inhibition). In contrast, extracellular dye concentrations are measured in efflux studies as an indicator of transporter activity. Here, the amount of dye effluxed is reduced under transporter inhibition conditions compared to no treatment conditions. For both studies, transporter activity can be compared with wild-type or parental cells that do not have transporter expression [165]. Some common fluorescent substrates used in P-gp functional studies include rhodamine 123, doxorubicin, daunorubicin, or calcein-AM [166]. Both doxorubicin and calcein-AM or specific dyes like BCECF can be used as substrates for MRP1 functional studies [163, 164]. It is worth noting that despite the effectiveness and

applicability of flow cytometry, most fluorescent reporters may be limited to specific transporter studies [166].

1.10.3 In Silico Molecular Docking Studies

In silico docking models are quick, economical and primary screening platforms for predicting ligand-receptor interactions for novel pharmacophores in drug development and discovery. The docking process involves generation of suitable conformations of both ligands and receptors which are then docked using simulations to discover lead compounds with the best binding interactions. Docking reveals both the quantitative and qualitative characteristics of ligand binding modes to active sites of receptor molecules using various applications [136]. Various docking models have been used to predict binding interactions of potential modulators for P-gp, MRP1 and BCRP which has allowed prediction of modulator binding interactions with their respective transporters [167-169].

1.10.4 Fluorescence Spectroscopy – FRET

Fluorescence spectroscopy is an indispensable molecular probing tool used to investigate structural-based interactions between cellular molecules. In fluorescence resonance energy transfer (FRET), the transfer of energy between two fluorophores is used as a measure of their interaction. One fluorophore (donor) in an excited state is able to transfer its energy to a nearby fluorophore (acceptor) in close proximity before returning to its ground state. The closer the distance (within10 nm) between the two

fluorophores (the donor and acceptor), the greater the energy transfer or FRET efficiency and greater the sensitivity to detect significant structural changes between the two molecules. There is also a spectral overlap between donor emission and acceptor excitation as shown in Figure 1.9.

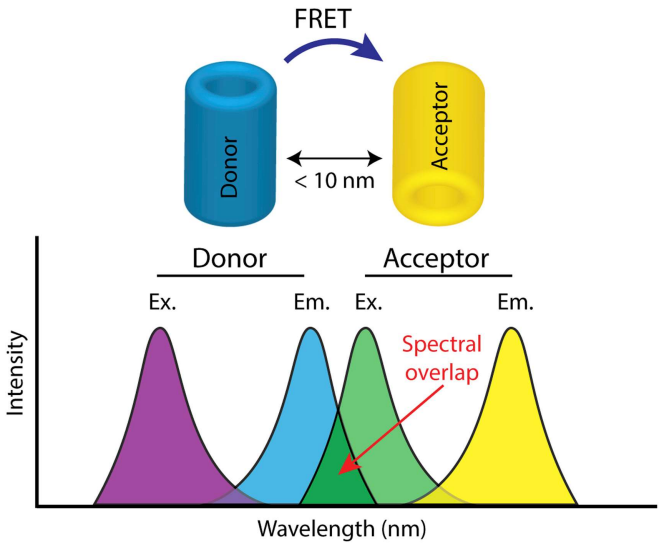

Figure 1.9 Diagram illustrating the fundamental principle of FRET [170]

FRET-based spectroscopy has been used as a key biochemical approach to study real time structural characteristics in ABC transporters using fluorescent proteins and powerful imaging techniques. A study by Verhalen et al. using FRET spectroscopy was able to elucidate the dynamics of ligand-induced structural rearrangements in the NBD catalytic cycle of P-gp [171]. Recently, our group was able to develop genetically engineered unique biosensors to identify novel modulators for MRP1 based on quantitative intramolecular FRET-based interactions [172]. This FRET-based approach

was also applied in this study to investigate CIEA compound interactions with P-gp and MRP1.

1.11 Research Strategy and Significance

Current generation P-gp and MRP1 inhibitors are limited by toxicity, lack of specificity and low therapeutic effect even though they have shown promising potential in some clinical studies. The absence of no successful P-gp or MRP1 inhibitor in clinical settings has prompted more research to focus on developing and discovering novel compounds from natural products as desirable alternatives due to the less toxic and structurally diverse properties of naturally-derived chemical entities. To discover novel drug candidates with superior and safe pharmacological outcomes towards specific cancers, the Halaweish group designed a library of estrone derivatives known as cucurbitacin-inspired estrone analogues (CIEA). In the context that the CIEA compounds have been developed as safe pharmacological candidates for cancer treatment and the fact that cucurbitacins are less explored as potential ABC transporter inhibitors, our lab collaborated with the Halaweish group with the aim to test the effect of the new CIEA compounds on selected ABC transporters. We posed the question whether these novel compounds will be more effective than currently known inhibitors of P-gp and MRP1. Our primary goal was to identify novel inhibitors for P-gp and MRP1 from the large library of CIEA. To address this goal, the following specific objectives were established:

Objective 1: Profiling a library of 81 CIEA compounds against P-gp and MRP1 overexpressing cell lines using an established high content fluorescence-based transporter

activity assay. The inhibition of P-gp by the CIEA compounds was evaluated by using calcein AM as fluorescent probe whereas MRP1 inhibition was detected using two different fluorescent probes, doxorubicin and calcein red orange. The percent inhibition by the CIEA compounds were compared with standard P-gp and MRP1 inhibitors to identify potential hits.

Objective 2: Validation of inhibition activity of the hit compounds identified from the HCS. In this objective, flow cytometry-based and confocal microscopy-based accumulation assays were used to confirm the functional activity of P-gp and MRP1 in the presence of the hit compounds.

Objective 3: Evaluation of the MDR reversing property of the hit compounds in multidrug resistant P-gp or MRP1 overexpressing cells towards selected anticancer agents using cell viability assays.

Objective 4: Investigating the effects of hit compounds on P-gp or MRP1 protein expression levels using western blot technique.

Objective 5: Investigation of direct interaction of the hit compounds with P-gp or MRP1 proteins using molecular docking and fluorescence spectroscopic studies.

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Chapter 2

Characterization of cucurbitacin-inspired estrone analogues as novel inhibitors for human permeability glycoprotein

Abstract

Multidrug resistance (MDR) remains a major challenge to cancer therapy and it is mainly attributed to the overexpression of membrane bound ATP-binding cassette (ABC) superfamily of transporters. Permeability glycoprotein (P-gp), a member of the ABC transporter family contributes to multidrug resistance in various cancers by reducing the efficacy and bioavailability of anticancer drugs. Therapeutic strategies to overcome P-gp have failed due to lack of specificity, low potency and severe toxicity. To identify novel inhibitors for P-gp, a high content fluorescence-based screening assay initially developed by our group was used to screen a library of 81 novel cucurbitacin-inspired estrone analogs (CIEAs). Defining a hit as a compound showing at least 50% percent inhibition, the assay identified 8 compounds as inhibitors of P-gp using calcein-AM as fluorescent reporter. The hit compounds demonstrated 50-85% range percent inhibition of P-gp-mediated calcein efflux. Flow cytometry and confocal microscopy-based accumulation assays were used to confirm inhibition of P-gp efflux activity. The ability of the compounds to reverse resistance of P-gp overexpressing cells against vincristine, paclitaxel and etoposide was also tested. The compounds were most effective in reversing etoposide resistance in HEK293/P-gp cells by reducing resistance fold from 173 to between 33-50 fold. Nonetheless, the identified compounds had no effect on P-gp expression. Using in silico molecular docking and FRET-based spectroscopic analyses showed that the identified inhibitor compounds directly interacted with P-gp protein. The

results presented in this study suggest the use of cucurbitacin-inspired estrone analogues as promising leads for future drug optimization against multidrug resistance in cancer.

Keywords: P-gp, ABC transporter, multidrug resistance, cancer therapy, anticancer drugs bioavailability, CIEA, promising leads, drug optimization

2.1 Introduction

A key problem leading to chemotherapeutic failure in cancer treatment is the development of multidrug resistance (MDR). MDR is the insensitivity of tumor cells to many anticancer agents which are different in structure, function or mechanism of action [1]. Several mechanisms including decreased drug uptake, increased drug efflux, drug inactivation, drug target alteration, DNA damage repair and cell death inhibition have been reported to promote drug resistance [2, 3]. However, the best studied and major underlying mechanism of MDR in malignant tumors is that due to overexpression of mammalian ATP-binding cassette (ABC) transporters [4]. ABC transporters represent the ubiquitous family of transmembrane efflux pumps that utilize energy derived from ATP binding and hydrolysis to drive the transport of molecules against their concentration gradient across the plasma membrane [5-7]. To date, 48 human ABC transporter genes have been reported and they are classified into seven subfamilies, ABCA-G [8]. Amongst the ABC transporters, P-gp (P-glycoprotein/ABCB1), MRP1 (multidrug resistance protein 1/ABCC1) and BCRP (breast cancer resistance protein/ABCG2) are the main drug transporters implicated in many MDR related cancers. All three transporters facilitate active efflux of structurally and pharmacologically unrelated chemotherapeutic agents across the plasma membrane limiting intracellular drug accumulation and drug-mediated cancer cell death. [3].

P-gp, the first member of the ABC transporters discovered and the most characterized transporter is a 170 kDa protein encoded by the human MDR1 gene. The P-gp polypeptide is made up of two homologous halves, each composed of one membrane spanning domain (MSD) and one nucleotide binding domain (NBD) [5, 9, 10]. The

distribution of P-gp plays a major role in protecting tissues from xenobiotics and harmful metabolites. P-gp is typically but not exclusively, localized at the apical membranes of epithelial cells of the colon, small intestines, liver, pancreas, kidney and uterus [11, 12]. Thus, intestinal P-gp has been suggested as the primary barrier for orally-administered drug substrates due to limiting the bioavailability of these drugs in the body [13]. Furthermore, high expression levels of P-gp have been reported in up to 50% of gastric cancers [14]. In humans, P-gp is biologically localized at important pharmacological barriers such as the blood-brain and blood-nerve barriers [5, 13, 18, 22]. Expression of P-gp at these sites is consistent with its anatomical drug-efflux role in protecting sensitive and vital tissues from toxic exogenous compounds [10, 15-19]. More critically, P-gp expression at the endothelial cells of the blood-brain barrier (BBB) is physically necessary to block the net penetration of xenobiotics into the brain tissue [20]. The absence of this can result in up to 10 to100 fold increase in drug penetration in the brain which is clinically undesirable [5, 17, 18, 21]. However, this could be of therapeutic relevance to clinically important drugs such as antiepileptics and antiemetics targeting the brain or central nervous system (CNS) [22]. In the blood-testis and fetal-maternal barriers, P-gp is expressed in stage-specific manner and plays an analogous function as in the BBB [5]. In general, P-gp overexpression is associated with poor chemotherapy outcome at both primary diagnosis and disease relapse stages of several cancers such as leukemia, lymphomas, adult and childhood sarcomas and neuroblastomas [23-26]. Apart from its drug-efflux function, P-gp also plays a significant role in other cellular processes such as intracellular cholesterol trafficking and lipid transport, cell immunity, apoptosis regulation and dendritic cell migration [27].

P-gp transporter has broad substrate polyspecificity and ability to distinguish many compounds from as tiny as 330 to 4000 Da [9, 28, 29]. Additionally, P-gp is able to extrude many relatively hydrophobic substrates partitioned in the lipid bilayer from the plasma membrane into the extracellular aqueous matrix [28, 30]. Two mechanistic models accounting for these two interesting features have been hypothesized. The first proposes a model where the transporter creates a hydrophilic pathway and drugs are effluxed from the cytosol to the extracellular matrix through the middle of a pore protecting the hydrophobic lipid phase [31] . The second model describes the transporter as a 'flippase' that intercepts a substrate and flips it from the inner cell leaflet to the outer and into the extracellular matrix as the substrate travels through the lipid membrane [32]. It is not surprising that P-gp transports various clinically significant drugs, including anticancer agents such as vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes, immunosuppressive drugs, cardiovascular agents, anthelmintics, and steroids out of the cell [22]. Considering that P-gp recognizes and transports such broad range of structurally unrelated compounds, finding the appropriate P-gp inhibitor to improve the pharmacological benefit of many commonly used xenobiotics still remains a challenge.

Several attempts to circumvent P-gp mediated multidrug resistance with small molecular entities, a process known as chemosensitization have given rise to various pharmacological classes of P-gp inhibitors [33, 34]. The first generation P-gp inhibitors were mostly drugs originally developed for other therapeutic applications such as immunosuppression, antibiotic and cardiacs [35]. These P-gp inhibitors including verapamil, cyclosporin, nifedipine, chlorpromazine, quinine and elodipine have remarkable MDR reversal activities both *in vitro* and *in vivo* [10] [36]. However, their

success was limited by toxicity and off-target effects [37, 38]. Verapamil, a calcium-channel blocker was reportedly associated with serious cardiotoxicity as it requires relatively high plasma concentrations for efficacy [39]. Also, some of these inhibitors were reported to interfere with the activity of CYP3A, an isoform of the drug metabolizing enzyme cytochrome P450 [40, 41], suggesting that these inhibitors may have similar substrates with CYP3A and therefore interfered with the pharmacokinetics of chemotherapeutic drugs in combinatorial therapy. The second generation of inhibitors such as dexverapamil, emopamil and valspodar [10, 42, 43] derived from the first generation compounds were introduced as an improvement on dose-potency and toxicity but still interfered with CYP3A activity [10]. The search for compounds to overcome the second generation limitations, resulted in compounds designed using combinatorial chemistry and structure-activity relationship approaches [10, 11, 44, 45]. These inhibitors, the third generation compared with the first- and second generations were more specific inhibiting P-gp function with no significant impact on CYP3A activity and include drugs such as tariquidar, zosuquidar and laniquidar [10]. Disappointingly, outcomes from clinical trials using these inhibitors were not clinically beneficial. A study done by Kelly et al, revealed that co-administration of tariquidar (XR9576) with docetaxel did not improve overall survival rate of patients with metastatic cancers even with no toxicity [46]. Another study showed zosuquidar to be ineffective towards newly diagnosed acute myeloid leukemia adults (older than 60 years) [47]. The reasons for the lack of therapeutic benefits from clinical trials have been attributed to patients selection not based solely on high P-gp expression, presence of other efflux mechanisms or lack of accounting for other drug efflux proteins or possible occurrence of transporter

polymorphisms that may lead to interpatient variability [48]. Thus, it appears future trials of current generation P-gp inhibitors will be limited by their therapeutic benefit hence alternative strategies should be sought.

Presently, the fall back to natural products as source for novel compounds has optimistically advanced in the search for MDR modulation agents. Natural products are important sources for new chemical entities (NCEs) in contemporary drug discovery and design due to their diversity and amenability to semi- and total synthetic modification [49]. Cucurbitacins (Cucs) are natural products with oxidized tetracyclic triterpenoids derived from different species of the plant family Cucurbitaceae; with the main types being cucurbitacins A to T [50]. Cucurbitacins are well-known to possess significant pharmacological properties that have been explored in the treatment of various illness. The hepatoprotective activity of cucurbitacins has been demonstrated by Bartalis and Halaweish on HepG2 HSC-T6 cell lines [51]. Accumulated data have shown cucurbitacins remarkable antitumor activities in various cancer profiles both in vitro and in vivo. Particularly, cucurbitacin B has potent cytotoxic and antitumor activity in more than 100 tumor panels e.g. breast, colon, leukemia and lung cancers [52, 53]. Cucurbitacin B has been reported to induce G2 arrest and caspase-dependent apoptosis in human colon adenocarcinoma SW480 cells in a reactive oxidative species (ROS) – dependent manner while activating caspases and suppressing the expression of cyclin B1 and cdc25C proteins [54]. Similarly, cucurbitacin D has reportedly shown to exert inhibitory activities in leukemic, hepatic, colon, lung, breast and central nervous system tumor cell lines [55]. In a recent study, cucurbitacin D suppressed metastasis in human prostate cancer cells via a glucose regulation mechanism [56]. Cucurbitacin E, was

reported to disrupt microtubule skeletal elements (actin and vimentin) responsible for tumor aggression in prostrate carcinoma [57, 58]. In a combination therapy with doxorubicin, cucurbitacin E enhanced the collective chemotherapeutic and antitumorigenic effects in gastric cancer both in vitro and in vivo [59]. Cucurbitacins C, I and Q are known to have antiproliferative and antitumor activities in various cancers [58, 60]. Collectively, these reports position cucurbitacins and their derivatives as prospective drug candidates in cancer treatment. However, evaluation of cucurbitacins and its derivatives as sources of ABC transporter (s) modulating agents is less explored. In this work, we reckoned that exploiting the anticancer properties of cucurbitacins-derived small compound analogues may position them as drug candidates for P-gp modulation against multidrug resistance in cancer.

Using molecular modeling and structural activity relationships (SAR), the Halaweish group synthesized novel compounds known as cucurbitacin-inspired estrone analogs (CIEAs) from cucurbitacin using estrone as starting scaffold. These compounds were designed to dual target epidermal growth factor receptor (EGFR) and mitogen activated protein kinase (MAPK) pathways involved in cancer proliferation. In this study, we investigated the effects of the CIEAs on P-gp activity. A library of 81 CIEA were screened using a high content fluorescence-based transporter activity assay previously developed in our lab [61]. From the high content fluorescence-based inhibition experiment using calcein-AM as P-gp substrate, 8 CIEAs were identified as potential P-gp inhibitors. Using established cell-based functional assays, we validated the inhibitory activity of the identified inhibitors and also demonstrated their ability to sensitize multidrug resistant P-gp overexpressing cells towards different anticancer agents using

cell viability assays. The direct interaction of the identified compounds with P-gp was also demonstrated.

2.2 Materials and Methods

2.2.1 Chemicals

Thiazolyl blue tetrazolium bromide (MTT), doxorubicin and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Calcein-AM was acquired from Corning Life Sciences (Corning, NY). Vincristine and etoposide were procured from Cayman Chemicals (Ann Arbor, MI) while paclitaxel was purchased from MedChemExpress (Monmouth Junction, NJ). Cucurbitacin-inspired estrone analogue (CIEA) compounds were generously provided by Dr. Fathi Halaweish (South Dakota State University, Brookings, SD).

2.2.2 Cell lines and cell culture

HEK293/pcDNA3.1 and HEK293/P-gp, were kindly provided by Dr. Suresh V. Ambudkar (NIH, Bethesda, MD). HEK293T cells were gifted by Dr. Adam Hoppe (South Dakota State University, Brookings SD). HEK293 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare Life Sciences, Logan, UT) complemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone^{TM}, GE Healthcare Life Sciences). Cell cultures were maintained at 37 °C in a humidified incubator supplied with 5% CO_2. These conditions were utilized for succeeding cell culture techniques.

2.2.3 High content fluorescence-based transporter activity assay for screening of CIEA library

The assay was previously developed and optimized as reported in [61]. Fluorescence-based inhibition screening of the 81 CIEA compound library was performed on HEK293/pcDNA3.1 and HEK293/P-gp cells using calcein-AM as fluorescent substrate and reversan as positive inhibitor control. Cells were seeded at density of 8 x 10^3 cells per well in 100 µL culture medium placed in a polymer-based 96-well optical-bottom plate (Thermo Fisher Scientific, Waltham, MA) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) and incubated overnight. The next day, the culture medium was removed and replaced with 80 µL serum-free medium. Drug treatment was performed by adding DMSO (0.2% final concentration) as a negative control, reversan (15 µM final concentration) as positive control or test compounds (10 µM final concentration) and incubated at 37 °C for 30 min. Fluorescent substrate treatment was performed by adding calcein-AM (0.25 µM final concentration). After 1 h, media containing treatment were removed and cells were washed once with PBS and suspended in 100 µL PBS containing 10 mM HEPES and 4.5% glucose prior to analysis.

An ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA) equipped with a 0.70 numerical aperture 60x objective was used to acquire fluorescent images. Two independent experiments were conducted. Images were obtained using an FITC filter for calcein-AM with excitation and emission wavelengths of 482/35 nm and 536/40 nm respectively with exposure time set for 100 ms. Negative and positive controls were included in every two columns to account for post treatment drop in intracellular calcein-AM fluorescence across the plate

over time. Fluorescent images were analyzed using the MetaXpress software (version 5.10.41, Molecular Devices) based on segmentation of fluorescent objects in the FITC channel. A custom application component depending on the 'Find Blobs' module was applied to differentiate fluorescent objects against background fluorescence by employing segmentation masks as per parameters set for object size.

2.2.4 Calcein-AM accumulation assay

The ability of the identified CIEA compounds to inhibit P-gp-mediated calcein-AM efflux was confirmed using flow cytometry. HEK293/pcDNA3.1 and HEK293/P-gp cells were prepared in serum free DMEM culture media at $7x10^5$ cell density per mL and treated with DMSO (0.2% final concentration), negative control, verapamil (10 μM final concentration) as positive control or test compounds (10 μM final concentration) and incubated at 37 °C for 10 min. Cells were then treated with calcein-AM (0.25 μM final concentration) and incubated for 30 min. Post incubation, the reaction was halted by addition of 3 mL ice-cold PBS. Cells were pelleted, washed twice with ice-cold PBS, resuspended in 1% paraformaldehyde (in ice cold PBS) and kept on ice for analysis. Intracellular fluorescence was determined using BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) equipped with 488 nm argon laser and detected with 533/30 nm band pass filter for calcein-AM. Each experimental treatment was duplicated and fluorescent values expressed as mean of 10,000 events. Results were collated from three independent experiments and presented as mean ± SEM with $p \leq 0.05$ compared to the control.

2.2.5 Doxorubicin accumulation assay

HEK293T cells were seeded in a 6-well plate, each well containing a poly-D-lysine coated (ethanol sterilized) cover glass with 2.5 x 10^5 cells in 2 mL culture medium. After incubation overnight, cells were transiently transfected with a two-color P-gp-GFP-RFP expression vector (GR 678) using jet Prime Transfection Reagent (Polyplus – transfection SA, Illkirch, France) in accordance with manufacturer's protocol. Following 48 h after transfection, cells were pretreated with test compounds (10 μM) for 30 min, before incubation with doxorubicin (10 μM) for 1 h. Cells were preserved in a buffer (4.5% glucose, 10mM HEPES and PBS containing Ca^{2+} and Mg^{2+}) before analysis.

Intracellular fluorescence accumulation was viewed 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 445 nm with emission bands at 537/26 nm and 605/64 nm respectively. RFP was viewed in the 605/64 nm band channel after excitation at 561 nm. Images were processed with ImageJ (NIH, Bethesda, MD).

2.2.6 Drug resistance reversal studies

The potential of the identified CIEA compounds to reverse resistance of HEK293/P-gp cells against P-gp anticancer substrates (vincristine, paclitaxel and etoposide) were tested using MTT colorimetric assay. HEK293/pcDNA3.1 and HEK293/P-gp cells at a density of 5 x 10^4 cells/well in 100 μL culture medium were seeded in a 96-well plate (NEST®, Rahway, NJ). After incubation overnight, cells were

treated with 50 µL (in culture medium) test compounds (at non cytotoxic concentrations) or 0.2% DMSO for 1 h before addition of 50 µL drug substrates (vincristine, paclitaxel and etoposide) at varying concentrations. Following incubation for 72 h, 100 µL of culture medium was removed and cells were treated with MTT dye (5 mg/mL in PBS) for 4 h to allow viable cells to change the yellow-colored MTT into dark-blue formazan crystals. The MTT formazan product was dissolved with 100 µL 15% SDS containing 10 mM HCl. Cell viability was determined by measuring absorbance at 540 nm with a plate reader, Hidex Sense Beta Plus (Turku, Finland). Three independent experiments were performed with each done in triplicates.

2.2.7 Western blot analysis

Briefly 7 x 10^5 HEK293/P-gp cells were seeded in a 6-well plate at 2 mL per well and incubated overnight before treatment with 10 µM test compounds. After 48 h, cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific, Waltham, MA) containing 1x Halt Protease Inhibitor Cocktail. Lysate protein concentrations were determined using Pierce bicinchoninic acid (BCA) Protein Assay. Proteins (20 µg) from each treatment were subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels before being transferred to a PVDF membrane (EMD Millipore, Burlington, MA). The membrane was blocked and incubated overnight at 4 °C with monoclonal P-gp antibody [C219] (GTX23364, GeneTex) and anti-α-tubulin antibody (Sigma-Aldrich) at 1:250 and 1:5000 dilutions, respectively. Incubation with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; H+L, ThermoFisher Scientific) at 1:5000 dilution was performed for 1 h at room

temperature. ABCB1 proteins were developed using western blotting luminol reagent (Santa Cruz Biotechnology, sc-2048) and imaged with LI-COR Odyssey Fc Imaging System. Protein band densities were analyzed with Image Studio Lite version 5.2 (LI-COR Biotechnology, Lincoln, NE) software. Uneven sample loading was corrected using the intensity of corresponding protein band relative to α -tubulin (loading control) band. Two independent experiments were conducted and statistical testing was performed at 5% level of significance.

2.2.8 In silico molecular docking studies

The identified CIEA compounds (ligands) were prepared for docking by generating three-dimensional conformations using Chem 3D Ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft Corporation, USA (2010)], accompanied by energy minimization using MMFF94 force field application. Human ABCB1 homology model based on refined mouse ABCB1 was obtained from protein data bank (PDB code: 4M1M). After bound molecules and ligands were eliminated, the protein drug-binding pocket was prepared for docking using OpenEye software. Docking was performed with FRED (version 2.5.1.4) and VIDA application (version 4.1.2) was used to visualize binding poses of the ligand-receptor complex.

2.2.9 Fluorescence spectroscopic analysis

Membrane vesicles were prepared as previously described [61, 62] with slight modifications. HEK293 cells stably transfected with 2-color P-gp (GFP and RFP)

plasmid, GR678 were first cultured in 150 mm cell culture dishes. After 7 days or about 98% cell confluency, the cells were pelleted and stored at −80 °C till ready for use. For membrane vesicle preparation, the frozen pelleted cells were initially thawed and maintained in 10 mL homogenization buffer (250 mM sucrose, 50 mM Tris.HCl, 0.25 mM CaCl2, pH 7.4) supplemented with 1 tablet protease inhibitor (Santa Cruz Biotechnology, Dallas, TX) and 10 µL benzamidine (200 mg/ml) before nitrogen bombed at 450 psi for 5 min. Membranes from disrupted cells were obtained through centrifugation (25,000 rpm) at 4 °C for 1 h. Membrane vesicles were formed by passing pelleted membranes 20 times through a 27-gauge needle. Membrane vesicles protein concentration was determined using Quick Start Bradford Protein Assay (BioRad, Hercules, CA). For fluorescence spectroscopic analysis, two reaction mixtures were set up: the membrane vesicle protein, 20 µg in Tris sucrose buffer (TSB) (250 mM Tris and 50 mM sucrose; pH 7.4) and the compound mixture consisting of 10 µM test compounds in TSB. The reaction mixtures were separately pre-incubated for 5 min in a 37 °C water bath, before they were mixed and incubated afterwards for 10 min to enable ligand binding. To stop the reaction, samples were kept on ice prior to analysis with a Fluorometer (model FL3-11; HORIBA Edison, New Jersey). Spectroscopic measurements were carried out using a 50-uL quartz glass cuvette. The two-color (GFP and RFP) P-gp protein was used as an acceptor fluorophore, therefore a previously cloned GFP protein was used as the donor fluorophore. Excitation and emission for GFP were attained at 465 nm and 480–650 nm respectively. Emission scans were recorded every 3 s at 5 nm interval for 10 min. The intensities of the 2-color P-gp for compound treatments were measured while monitoring quenching of the donor fluorophore. The FRET

efficiency was determined by normalizing the intensity of the 2-color P-gp acceptor ligand-free (apo) treatment with that of the donor fluorophore using the equation below:

$$1 - (\frac{I_{DA}}{I_D})x\ 100$$

where I_D and I_{DA} represent the intensities (counts per second, cps) of the donor and acceptor fluorophores, respectively. The percent change in FRET was obtained from the difference in FRET efficiencies of the ligand-free and the compound-induced treatments.

2.2.10 High content fluorescence inhibition screening data analysis

Analysis of fluorescence inhibition was done using MetaXpress software. The software uses segmentation masks to determine the average fluorescent intensities from four images captured in each well. Incorporation of segmentation mask subtracts background fluorescence. The relative inhibition of calcein-AM efflux by test compounds were calculated for each well using the equation below:

$$\%\ inhibition = \frac{X_T - X_{calcein-A}}{X_{positive\ inhibito} - X_{calcein-AM}}\ x\ 100$$

where X represents the average fluorescence intensities and T represents the test compound. 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 assay quality and validation were assessed using Z'-factor and calculated as follows:

$$Z' - factor = 1 - \frac{3(\sigma_{positive\ control} + \sigma_{calcein-AM})}{|\mu_{positive\ control} - \mu_{calcei}|}$$

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

2.2.11 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.4.3 software (GraphPad Software, La Jolla, CA). Analysis of differences between values were determined by application of linear mixed model. Dunnett correction was used for multiple comparisons. Significant differences were considered at p-value less than 0.05.

2.3 Results

2.3.1 CIEA compound library screening for P-gp inhibitors

To identify novel P-gp inhibitors, a compound library of 81 CIEAs were screened using a high content fluorescence-based transporter activity assay previously developed by our group. Screening was performed on P-gp overexpressing cell line, HEK293/P-gp using calcein-AM as fluorescent reporter. The positive control inhibitor (reversan 15 μM) was considered as baseline (100% inhibition) for calculating the relative percent inhibition for the test compounds. As shown in Figure 2.1A, the 2D scatter plot represents the relative inhibition activity of each CIEA compound from two independent experiments. The cut-off value defined for a positive hit was a compound with ≥50% mean percent inhibition. With this cut-off value, 8 CIEA compounds were identified as hits. The chemical structures of these compounds are shown in Figure 2.1B. The hit compounds and their mean percent inhibition values are shown in Table 2.1. The screening assay yielded a correlation value of 0.87 indicating that the two experiments had good reproducibility. The assay also yielded an average Z'-factor value of 0.65 across all plates indicating a good assay performance.

Table 2.1 Percentage calcein control inhibition for identified P-gp inhibitors

Compound	% Calcein Control Inhibition [a]
Reversan (Positive inhibitor)	100.00 ± 0.40
HL 4	78.29 ± 1.94
HL 15	62.27 ± 13.97
HL 16	65.35 ± 2.07
HL 20	50.41 ± 2.92
HL 21	85.32 ± 2.71
HL 22	83.27 ± 7.27
HL 77	59.73 ± 2.45
HL 80	58.68 ± 25.22

[a] Mean ± SEM of n = 2 independent experiments

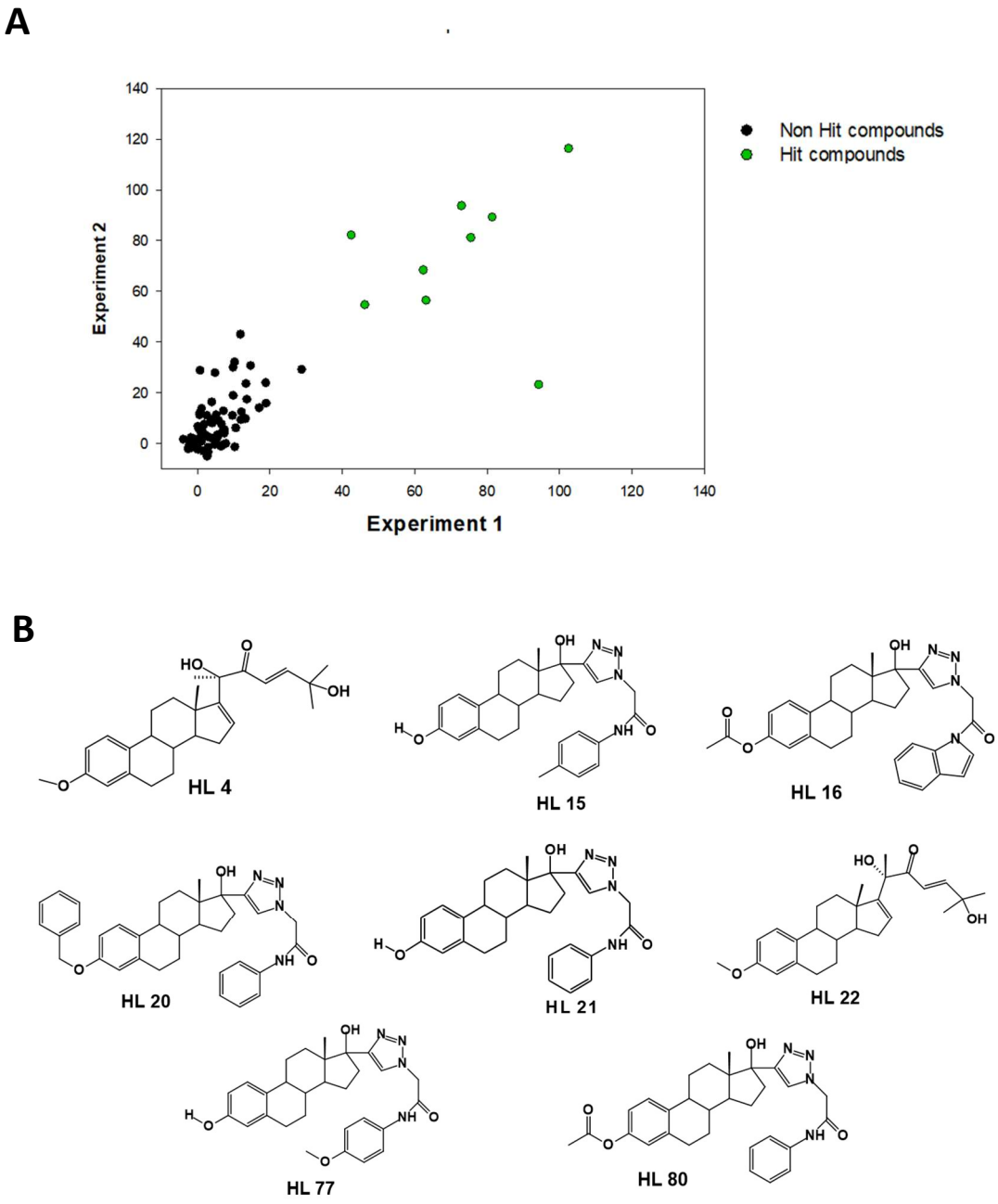

Figure 2.1 CIEA compounds screening and selection. (A) Representation of the cell-based P-gp mediated calcein efflux inhibition screening assay. Screening of a library of 81 CIEA compounds was conducted in two independent experiments at a compound concentration of 10 µM. The 2D plot displays the calculated relative inhibitory activity of compounds in the two experiments with a correlation coefficient value of 0.87. Green

dots denote compounds with mean percentage inhibition of ≥ 50%. 2D scatter plot was generated using Sigma Plot 12.0 and correlation coefficient was calculated using MS Excel. (B) Chemical structures of the 8 CIEA compounds identified hits.

2.3.2 Validation of identified P-gp inhibitors

Based on the results from the high content screening assay, we selected to use two well-established cell-based functional assays to confirm the inhibition action of the hit compounds and also eliminate false positives. To this end, we evaluated the compounds effect on P-gp mediated calcein-AM efflux using flow cytometry. HEK293/pcDNA3.1 and HEK293/P-gp cells were treated with positive control or test compounds for 10 min and with calcein-AM for 30 min. As shown in Figure 2.2A, verapamil at 10 μM enhanced calcein accumulation in HEK293/P-gp cells by 10.9 fold compared to the untreated cells (control). Moreover, all the hit compounds significantly enhanced calcein accumulation in HEK293/P-gp cells at varying degrees (4.0 – 9.6 fold). Compounds HL 16, HL 21, HL 22 and HL 80 were very potent in inhibiting P-gp, by increasing calcein accumulation considerably by 9.2-9.6 fold. On the other hand, no modulatory effect on calcein accumulation was observed for the compounds in HEK293/pcDNA3.1 cells.

Our next direction was to confirm inhibition of P-gp activity by the hit compounds on the efflux of another P-gp substrate. The effect of the compounds on P-gp mediated doxorubicin (Dox) efflux was observed using confocal microscopy. A two-color P-gp-GFP-RFP expression vector (GR 678) was transiently transfected into HEK293T cells. The accessibility of mixed population of cells derived from transient

transfection provides the means to visualize cells that pick up the vector DNA in the cell membrane against non-transfected cells in a single image. After treatment with the positive inhibitor or test compounds and incubation with doxorubicin, images acquired from the GFP, RFP, and Dox channels were merged and analyzed. P-gp transport of doxorubicin was viewed in the nucleus. As shown in Figure 2.2B, the control (DMSO) treated cells exhibited high doxorubicin accumulation in the nuclei of non-transfected cells, whereas doxorubicin fluorescence was undetectable in cells expressing P-gp-GFP-RFP. Treatment with positive control, verapamil (10 μM), restored doxorubicin accumulation in P-gp-GFP-RFP expressing cells. The compounds HL 15, HL 16, HL 20, showed strong doxorubicin accumulation and hence strong inhibition similar to verapamil. Moreover, moderate doxorubicin accumulations were observed in cells treated with HL 4, HL 21, HL 22, HL 77 and HL 80.

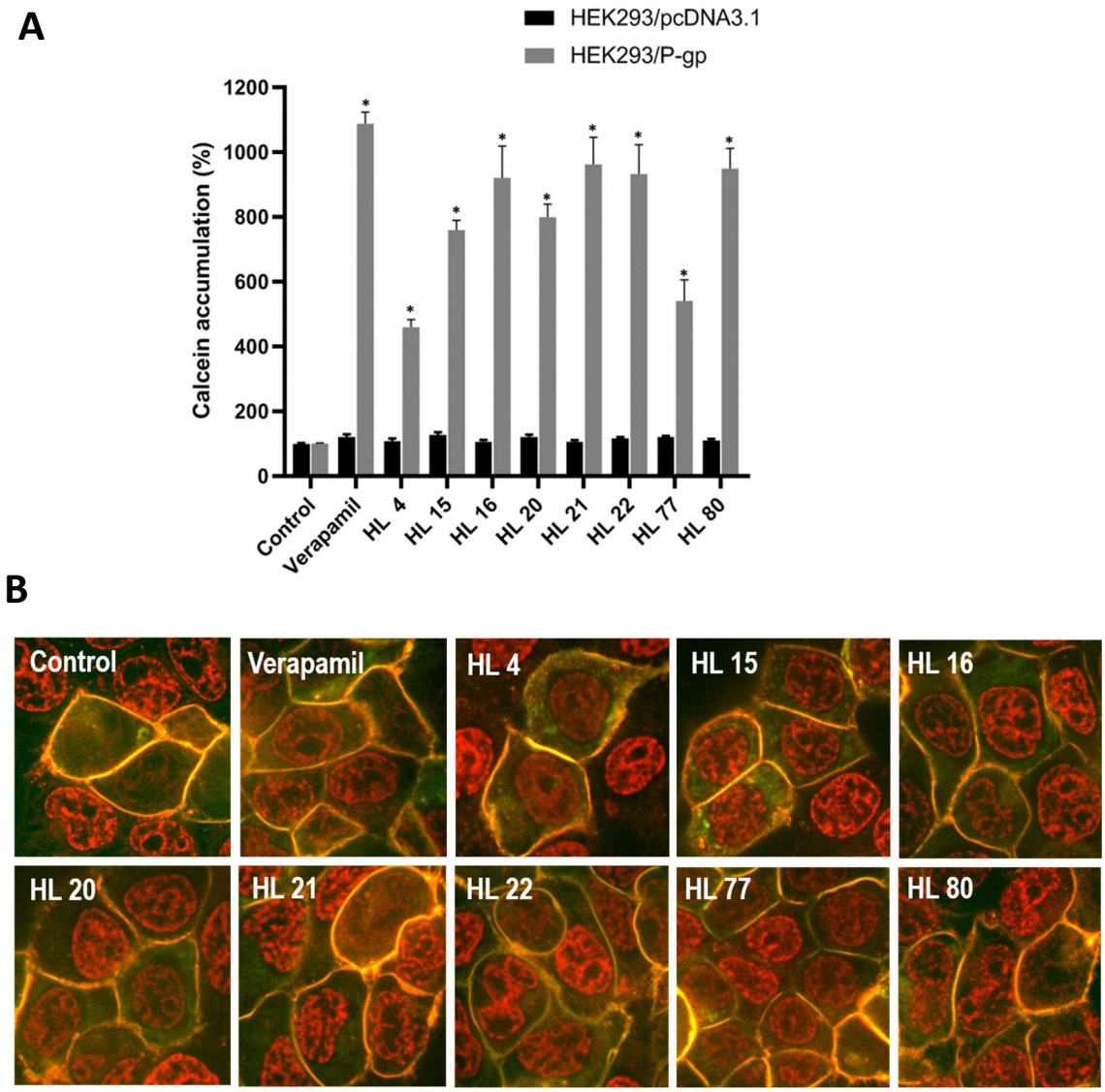

Figure 2.2 Confirmation of inhibitory effect of identified compounds on P-gp activity. (A) HEK293/pcDNA3.1 and HEK293/P-gp cells were treated with 0.25 μM calcein-AM at 37 °C for 30 min after pretreatment with 10 μM verapamil or test compounds for 10 min. Collated results from three independent experiments (performed in duplicates) and presented as mean ± SEM with *$p < 0.05$ compared with control. (B) A two-color P-gp-

GFP-RFP expression vector (GR 678) was transiently transfected into HEK293T cells. After 24-48 h, cells were incubated with 10 μM test compound for 30 min, and then treated with doxorubicin (10μM) for 1 h. Images were viewed using confocal microscope equipped with a 1.35 numerical aperture 60x oil-immersion objective. GFP and Doxorubicin were excited at 488 nm with emission bands at 537/26 nm and 605/64 nm respectively. RFP was excited at 561 nm with emission band at 605/64 nm.

2.3.3 Reversal of drug resistance by identified P-gp inhibitors

The CIEA compound library under study in this work were designed to target EGFR and MAPK pathways which play significant roles in cancer proliferation and differentiation. Nonetheless, we decided to evaluate the capability of these compounds to re-sensitize P-gp overexpressing cells to chemotherapeutic agents. Many reports have established the ability of P-gp inhibitors to reverse drug resistance as crucial to the performance of chemotherapeutic agents in cancers where P-gp is overexpressed. The cytotoxic effect of each identified inhibitor compound on HEK293/P-gp cells was first determined using a dose-response curve (data not shown). The non-cytotoxic concentrations of the compounds were then selected and combined with various concentrations of vincristine, paclitaxel or etoposide (common P-gp chemotherapeutic substrates) and evaluated for drug resistance reversal. HEK293/pcDNA3.1 was used as the negative control cell line. As shown in Figure 2.3A-C, The P-gp overexpressing cell line, HEK293/P-gp, (solid red) demonstrated considerable drug resistance as anticipated against vincristine, paclitaxel and etoposide compared with the control cell line

HEK293/pcDNA3.1 (dotted green). Treatment with verapamil reversed resistance of HEK293/P-gp (solid blue) towards all three chemotherapeutic drugs. Cell treatments with vincristine, paclitaxel and etoposide with their IC_{50} values and fold resistance are represented in Table 2.2. In vincristine treatment, HEK293/P-gp cells showed 290-fold greater resistance compared with the control, HEK293/pcDNA3.1. After treatment with the known P-gp inhibitor, verapamil (25 μM), the fold resistance of HEK293/P-gp was significantly reduced to 0.27-fold. Amongst the 8 hits, compound HL 77 reduced the fold resistance of HEK293/P-gp by more than half (111-fold). Compounds HL 4, HL 15, HL 20 and HL 22 reduced resistance of HEK293/P-gp between 149 and 189-fold while compounds HL 16, HL 21 and HL 80 showed modest reduction (between 217 – 256) in fold resistance. The CIEA compounds at their non-cytotoxic concentrations reversed the resistance of HEK293/P-gp cells towards paclitaxel to various extents. Compounds HL 4, HL 16, HL 21, HL 77 and HL 80 reduced the fold resistance of HEK293/P-gp (845-fold) to more than half (< 422.5-fold). HL 15, HL 20 and HL 22 demonstrated modest reversal of resistance of HEK293/P-gp towards paclitaxel. Treatment of HEK293/P-gp cells with etoposide in the absence of inhibitor, produced 173-fold more resistance than the control HEK293/pcDNA3.1.In comparison to vincristine and paclitaxel, all the test compounds were most effective in sensitizing HEK293/P-gp towards etoposide (reduced resistance fold of HEK293/P-gp cells from 173 to between 33-50-fold).

Table 2.2 Effect of the identified inhibitors on the $1C_{50}$ values of vincristine, paclitaxel and etoposide in HEK293/pcDNA3.1 and HEK293/P-gp cells.

Cell line/Treatment	IC_{50}[a] (μM)					
	Vincristine	**Fold resistance[b]**	**Paclitaxel**	**Fold resistance[b]**	**Etoposide**	**Fold resistance[b]**
HEK293/pcDNA3.1	0.0038 ± 0.0007	1.00	0.0016 ± 0.0002	1.00	0.0710 ± 0.0574	1.00
HEK293/P-gp	1.10 ± 0.09	290.16	1.36 ± 0.06	848.57	12.20 ± 1.96	172.68
HEK293/P-gp + Verapamil [25 μM]	0.0010 ± 0.0005	0.27	0.0031 ± 0.0024	1.92	0.6570 ± 0.0704	9.31
HEK293/P-gp + HL 4 [2 μM]	0.57 ± 0.13	148.56	0.57 ± 0.13	355.26	2.67 ± 0.57	37.58
HEK293/P-gp + HL 15 [2 μM]	0.72 ± 0.04	189.03	0.94 ± 0.06	589.89	2.50 ± 0.18	35.34
HEK293/P-gp + HL 16 [10 μM]	0.97 ± 0.05	255.70	0.39 ± 0.01	241.93	2.65 ± 0.15	37.53
HEK293/P-gp + HL 20 [2 μM]	0.63 ± 0.22	164.72	0.79 ± 0.12	496.85	2.45 ± 0.19	34.62
HEK293/P-gp + HL 21 [2 μM]	0.95 ± 0.11	250.71	0.47 ± 0.19	296.79	3.51 ± 0.31	49.64
HEK293/P-gp + HL 22 [2 μM]	0.68 ± 0.08	178.36	1.14 ± 0.12	712.35	2.38 ± 0.50	33.66
HEK293/P-gp + HL 77 [2 μM]	0.42 ± 0.09	110.57	0.40 ± 0.07	251.26	3.25 ± 0.71	46.04
HEK293/P-gp + HL 80 [10 μM]	0.83 ± 0.14	217.46	0.59 ± 0.10	370.99	2.98 ± 0.70	42.20

[a] Mean ± SD of at least three independent experiments performed in triplicates.
[b] Fold resistance determined by dividing the IC_{50} value for each treatment by the IC_{50} value of HEK293/pcDNA3.1 treatment with vincristine, paclitaxel or etoposide alone.

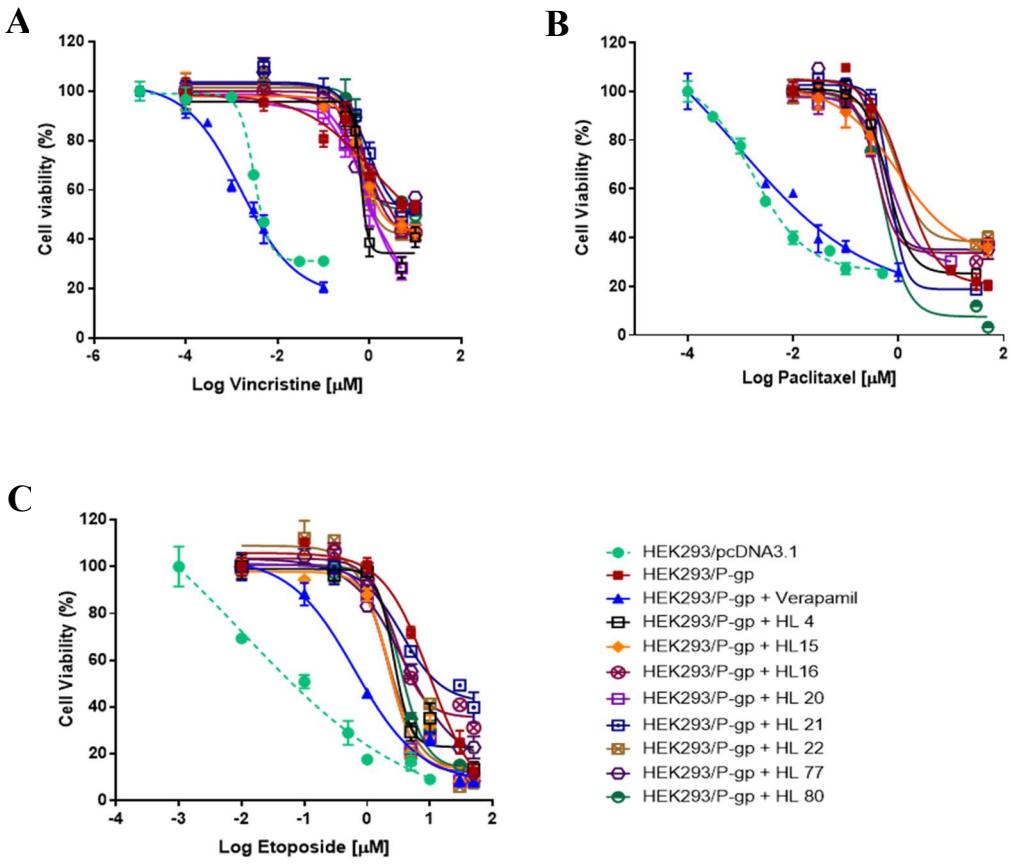

Figure 2.3 Reversal of drug resistance of P-gp overexpressing cells by identified compounds towards P-gp anticancer substrates. HEK293/ P-gp cells were treated with vincristine (A), paclitaxel (B) and etoposide (C) at increasing concentrations in the absence or presence of verapamil (positive inhibitor) and test compounds for 72 h. Verapamil concentration was at 25 µM; HL 4, HL 15, HL 20, HL 21, HL 22 and HL 77 were at 2 µM. HL 16 and HL 80 concentrations were at 10 µM. HEK293/pcDNA3.1 cells was used as negative control. Cell viability was evaluated with MTT colorimetric assay. Data are representative of three independent experiments and presented as mean ± SD (n = 3).

2.3.4 The effect of the identified inhibitors on P-gp protein expression

Since inhibition of ABC transporter-mediated multidrug resistance mechanisms may arise from down-regulation at the protein level, we performed western blot to determine whether the identified P-gp inhibitors affected the expression of P-gp. As shown in Figure 2.4, the expression levels of P-gp (170 kDa) in P-gp overexpressing HEK293/P-gp cells were not significantly altered after treatments with 10 μM compounds.

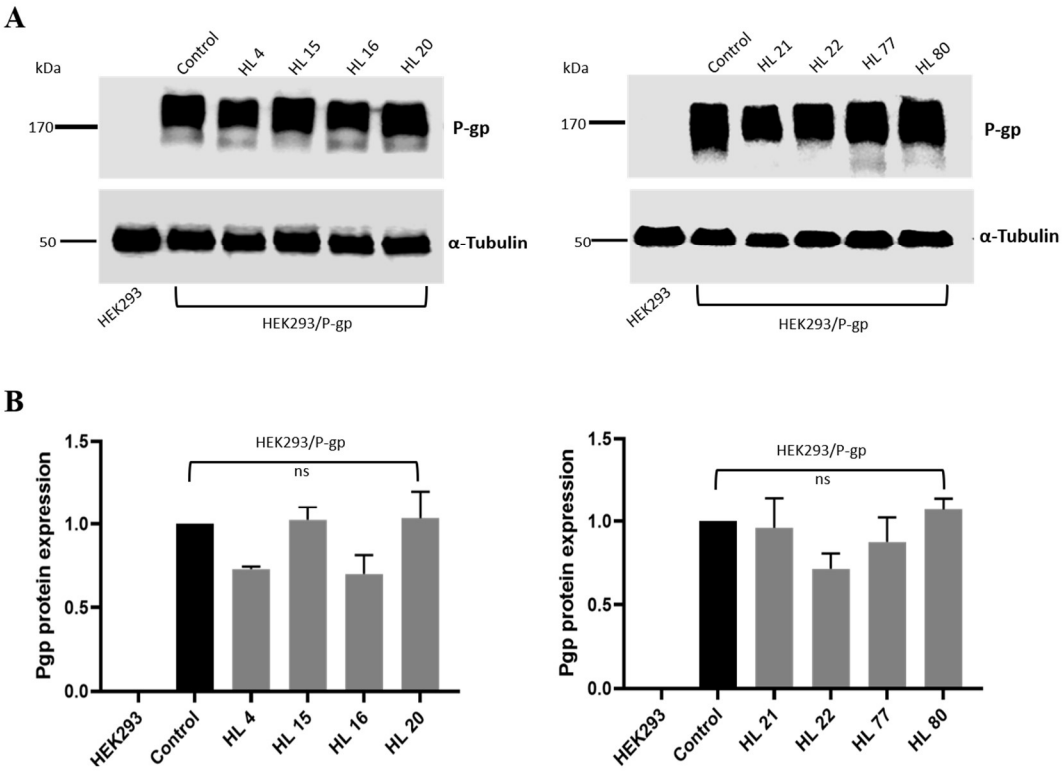

Figure 2.4. Expression pattern of P-gp protein in HEK293/P-gp cells treated with identified inhibitor compounds. (A) Immunoblots of whole cell lysates prepared from HEK293/P-gp cells treated with 10 μM test compounds for 48 h with α-tubulin used as

loading control. Blots are representative of two independent experiments. (B) Protein band densities were quantified using Image Studio Lite (LI-COR Biotechnology, Lincoln, NE) software with uneven sample loading and transfer corrected per individual protein band relative to α-tubulin. P-gp expression presented as mean ± SEM with $p < 0.05$ compared with control, calculated using linear mixed model and Sidak post hoc test.

2.3.5 Docking of identified P-gp inhibitors with refined mouse P-gp structure

To investigate the binding interaction of the identified inhibitors with P-gp protein, a docking grid at the drug binding pocket of the human ABCB1 homology based on refined mouse ABCB1 model (PDB code: 4MIM) was created and prepared for docking using OpenEye software. The software generates consensus scoring which signifies the virtual binding affinity of the ligands to their receptor. A lower consensus scoring means good binding affinity of the ligand towards the receptor. From the docking studies, all the CIEA compounds displayed better binding affinities (consensus scores: < 27) towards P-gp than the known P-gp inhibitor, verapamil (consensus score: 27) (Table 2.3). Compound HL 20 had the lowest consensus score (consensus score:1), representing the compound with the strongest binding affinity towards the P-gp binding pocket though with no apparent hydrogen bond interaction. Additionally, no hydrogen bond interactions were observed for compounds HL 21 and HL 80, though both exhibited equal binding affinities (consensus score: 19) toward P-gp. The nitrile group of verapamil hydrogen bonded with TYR:306:A at the binding pocket (Figure 2.5A). Hydrogen bond interactions were observed for compounds HL 4, HL 16 and HL 22 between their

hydroxyl substituent groups and specific amino acid residues (Figures 2.5B, 2.6B, 2.8A). Both HL 4 and HL 22 interacted with TYR:303:A. GLN:721:A residues which may be attributed to the close similarity in their structures. HL 16 interacted with TYR:949:A residues at the P-gp binding pocket. The amino phenyl side chain of HL 15 (Figure 2.6A) and the hydroxyl group on C-1 of HL 77 (Figure 2.8B) were also involved with hydrogen bond interactions with ALA:983:A and ASN:717:A respectively.

Table 2.3 Molecular docking consensus scores, and H-bonding interactions for CIEA analogs. Verapamil used as reference drug

Compound	**Mol. Wt.**	**Consensus score**	**Target H-bonding residues**
Verapamil	454.602	27	TYR: 306:A
HL 4	424.572	21	TYR: 303:A; GLN 721:
HL 15	486.605	16	GLN:986:A
HL 16	538.63676	11	TYR:949:A
HL 20	562.70122	1	-
HL 21	472.57868	19	-
HL 22	424.57234	18	TYR:303:A; GLN:721:A
HL 77	502.60466	25	ASN:717:A;
HL 80	514.61536	19	-

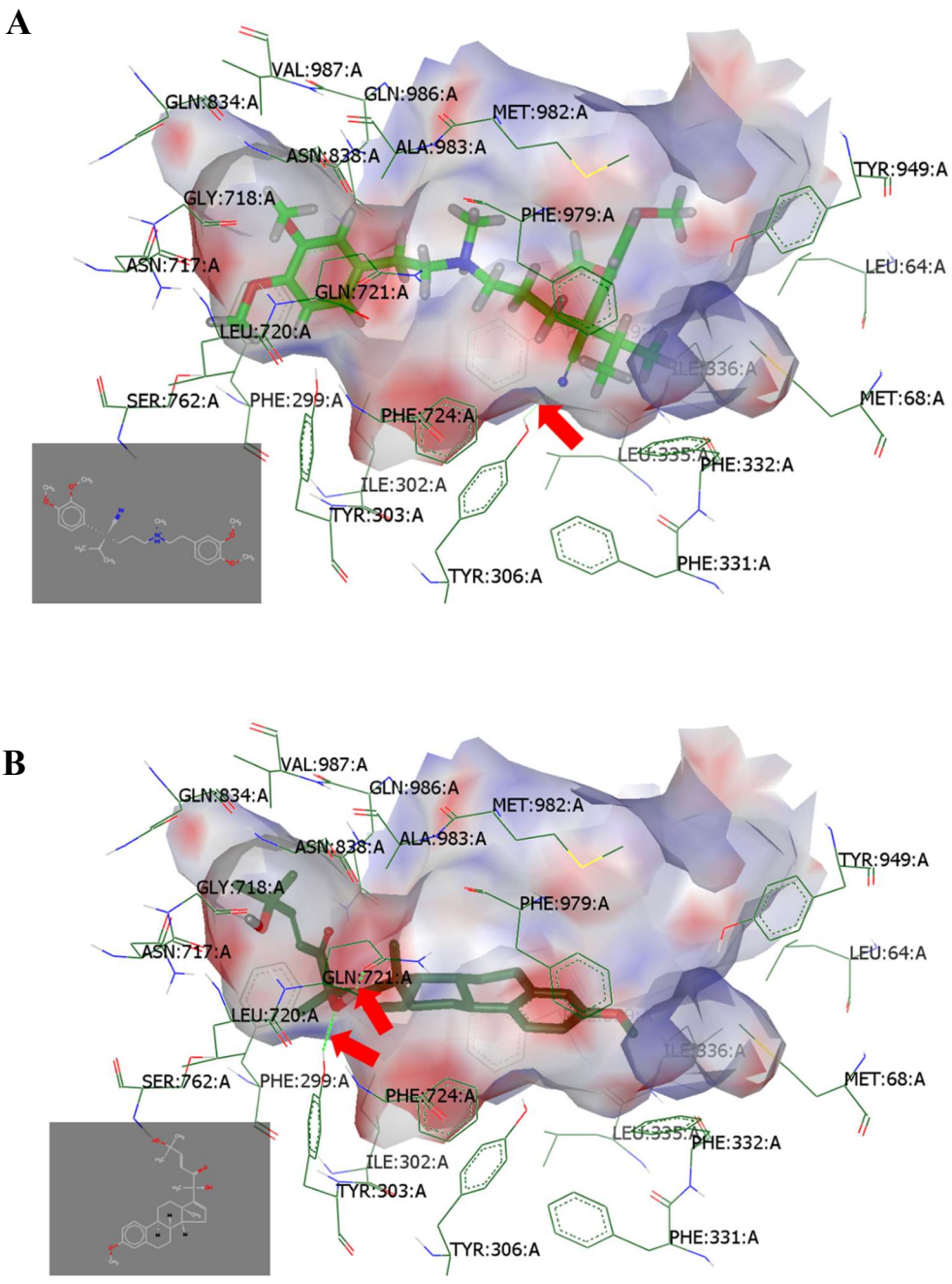

Figure 2.5 Visual representation for (A) Verapamil and (B) HL 4 within the binding pocket of P-gp (PDB ID: 4MIM). Red arrows indicating location of hydrogen bonds (green dashed lines).

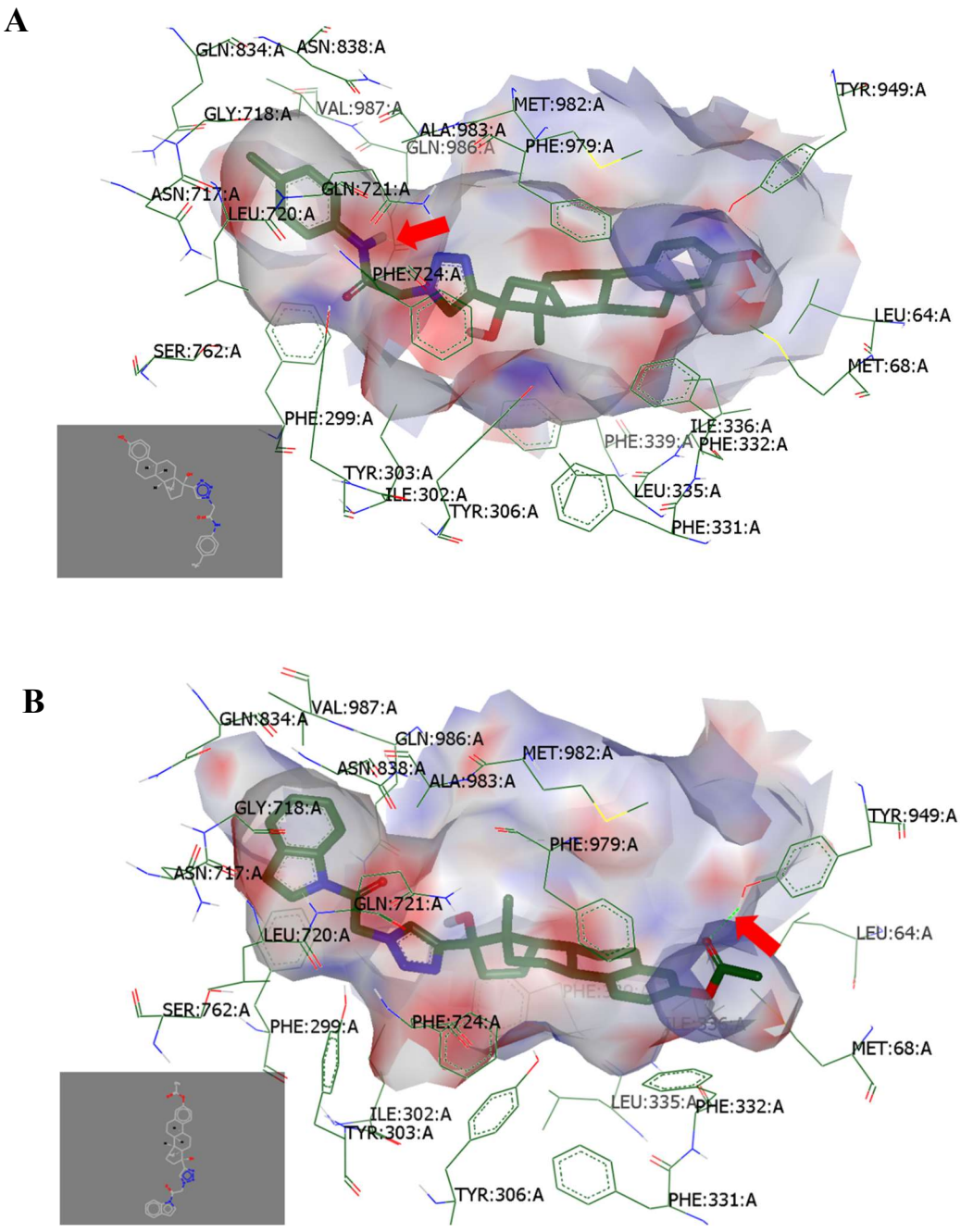

Figure 2.6 Visual representation for (A) HL 15 and (B) HL 16 within the binding pocket of P-gp (PDB ID: 4MIM). Red arrows indicating location of hydrogen bonds (green dashed lines).

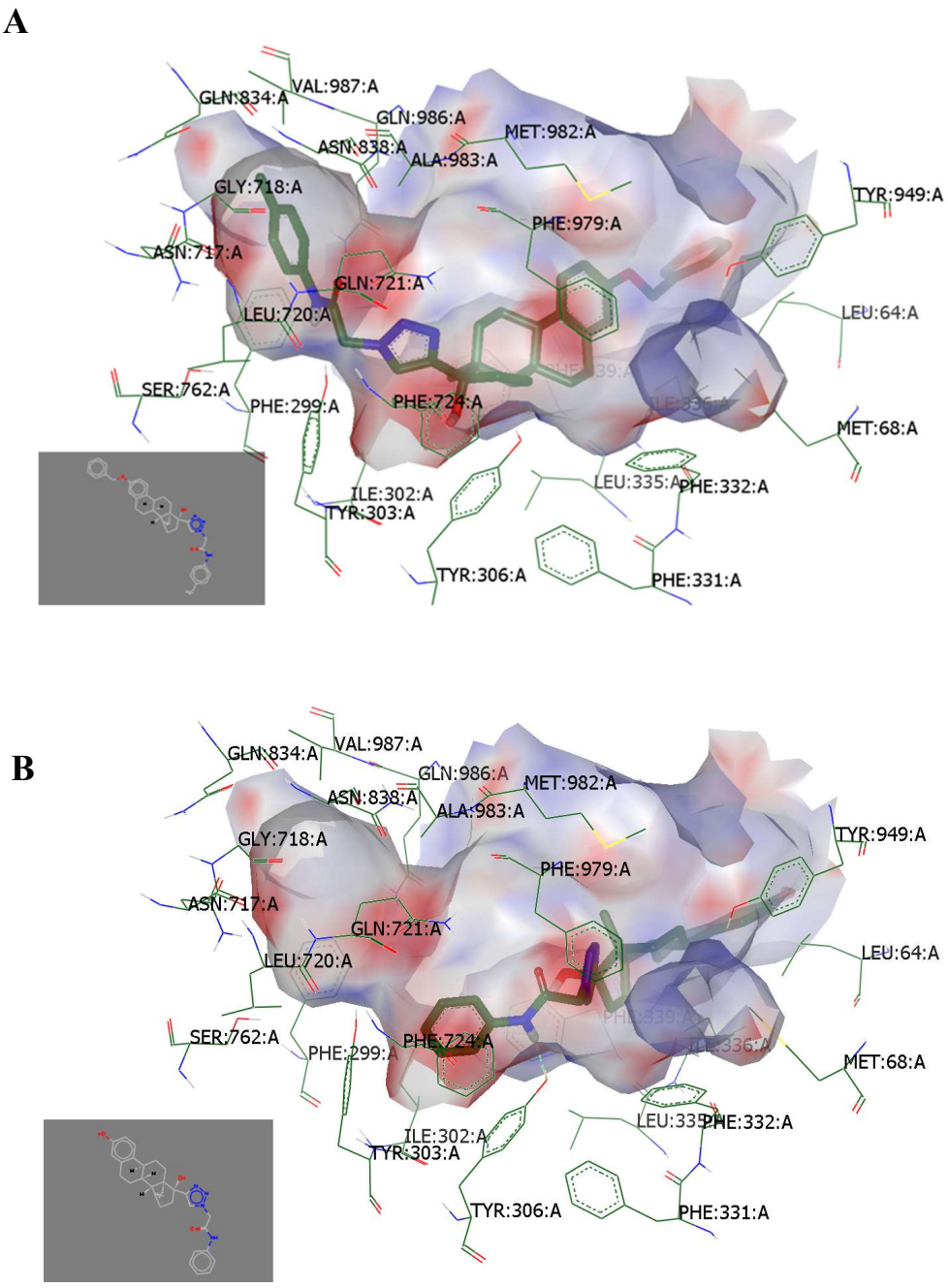

Figure 2.7 Visual representation for (A) HL 20 and (B) HL 21 within the binding pocket of P-gp (PDB ID: 4MIM).

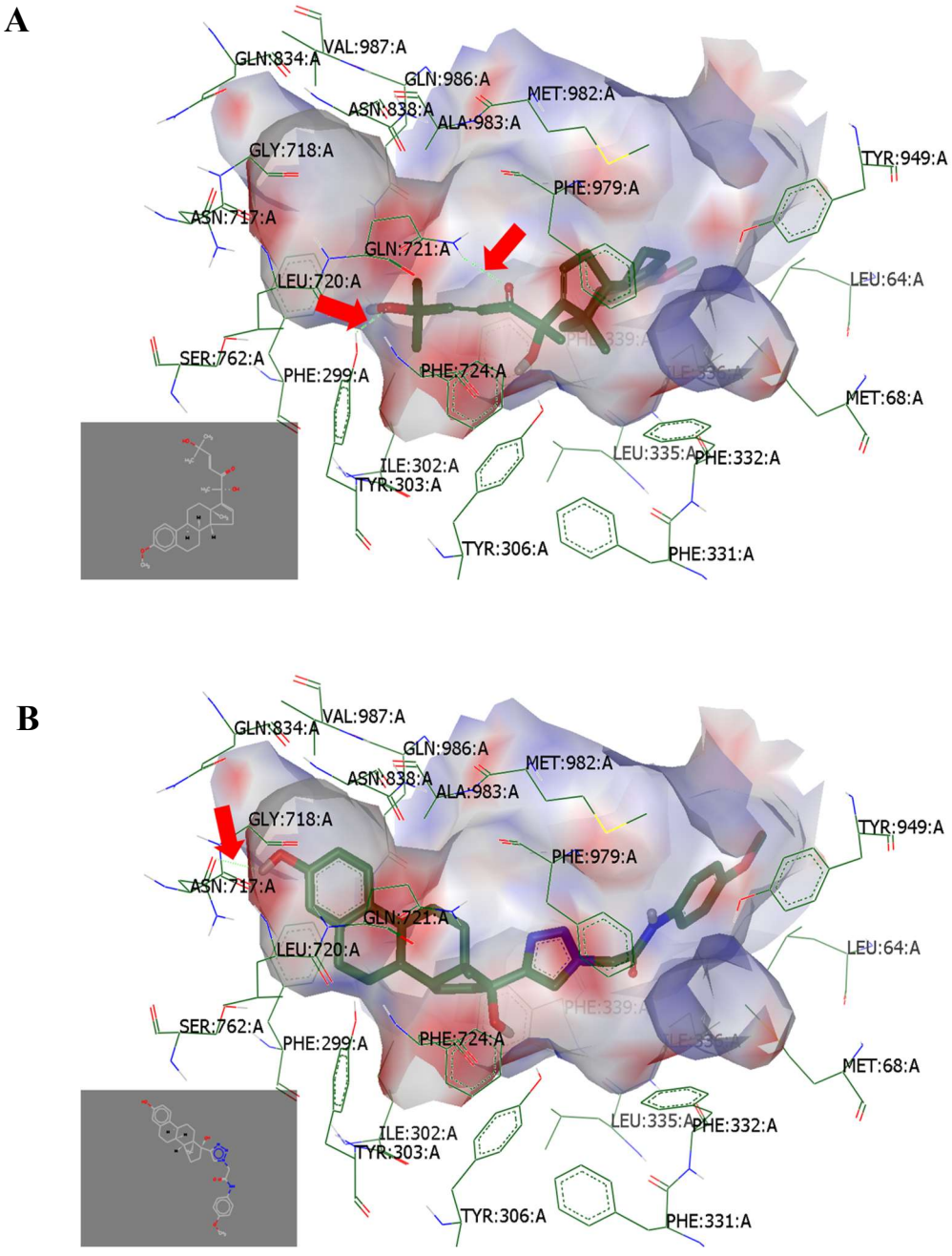

Figure 2.8 Visual representation for (A) HL 22 and (B) HL 77 within the binding pocket of P-gp (PDB ID: 4MIM). Red arrows indicating location of hydrogen bonds (green dashed lines).

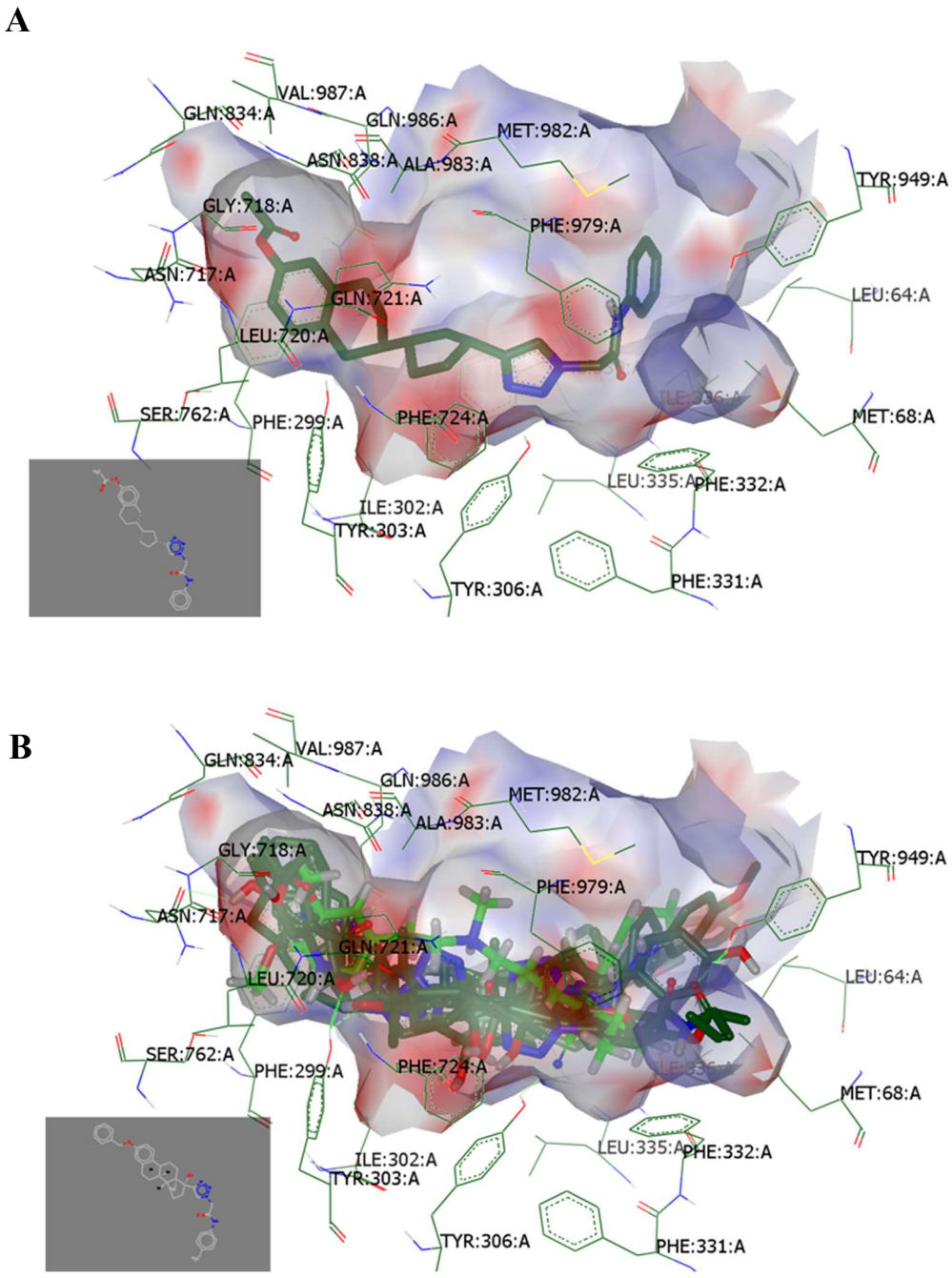

Figure 2.9 Visual representation for (A) HL 80 and (B) Overlay of the binding modes of all CIEA compounds (gray) and reference compound verapamil (green) within the binding pocket of P-gp (PDB ID: 4MIM).

2.3.6 Identified P-gp inhibitors induce dynamic FRET changes

FRET spectroscopy is considered an important biophysical tool for probing interactions between cellular molecules at nanoscale distances allowing for accurate structural information of molecules. We used the structural-based intramolecular FRET approach to investigate our inhibitor compounds interaction with P-gp and also to validate the results obtained from the molecular docking studies. The fluorescence spectra representation of two-color P-gp in the presence or absence of ligands (test compounds) and compound-induced FRET changes are shown in Figures 2.10A and B respectively. Compounds HL 4 and HL 15 produced FRET efficiency changes (~1.5 and 1.7% respectively) which were close to that of verapamil (~1.5%). Compound HL 20 produced the highest increase in FRET efficiency (~15.3%) followed by HL 80 (~9.2%) whiles the lowest change in FRET (~0.8%) was observed for HL 16. HL 21, 22 and 77 yielded compound-induced FRET efficiencies between ~2.7 – 5.3%.

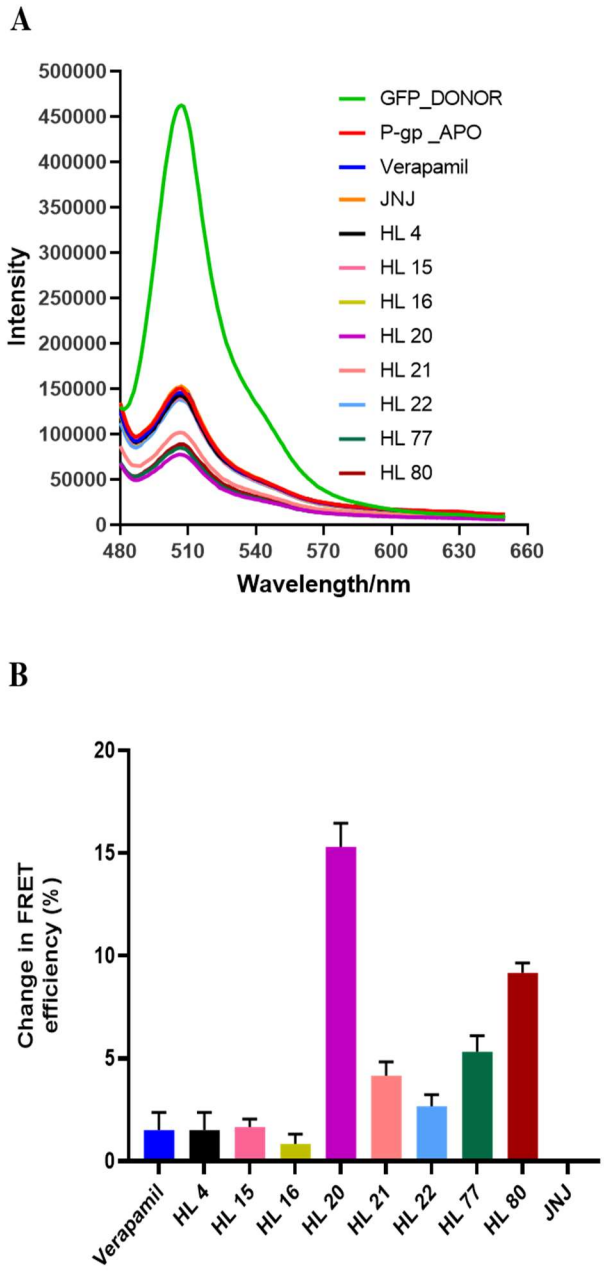

Figure 2.10 Identified inhibitor (CIEA) compounds interaction with P-gp protein. (A) Fluorescence spectra representation of two-color P-gp protein (P-gp-GFP-RFP) in the absence or presence of ligands (identified P-gp inhibitors). GFP protein was used as donor control. (B) Inhibitor compounds-induced FRET changes with two-color P-gp protein. Membrane vesicles (20 µg) prepared from HEK 293T cells stably transfected

with a two-color P-gp, GR 678 were treated with 10 μM of test compounds for 10 min before spectroscopic analysis with the fluorometer. Verapamil and JNJ-26854165 were included as positive and negative controls, respectively. GFP was excited at 480 nm and RFP at 530 nm. Emission for both was achieved at 480-650 nm. Data are representative of two independent experiments performed in triplicates. Results are presented as mean ± SEM.

2.4 Discussion

Cancer is the second leading cause of mortality globally accounting for 1 in every 6 deaths in 2018 [63]. Multidrug resistance mediated by the overexpression of ABC transporters is responsible for low patient survival rates in many malignancies. P-gp, the most intensively studied MDR-transporter is recognized for its role in MDR due to its ability to expel a broad array of drug substrates including anticancer agents out of cells. In clinical settings, this function of P-gp serves as a first-line defense mechanism for malignant tissues to escape the cytotoxic effects of chemotherapeutic agents. To advance patient clinical outcomes in chemotherapy treatments, it is important to overcome P-gp mediated MDR through inhibiting its function or down regulating its expression. To date, no P-gp inhibitor has gone past the clinical trial stages as a result of unfavorable drug effects [48]. Developing and discovering drugs and new compounds that target P-gp function is therefore imperative.

The various types of cucurbitacins and their analogs have shown potential inhibitory effects in many types of cancers [64, 65]. However, the effects of cucurbitacins

and their derivatives on the activity of ABC drug efflux transporters in MDR is less studied. Novel compounds known as cucurbitacin-inspired estrone analogs (CIEAs) have currently been proven to have antiproliferative activity in hepatocellular carcinoma via inhibiting phosphorylation pathways of EGFR and MAPK [66]. In this study, we assessed for the first time the potential of the CIEAs to overcome P-gp-mediated MDR in vitro. We first employed a high content fluorescence-based transport activity screening assay to detect the inhibition of P-gp mediated efflux of calcein-AM, a known P-gp substrate by the CIEA compounds. Calcein-AM is a cell permeable, non-fluorescent compound that is popularly used in many modulation studies to evaluate the activity of P-gp [67, 68]. The high content fluorescence-based screening is suitable for screening large libraries of natural and synthetic drugs. In addition to being a highly sensitive, in situ cell-based technique, it also incorporates an imaging-based system to directly detect cytotoxic effects. The assay identified 8 CIEA compounds as hits for P-gp inhibition with good reproducibility (0.87 correlation value) and performance (0.65 Z-factor).

To validate the results from the high content screening assay, the inhibition activity of the hit compounds were further evaluated on P-gp transport activity. All the compounds displayed strong inhibition (in comparison to verapamil) of P-gp-mediated calcein-AM efflux using flow cytometry. They also demonstrated inhibition of P-gp-mediated efflux of doxorubicin, in confocal microscopy studies. Furthermore, in drug reversal studies, the CIEA compounds demonstrated varying degrees of selectivity towards reversal of resistance of the chemotherapeutic drugs vincristine, paclitaxel and etoposide in P-gp overexpressing cells. For instance, all the compounds were very effective in sensitizing HEK293/P-gp cells towards etoposide but not vincristine or

paclitaxel. Compounds HL 16, HL 21 and HL 80 only showed modest modulatory effect in sensitizing HEK293/P-gp cells towards vincristine while compounds HL15, HL 20 and HL 22 displayed modest reversal effect in paclitaxel resistance. Based on these findings, we think that these CIEA compounds can specifically improve the chemotherapeutic response of etoposide in P-gp mediated MDR cancers where P-gp is highly expressed.

To explore the potential mechanisms of inhibition and drug resistance reversal effect portrayed by the CIEA compounds, P-gp protein expression studies were performed using western blot. The results indicated that the CIEA compounds have no effect on P-gp expression at dose (10 μM) that inhibited P-gp efflux suggesting that these compounds affect P-gp by mainly inhibiting its efflux function. It has been reported that known P-gp inhibitors such as verapamil inhibit P-gp activity by also decreasing its expression [69] and this might be useful in treating malignancies where P-gp is prominently expressed. However, it is worth noting that the normal physiological defense mechanism of P-gp in tissues such as the brain might require typical P-gp expression levels and therefore should not be compromised. In this case it will be safe to use compounds that only inhibit the transport function of P-gp.

The molecular basis for the polyspecificity nature of P-gp interaction with substrates or inhibitors have been determined by Aller et al.,2009 [9]. In our quest to investigate the CIEA compounds interaction with P-gp, molecular docking studies were conducted. Our results revealed that all the compounds have better binding affinities (based on consensus scoring, Table2.3) towards P-gp at the drug binding pocket than verapamil though the overlay of the docking poses (Figure 2.9B) show the compounds

and verapamil all docked at similar positions in drug binding pocket. Aller et al determined co-crystal structures of P-gp complex with the stereoisomeric cyclic hexapeptide inhibitors (QZ59-RRR and QZ59-SSS). They reported that the ligand in close proximity to the C-terminal half of the TMD was surrounded by three polar residues GLN-721, GLN-986, and SER-989 [9]. Our compounds HL 4 and HL 22 interacted through hydrogen bonding with GLN-721 residue, whereas HL 15 hydrogen bonded with GLN-986 at the drug binding pocket of P-gp. In addition, HL16 interacted with TYR-949 which was also reported to be in close proximity to TM12. Based on our results, we think that inhibition of P-gp transport function by the CIEA compounds may be due to strong interaction with C-terminal transmembrane residues at the drug binding pocket of P-gp.

From the docking studies, our attention was then directed to validate the CIEA compounds interaction with P-gp protein. Inhibition whether competitive, noncompetitive or uncompetitive will require direct binding of the ligand to its target. Using structural-based fluorescent spectroscopic technique which can measure ligand-induced intramolecular FRET changes in the P-gp protein, we showed that all CIEA compounds directly interact with P-gp and induced varying degrees of conformational changes. This together with our observations from the docking results reinforces our notion that the CIEA compounds inhibitory effects on P-gp are due to direct interaction with the transporter protein. Nevertheless, further studies are recommended to confirm and map the precise binding site for each of the CIEA compounds on P-gp protein.

2.5 Conclusion

Overall, we have used a high content fluorescence-based transporter activity assay to identify cucurbitacin-inspired estrone analogues (CIEA) as potential P-gp inhibitors. Validation of their inhibitory activity has also been demonstrated. In addition, these compounds were able to reverse drug resistance in P-gp overexpressing cells. Finally, we demonstrated that these compounds directly interacted with P-gp protein. This study provides vital information on CIEA as sources of new drug discoveries for optimization as treatment strategies to overcome MDR in cancer.

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Chapter 3

Novel compounds from cucurbitacin-inspired estrone analogues as inhibitors for multidrug resistance protein 1.

Abstract

Multidrug resistance protein 1 (MRP1/ABCC1), a member of the ATP-binding cassette (ABC) transporter family, actively transports diverse endogenous and xenobiotic substrates including anticancer drugs across membrane barriers. MRP1 is widely associated with multidrug resistance in cancer and reportedly responsible for poor patient outcome in many hematological malignancies due to its ability to diminish the efficacy of a broad range of anticancer agents. In this study, we identified novel inhibitors for MRP1 by utilizing a high content fluorescence-based transporter activity assay initially developed in our lab. From screening a library of 81 novel cucurbitacin-inspired estrone analogs (CIEAs) employing doxorubicin and calcein red orange as fluorescent reporters, 5 compounds were identified as inhibitors of MRP1-mediated doxorubicin efflux and 3 compounds were identified to inhibit calcein red orange efflux. Out of the 3 compounds identified as inhibitors of calcein red orange efflux, 2 (HL 15 and HL 21) were also identified as inhibitors from the doxorubicin inhibition assay. Four of the identified CIEA compounds were selected for further validation on MRP1 inhibition using well-established ABC transporter cell-based functional assays. All the selected CIEA compounds significantly increased the accumulation of MRP1 substrates, doxorubicin and calcein red orange. Drug sensitivity studies also showed selective sensitization of MRP1 overexpressing H69AR cells by the compounds towards the anticancer drugs,

vincristine and SN38, however, HL 15 demonstrated good sensitization towards both anticancer agents. Moreover, inhibition of MRP1 activity by the CIEA compounds did not significantly alter the protein expression levels of MRP1. In silico molecular docking and fluorescence spectroscopic studies showed inhibitor compounds directly interacted with MRP1 protein suggesting that the CIEA compounds affect the activity of MRP1. In summary, the findings in this work provide a novel insight in the utilization of CIEAs as promising drug candidates against MRP1-mediated multidrug resistance in cancer.

Keywords: MRP1, ABC transporter, multidrug resistance, diminished drug efficacy, CIEA, promising drug candidates

3.1 Introduction

Multidrug resistance protein 1 (MRP 1) is a membrane-associated efflux pump of the ATP-binding cassette (ABC) transporter family, an evolutionarily conserved superfamily of ATP-dependent transporters that contribute to key cellular processes in all organisms. In humans, there are 48 ABC transporter genes divided into seven unique subfamilies designated ABCA through ABCG according to their domain organization and sequence homology [1]. MRP1 (ABCC1) is the first member of the 'C' subfamily, known for containing several drug transporters as well as atypical ABC transporters including cystic fibrosis transmembrane regulator (CFTR/ABCC7) and sulfonylurea receptor (SUR/ABCC8/9) [2-4]. Like several of the 'C' subfamily members, the structural architecture of the 190 kDa MRP1 protein is characterized by a proximal NH2-terminal membrane spanning domain (MSD0) in addition to the typical four core domains: two MSD (MSD1 and MSD2) and two cytoplasmic nucleotide binding domains (NBD1 and NBD2) observed in most ABC proteins [5-8]. The MSD1 and MSD2 contain six transmembrane (TM) helices each and form the substrate translocation 'pore' through which substrates are transported [9]. The transport process is powered by ATP binding and hydrolysis at the catalytic core of the 2 NBDs, which is defined by a distinct functional asymmetry [10, 11]. The function of the MSD0 although proposed to play a role in dimerization still remains poorly understood [12].

MRP1 is the primary transporter of endogenous anionic substrates such as glutathione (GSH), cobalamin, bilirubin, estradiol glucuronide (E217βG) and cysteinyl leukotriene (LTC_4 [13-15]. The ATP-dependent transport of the antioxidant GSH and the

pro-inflammatory molecule, LTC_4, makes MRP1 an essential mediator in cellular redox homeostasis, cell differentiation, proliferation and immunological reactions. Owing to these essential cellular functions, it is not surprising that MRP1is associated with the pathophysiology of many metabolic, cardiovascular, neurological, immunological and tumor related diseases in humans [16-19]. MRP1 also mediates the efflux of sulfate-conjugated drugs, or unconjugated drugs concertedly with free glutathione many of which include antineoplastic agents like vinca alkaloids, epipodophyllotoxins, methotrexate, camptothecins and many clinically important therapeutics e.g. antibiotics, antivirals, antidepressants, statins and metal oxyanions [20, 21].

In normal tissues, MRP1 is distributed in the lung, gastrointestinal tract, cardiac, liver, pancreas, adrenal cortex, skin and skeletal muscle but mainly localized to basolateral membranes of polarized epithelia [22]. Importantly, MRP1 is expressed in pharmacological sensitive barriers and therefore exerts chemoprotective functions in tissues of the brain, testes and placenta [23]. Notwithstanding, MRP1 is the most prevalent drug efflux transporter implicated in multidrug resistance (MDR) in cancer besides P-gp. Since its discovery in a doxorubicin-selected lung cancer cell line, MRP1 overexpression in malignant diseases especially hematologic and solid tumors have also been confirmed [24-27]. MRP1 confers resistance to tumor cells by actively extruding a large number of structurally unrelated chemotherapeutics out of the cells thereby affecting the overall drug absorption, disposition and efficacy. The consequence is poor clinical outcome in many patients particularly with non-small lung cancer, acute myeloid leukemia, (AML), and breast cancer [26, 28, 29]. Clinical investigations have also shown a high correlation between overexpression of MRP1 and aggressive or multidrug

resistance traits in tumors [28]. Particularly, various studies on childhood neuroblastoma have shown evidence of high MRP1 expression levels as the key individual prognostic biomarker of poor patient outcome [30, 31]. Furthermore MRP1, is reported as the target of MYCN, the main oncogene frequently amplified in human neuroblastoma [32].

Generally, the strategy to overcome ABC transporter-mediated MDR in cancer is the pharmacological suppression of drug transporters to restore sensitivity of cancer cells to anticancer drugs. A number of inhibitors have been developed and progressed to the clinical trial stages for the first drug efflux transporter, P-gp since its discovery over 4 decades ago [33, 34]. In contrast, the search for MRP1 inhibitors is still at the infancy stage and prominent MRP1 inhibitors such as leukotriene D_4 (LTD_4) receptor antagonist, MK571 and ONO-1078 [35, 36]; the anion transport inhibitor, probenecid [37] and the nonsteroidal anti-inflammatory drug indomethacin [38, 39] are still currently being studied for their activity on MRP1 function. In addition, known P-gp inhibitors such as cyclosporine A, PC-883 (valspodor), and verapamil [40] and natural products e.g. agosterol and flavonoids [41-44] have all been reported to have inhibitory action on MRP1. Most recently, tyrosine kinase inhibitors (TKIs) such as ibrutinib and lapatinib have also demonstrated potent MRP1 inhibition [45, 46]. Unfortunately, the action of some of these MRP1 inhibitors have been far from satisfactory due to toxicity and severe side effects among other reasons [47-49]. The development of more structurally diverse, highly potent, less toxic and selective MRP1 inhibitors is thus desirable.

In our continuous effort to search for novel MRP1 inhibitors, we explored novel compound library known as cucurbitacin-inspired estrone analogs (CIEAs) as potential MRP1 inhibitors. CIEAs are natural products derived from cucurbitacins D, a class of

oxidized tetracyclic triterpenoids isolated from the plant family *Cucurbitaceae*. Cucurbitacins are well-known for their anticancer, antiproliferative and anti-inflammatory properties in many types of cancers [50-52]. The CIEAs were designed using estrone as starting steroidal scaffold targeting the EGFR and MAPK pathways involved in the proliferation activity of hepatocellular cancer [53]. In the present study, we focused on using various in vitro ABC transporter functional evaluation assays and in silico molecular docking studies to assess the effects of the CIEA compounds on MRP1 activity.

3.2 Materials and Methods

3.2.1 Chemicals

Doxorubicin, calcein red orange, poly-D-lysine and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Vincristine and MK571 were procured from Cayman Chemicals (Ann Arbor, MI). SN38 was purchased from MedChem Express (Monmouth Junction, NJ). Novel compound library consisting of 81 CIEAs were generously provided by Dr. Fathi Halaweish (South Dakota State University, Brookings, SD).

3.2.2 Cell lines and cell culture

MRP1 overexpressing cells, H69AR and its parental H69 were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and were cultured in RPMI 1640 (ATCC) medium. HEK293T cells were a kind gift from Dr. Adam Hoppe (South

Dakota State University, Brookings, SD) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare, Marlborough, MA). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone^{TM}, GE Healthcare Life Sciences). All cell lines were maintained at 37 °C in a humidified incubator supplied with 5% CO_2. H69AR cells were challenged with doxorubicin (0.8 μM) once monthly and cultured for 7 days drug-free before use.

3.2.3 Fluorescence-based transporter activity assay via high content screening

Assay development and optimization were conducted as previously reported [54]. The assay was performed on H69AR cells using doxorubicin and calcein red orange as fluorescent substrates. Briefly, cells were seeded onto a 96-well optical-bottom plate with polymer base (Thermo Fisher Scientific, Waltham, MA) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) at a final concentration of 6 x10^4 per well in 100 μL culture medium. After incubation overnight, media was replaced with 80 μL serum free media prior to treatment with test compounds and fluorescent substrates. Treatments were done using DMSO as negative control at 0.2% final concentration, MK571 as positive control at 50 μM final concentration and 10 μM final concentration for test compounds. For doxorubicin inhibition screening, cells were initially treated with test compounds or positive control for 30 min before incubation with doxorubicin (10 μM final concentration) for 2 h. For calcein red orange screening, cells were similarly treated with test compound or positive control for 30 min, calcein red orange (0.25 μM final concentration) was added and incubated for additional 30 min. Post incubation with fluorescent substrates, cells were rinsed once with PBS and loaded with 100 μL of PBS containing 10 mM HEPES and 4.5% glucose for analysis.

Fluorescent images were acquired using ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices, Sunnyvale, CA) equipped with a 0.70 numerical aperture 60x objective. Four images were acquired from each well in the Texas red channel for both fluorescent reporters with excitation and emission at 562/40 nm and 624/40 nm respectively, and exposure time set for 100 ms. A possible limitation of fluorescence experiments in 96-well plates is the gradual decrease in fluorescence across the plate due to drop in intracellular fluorescence over time. To account for this, negative and positive controls were included in every two columns across the plate. The screening assay was conducted in two independent experiments. Fluorescent intensity values from images were analyzed using MetaXpress software (version 5.10.41, Molecular Devices). The analysis involved segmentation of fluorescent objects in the Texas red channel by a custom application model built from the 'Find Blobs' module. The model distinguishes fluorescent accumulation against a background by applying segmentation masks depending on specifications set for object size.

3.2.4 Fluorescence accumulation assay by flow cytometry

Using the same fluorescent substrates (doxorubicin and calcein red orange) employed in the high content screening assay, we validated the inhibition activity of the identified hit compounds employing flow cytometry. H69 and H69AR cells at a density of 7 x10^5 were suspended in 1 mL serum free media and treated with negative control (DMSO at 0.2% v/v) or positive control (MK571 at 50 μM final concentration) or test compound (10 μM final concentration). For MRP1-mediated doxorubicin efflux, treatments were incubated for 1 h at 37 °C after which doxorubicin (10 μM final

concentration) was added and further incubated for 2 h. MRP1-mediated calcein red orange efflux assay involved pretreatment with negative, positive controls and test compounds for 10 min at 37 °C before addition of calcein red orange dye (0.25 μM final concentration) and further incubation for 30 min. After incubation, the reaction was ceased by addition of 3mL ice-cold PBS to each treatment. Cells were pelleted, washed twice with ice-cold PBS and then suspended in 1% paraformaldehyde (in ice cold PBS) for analysis. Accumulated intracellular fluorescence was determined using BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) equipped with 488 nm argon excitation laser. Doxorubicin and calcein red orange were both detected with > 670 nm band pass filter. Two independent experiments were conducted with each sample duplicated. For all samples, 10,000 events were collected and mean fluorescent intensities expressed as percentage of the control.

3.2.5 Doxorubicin localization assay using confocal microscopy

To further validate the inhibition activity of the identified compounds, we used confocal microscopy to qualitatively demonstrate the effect of the compounds on MRP1-mediated efflux of an anticancer drug. We elected to use doxorubicin as the common anticancer and fluorescent substrate of choice since the compounds identified as inhibitors using calcein red orange were also detected with doxorubicin in the high content screening. HEK293T cells at a density of 2.5×10^5 cells/well in 2 mL complete media were plated into a 6-well plate containing a poly-D-lysine coated (ethanol sterilized) cover glass and incubated at 37 °C. At 90 % confluency, cells were transiently transfected with an MRP1-GFP expression vector using jetPrime Transfection Reagent

(Polyplus – transfection SA, Illkirch, France) according to manufacturer's protocol. After 48 h, cells were treated with negative control (DMSO at 0.2% v/v) or positive control (MK571 50 μM final concentration) or test compound (10 μM final concentration) for 30 min, and then with doxorubicin (10 μM) for 1 h. Prior to microscopic analysis, cells were maintained in a buffer (4.5% glucose, 10mM HEPES and PBS) containing Ca^{2+} and Mg^{2+}. MRP1 and doxorubicin localization were imaged using an iMIC digital microscope (TILL Photonics GmbH, Gräfelfing, Germany) equipped with a 1.35 numerical aperture 60x oil-immersion objective. Both GFP and doxorubicin were excited at 445 nm with emission attained at 537/26 nm and 605/64 nm respectively. Images were processed using ImageJ (NIH, Bethesda, MD).

3.2.6 Drug sensitivity studies

The identified MRP1 inhibitors were investigated for their ability to re-sensitize multidrug resistant cells to chemotherapeutic drugs using MTT colorimetric assay. H69 and H69AR cells were plated in a 96-well plate (NEST®, Rahway, NJ) at a concentration of 2.5 x 10^4 cells/well in 100 μL culture medium. The next day, 50 μL of medium containing test compounds at non- cytotoxic concentrations were added to cells 1 h prior to treatment with 50 μL of the chemotherapeutic drugs (vincristine and SN38) at varying concentrations. After incubation for 96 h, 100 μL of culture medium was removed and cells were treated with MTT dye (5 mg/ml in PBS) for 4 h. The MTT formazan product was dissolved in 100 μL 15% SDS containing 10 mM HCl. Cell viability was determined by measuring absorbance at 570 nm with a plate reader, Hidex Sense Beta Plus (Turku, Finland).

3.2.7 Immunoblot analysis

H69 and H69AR cells were seeded in a 6-well plate at 1 x 10^6 cell density in 2 mL culture medium per well. After 24 h, cells were treated with or without 10 µM test compounds. H69 was used as negative control cell line. Cell lysis was performed in radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific, Waltham, MA) containing 1x Halt Protease Inhibitor Cocktail after 48 h. Lysate protein concentrations were determined using Pierce bicinchoninic acid (BCA) Protein Assay. Proteins at 20 µg were subjected to electrophoresis on 8.0 % sodium dodecyl sulfate polyacrylamide gels before being transferred to PVDF membranes (Immobilon® Millipore, Burlington, MA). Membranes were blocked prior to antibody treatments with rabbit monoclonal anti MRP1 antibody [EPR21062] (1:250; Abcam, ab233383) and anti α-tubulin antibody (1:5000; Sigma-Aldrich) and incubated overnight at 4 °C. This was followed by treatment with horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) (1:1000; ThermoFisher Scientific) secondary antibody for 1 h at room temperature for both MRP1 and α-tubulin. MRP1 proteins were developed using Western Blotting Luminol reagent (Santa Cruz Biotechnology, sc-2048) and immunoblots visualized with an LI-COR Odyssey Fc Imaging System. Resulting protein bands were analyzed with Image Studio Lite version 5.2 (LI-COR Biotechnology, Lincoln, NE), and the correction for uneven sample loading and transfer achieved by applying the relative bands for the loading control (α-tubulin). Three independent experiments were conducted.

3.2.8 Molecular docking studies

The identified inhibitors were subjected to in silico molecular docking with MRP1 protein structure. The structures of the inhibitors (ligands) were prepared for docking through energy minimizations using MMFF94 force field application followed by ligand conformations utilizing OMEGA application. The binding pocket of the receptor, bovine MRP1 (PDB code: 5UJ9) was generated via OpenEye software and docking was conducted using FRED (version 2.5.1.4). Docked poses were visualized using Vida application (version 4.1.2).

3.2.9 Fluorescence spectroscopic analysis

Membrane vesicles were prepared from HEK293 cells stably transfected with GFP- MRP1 (donor control containing only GFP) or a two-color (GFP and RFP) MRP1 plasmids. Steady-state fluorometry using experimental conditions as previously described [55] with slight modifications was performed to determine the intramolecular fluorescence resonance energy transfer (FRET) changes of apo (ligand-free) and ligand-bound MRP1. Briefly, 10 µg MRP1- enriched membrane vesicles in Tris sucrose buffer (250 mM Tris and 50 mM sucrose; pH 7.4) initially incubated for 2 min in a 37 °C water bath were treated with 10 µM of test compounds and re- incubated for 10 min. Fluorescent spectroscopic measurements were carried out using a 50-uL quartz glass cuvette with the Fluorometer model FL3-11 (HORIBA Edison, New Jersey). GFP excitation and emission was achieved at 465 nm and 480–650 nm respectively, with excitation and emission slit widths of 5 nm. The GFP-MRP1 (donor) emission scan was

collected whiles monitoring quenching of the emission peaks from the two-color MRP1 (acceptor) in the absence or presence of a ligand. Emission scans were collected every 3 s at 5 nm interval over a period of 10 min. FRET efficiency was calculated using the following equation:

$$1 - (\frac{I_{DA}}{I_D})x\ 100$$

where I_D and I_{DA} represent the intensities (counts per second, cps) of the donor and acceptor fluorophores, respectively. The transfer efficiencies of compound-induced samples were normalized with that of the apo condition to obtain the percent change in FRET.

3.2.10 High content fluorescence inhibition screening data analysis

Analysis of fluorescence inhibition was performed with MetaXpress software. For each well, four images were captured. Using segmentation masks to subtract background fluorescence, the mean fluorescent intensity of each well was determined from the average fluorescent values of the four images. The relative efflux inhibition of the fluorescent substrates (doxorubicin or calcein red orange) by each test compound was determined for each well using the following equation:

$$\%\ inhibition = \frac{X_T - X_{fluorescent\ substrate}}{X_{MK571} - X_{fluorescent\ substrate}}\ x\ 100$$

with X representing the average fluorescence intensities of the test compound (T). Positive and negative controls were placed in every two columns and used for the

determination of the percent inhibition for compounds within the same column. The assay quality and validation were assessed using Z'-factor and calculated as follows:

$$Z' - factor = 1 - \frac{3(\sigma_{positive\ control} + \sigma_{fluorescent\ substrate})}{|\mu_{positive\ control} - \mu_{fluorescent\ substrate}|}$$

where σ and μ represents standard deviations and means, respectively.

3.2.11 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.4.3 software (GraphPad Software, La Jolla, CA). Differences between values were analyzed by applying linear mixed model and Dunnett correction used for multiple comparisons. Significant differences were considered at p-value less than 0.05.

3.3 Results

3.3.1 Fluorescence imaging-based high-content screening of CIEA library for MRP1 inhibitors

A high content fluorescence inhibition screening assay originally developed in our lab was used to screen a library of 81 novel CIEA compounds to identify inhibitors for MRP1 using doxorubicin and calcein red orange fluorescent substrates which our lab have also established as suitable for identifying inhibitors for MRP1. The 2D scatter plots represent the relative percent inhibition by the test compounds on MRP1-mediated efflux of doxorubicin and calcein red orange respectively (Figures 3.1A and 3.1B) determined from two independent experiments. The Z'-factor which demonstrates the assay

performance and reproducibility obtained for doxorubicin and calcein red orange inhibition assays were 0.50 and 0.59 respectively. The screening was conducted on doxorubicin resistant lung cancer cell line, H69AR. A positive hit was defined as a compound with mean percent inhibition of at least 45 % from the two experiments. MK571 (50 μM) was used as positive control baseline (100 % inhibition) for determining the relative percent inhibition for the test compounds (10 μM). Five compounds were identified as inhibitors of MRP1-mediated doxorubicin efflux and three compounds identified as calcein red orange efflux inhibitors, including 2 compounds (HL 15 and HL 21), which were also identified from the doxorubicin inhibition assay (Table 3.1). From the hit compounds obtained from both screening assays, four compounds (HL 15, HL 21, HL 38 and HL 53) with chemical structures shown in Figure 3.2) were selected for further characterization.

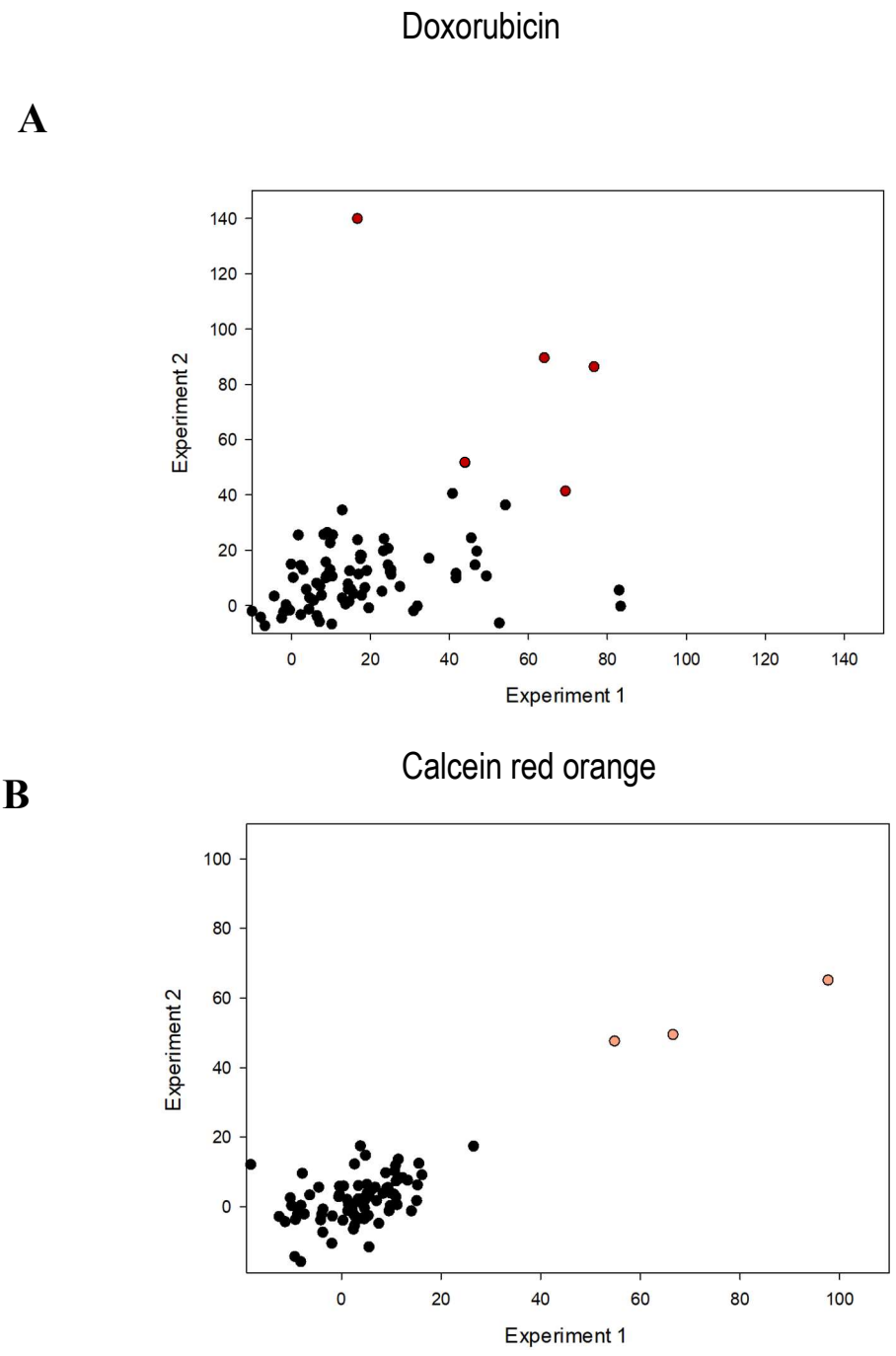


Figure 3.1. Performance of the high content fluorescence-based MRP1-mediated doxorubicin and calcein red orange inhibition screening assay. Screening of a library of 81 CIEA compounds was conducted in two independent experiments at a compound

concentration of 10 μM using doxorubicin (A) and calcein red orange (B) as fluorescent substrates. The 2D plot displays the calculated percentage inhibition of compounds in the two experiments. Red and Orange dots denote compounds with mean percentage inhibition of $\geq 45\%$ for doxorubicin and calcein red orange, respectively. 2D scatter plots were generated using Sigma Plot 12.0.

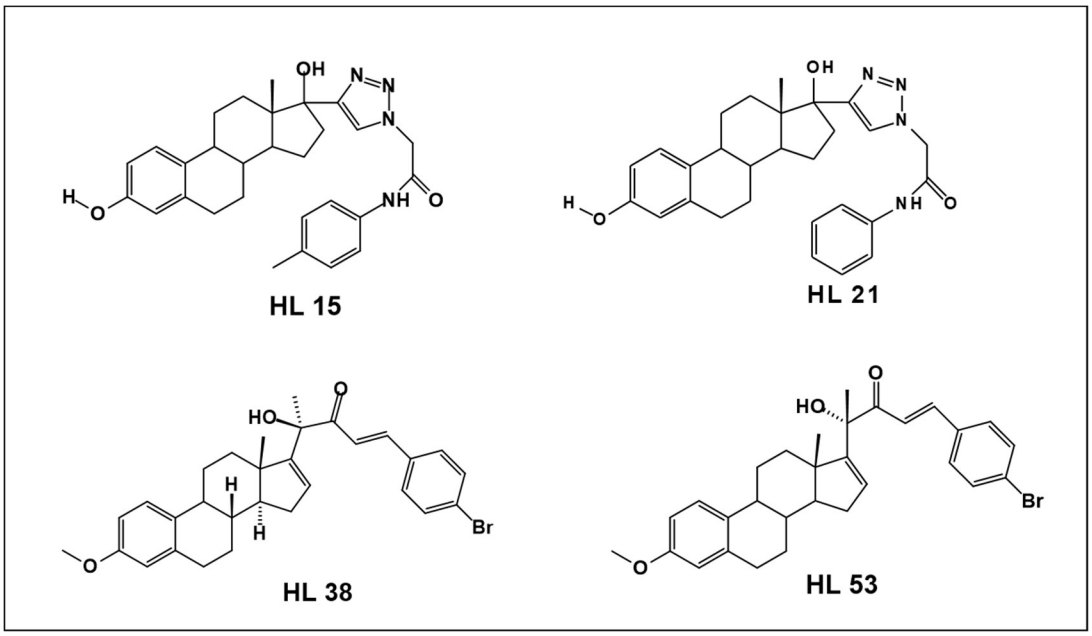


Figure 3.2 Chemical structures of 4 selected CIEA compounds identified as hits from both doxorubicin and calcein red orange screening assays.

Table 3.1 Percentage doxorubicin and calcein red orange control inhibition for identified MRP1 inhibitors

Compound	% Doxorubicin control inhibition [a]	% Calcein Red Orange control inhibition [a]
MK571	100.0 ± 0.0	100.0 ± 0.0
HL 10	78.3 ± 61.5	
HL 15	55.4 ± 14.1	58.0 ± 8.6
HL 21	45.2 ± 8.9	81.4 ± 16.4
HL 27		51.2 ± 3.7
HL 38	81.5 ± 4.7	
HL 53	76.8 ± 12.7	

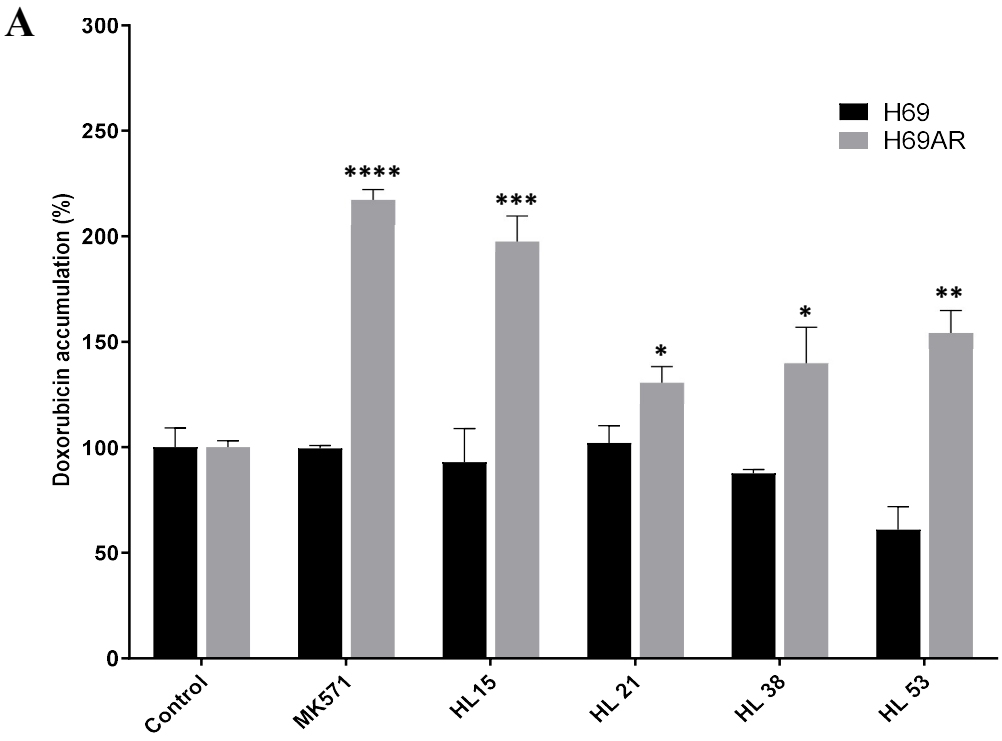
[a] Mean ± SEM of n = 2 independent experiments

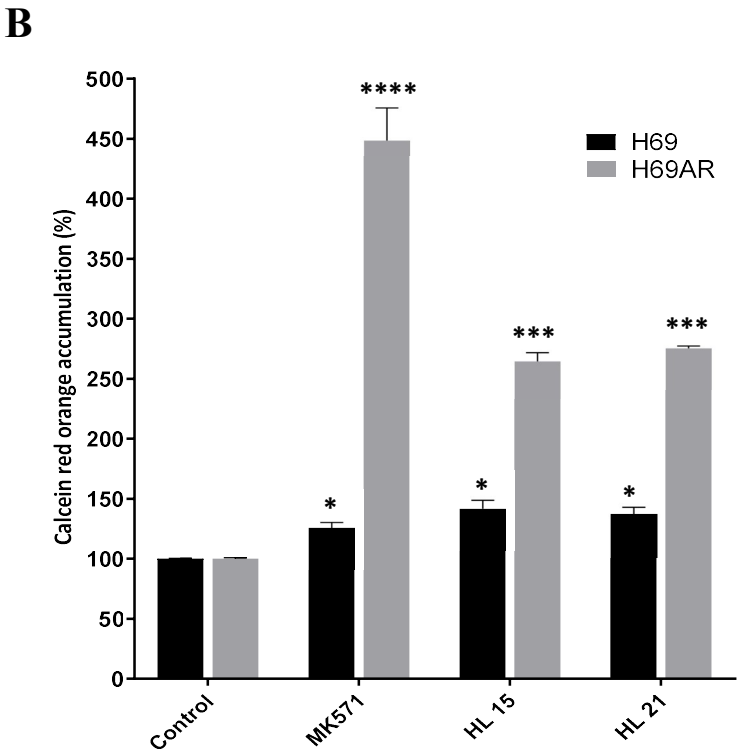
3.3.2 Validation of identified CIEA hit compounds

The selected compounds were validated for their inhibition activity on MRP1 function by employing two well-established ABC transporter functional assays. First, the compounds effect on MRP1 efflux of doxorubicin and calcein red orange were determined using flow cytometry. H69 and H69AR cells were incubated with the test compounds and the fluorescent substrates at specific time points (as indicated in the Materials and Methods section). As shown in Figure 3.3A, all the identified compounds (10 μM) significantly inhibited MRP1-mediated efflux of doxorubicin and thus enhanced doxorubicin accumulation in drug resistant H69AR cells but had no significant effect on H69, the drug sensitive cell line. The positive control MRP1 inhibitor, MK571 (50 μM) increased intracellular levels of doxorubicin by 2.2 fold. Amongst the compounds, HL 15

was the most effective doxorubicin efflux inhibitor, increasing doxorubicin accumulation by ~ 2.0 fold. Modest doxorubicin accumulations (1.3-1.5 fold enhancement) were observed for compounds HL 21, HL 38 and HL 53. From the results of the calcein red orange accumulation assay (Figure 3.3B), treatment of H69AR cells with HL 15 and HL 21 also enhanced intracellular calcein red orange levels by ~2.7 fold although MK571 (50 μM) was seen to increase calcein accumulation significantly by 4.5 fold. The test compounds on the other hand, showed only weak modulatory effect in H69 cells.

In the next validation assay, the effects of the compounds on MRP1-mediated efflux of doxorubicin in live cells were qualitatively assessed using confocal microscopy. This is because doxorubicin identified more hit compounds amongst which 2 compounds were also identified with calcein red orange in the high content screening assay. An MRP1-GFP encoding vector was transiently transfected into HEK293T cells. After cells were treated with controls or test compounds, localization of doxorubicin in the nucleus was visualized. As shown in Figure 3.3C, the nuclei of non-transfected cells in the control showed high accumulation of doxorubicin whiles doxorubicin was not detectable in MRP1-GFP transfected cells. Treatment with MK571 (50 μM) restored doxorubicin localization in MRP1-GFP expressing cells. Compounds HL 15, HL 21, HL 38 and HL 53 all enhanced doxorubicin accumulation in cells expressing MRP1 similar to MK571.


A
Doxorubicin accumulation (%)
300
250
200
150
100
50
0
H69
H69AR
****
***
*
*
**
Control
MK571
HL15
HL 21
HL 38
HL 53


B
Calcein red orange accumulation (%)
500
450
400
350
300
250
200
150
100
50
0
H69
H69AR
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Control
MK571
HL 15
HL 21

C

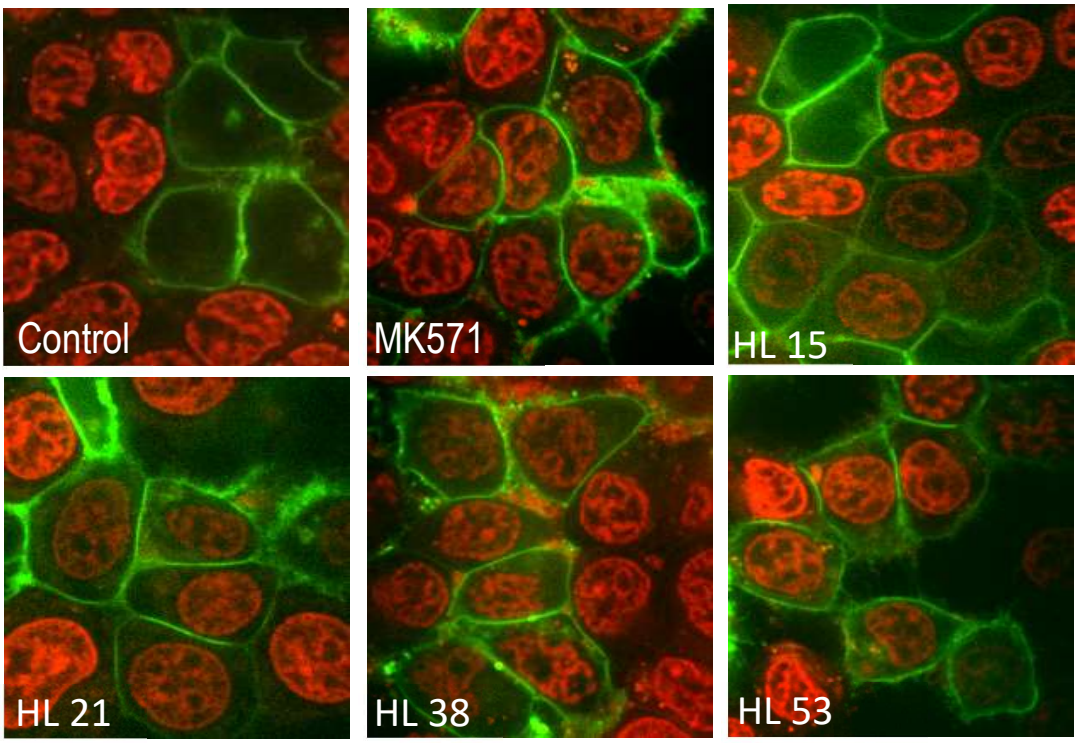

Figure 3.3 Validation of identified compounds inhibitory action on MRP1 efflux activity. (A) Doxorubicin accumulation assay. H69 and H69AR cells were pre-treated with 50 μM MK571 or test compounds for 2 h before incubation with 10 μM doxorubicin at 37 °C for 2 h. Fluorescence intracellular doxorubicin accumulation was measured using flow cytometry, with excitation and emission wavelengths of 480 and 670 (LP) nm, respectively. (B) Calcein red orange accumulation assay. H69 and H69AR cells were pre-treated with 50 μM MK571 or test compounds for 10 min before incubation with 25 nM calcein red orange at 37 °C for 30 min. Intracellular calcein red orange accumulation was quantified using flow cytometry, with excitation and emission wavelengths of 480 and 585/40 nm, respectively. (C) Doxorubicin localization assay. An MRP1-GFP expression vector (green) was transiently transfected into HEK293T cells. After 48 h, cells were

incubated with 10 μM test compound for 30 min and then with 10μM doxorubicin (red) for 1 h. Images were obtained using confocal microscope. GFP and Doxorubicin were excited at 488 nm with emission bands of 537/26 nm and 605/64 nm, respectively.

3.3.3 Effects of the identified MRP1 inhibitors (CIEAs) on the efficacy of anticancer drugs

MTT colorimetric assay was used to evaluate the ability of the identified MRP1 inhibitors (CIEA compounds) to reverse resistance of MRP1 overexpressing cancer cells (H69AR) to selected anticancer agents. The cytotoxic effect of the test compounds on H69AR cells were first analyzed to select compound concentrations that did not significantly affect cell viability (data not shown). The non-cytotoxic compound concentrations were used in combination with various concentrations of vincristine and SN38, prominent MRP1 substrates. Presented in Table 3.2 are the IC_{50} and fold resistance values of the drug resistant cell line, H69AR and its control parental cell line, H69 treated with vincristine and SN38 in the presence or absence of test compounds. As expected, MRP1 overexpressing H69AR cells showed higher resistance to vincristine (Figure 3.4A, red solid line) and SN38 (Figure 3.4B, red solid line) alone compared to H69 (green dash line) and produced resistance folds of 12.3 and 206.4 respectively. Addition of positive inhibitor, MK571 (10 μM) lowered the resistance folds of H69AR cells to vincristine and SN38 from 12.3 to 5.2 fold and 206.4 to 69.5 fold respectively. Compounds HL 15, HL 21 and HL 38 re-sensitized H69AR cells to vincristine by reducing resistance from 12.3 fold to the range of 8.2 to 9.6 fold. In contrast, HL 53 did not affect the sensitivity of H6AR to vincristine. All of the test compounds significantly reversed resistance of

H69AR to SN38 by reducing resistance fold from 206 to 60 -100 fold range. However, HL 15 and HL 38 were the most potent sensitizing H69AR to SN38, reducing resistance fold from 206.4 to 60.6 and 78 fold respectively.

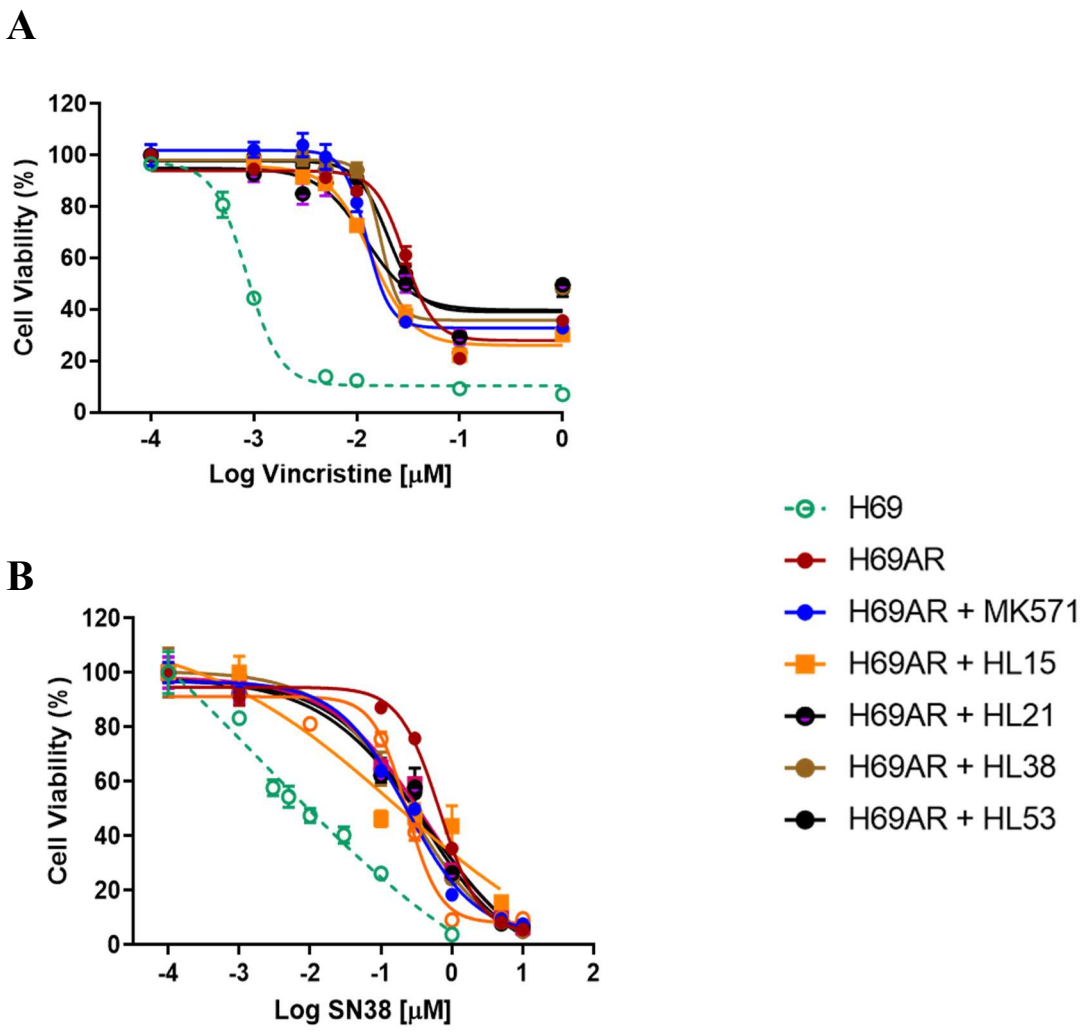

Figure 3.4 Reversal of drug resistance in H69AR cells by the identified MRP1 inhibitors towards anticancer substrates. H69 and H69AR cells were treated with vincristine (A) and SN38 (B) at increasing drug concentrations in the absence or presence of MK571 and test compounds for 72 h . MK571 concentration was at 10 µM; HL 15, HL 38 and HL 53 were at 2 µM while HL 21 concentration was at 0.4 µM. Cell viability was evaluated

using MTT colorimetric assay. Data are representative of at least three independent experiments and presented as mean ± SD.

Table 3.2 The effects of identified MRP1 inhibitors on the $1C_{50}$ values of vincristine and SN38 in H69 and H69AR cells

Cell line/Treatment	**IC_{50}[a] (nM)**			
	Vincristine	Fold resistance[b]	SN38	Fold resistance[b]
H69	1.90 ± 0.16	1.00	3.57 ± 1.28	1.00
H69AR	23.35 ± 5.92	12.30	736.40 ± 61.62	206.41
H69AR + MK571 [10	9.91 ± 3.11	5.22	247.80 ± 19.85	69.46
H69AR + HL 15 [2 μM]	16.69 ± 1.99	8.74	216.27 ± 58.12	60.62
H69AR + HL 21 [0.4 μM]	15.56 ± 3.05	8.20	631.67 ± 139.21	101.37
H69AR + HL 38 [2 μM]	18.12 ± 1.11	9.55	278.30 ± 78.24	78.01
H69AR + HL 53 [2 μM]	24.92 ± 4.95	13.13	357.03 ± 25.54	100.07

[a] Mean ± SD of at least three independent experiments performed in triplicates.
[b] Fold resistance determined by dividing the IC_{50} value for each treatment by the IC_{50} value of H69 cells treated with vincristine or SN38 alone.

3.3.4 The effects of identified CIEA hits on MRP1 protein expression

We assessed whether the inhibitory action demonstrated by the identified compounds had any effect on MRP1 expression at the protein level. Immunoblot analysis (Figure 3.5A- B) conducted on MRP1 overexpressing H69AR cells showed that the identified CIEA compounds (10 μM) have no significant effect on MRP1 protein expression.

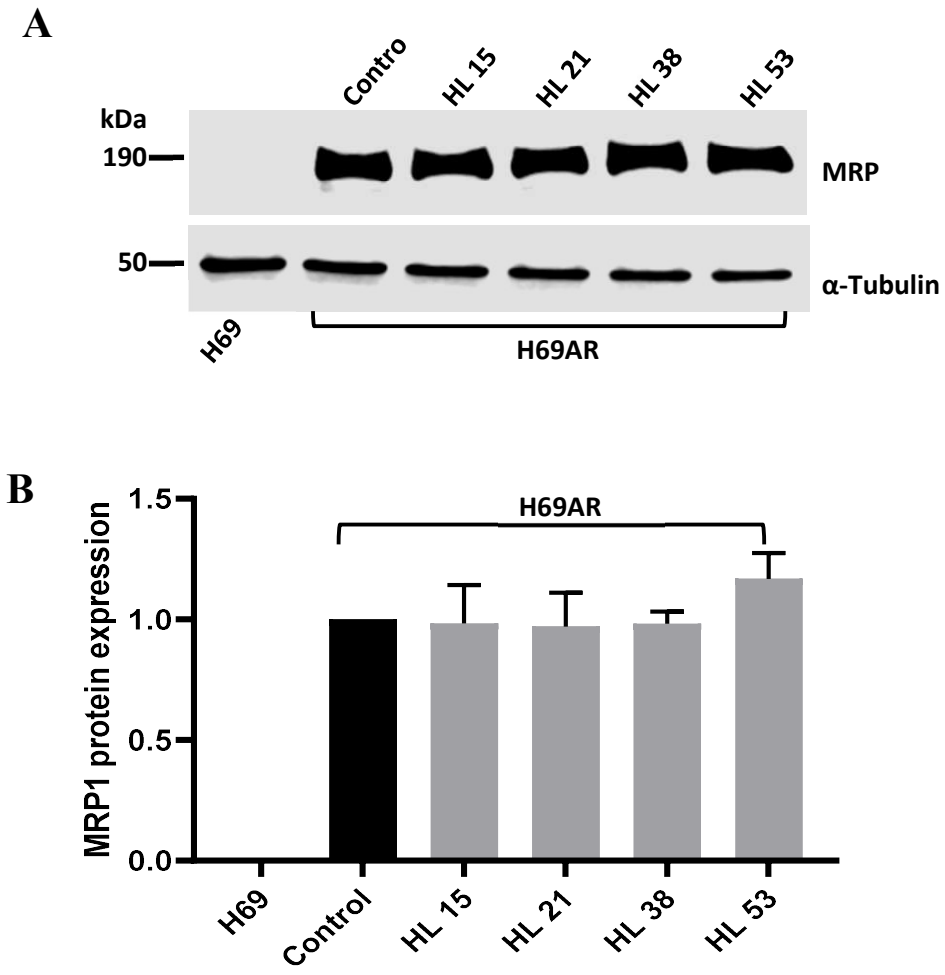

Figure 3.5 The effect of CIEA compound inhibitors on MRP1 protein expression. (A) Representative immunoblot of whole cell lysates prepared from H69AR cells treated with or without 10 μM test compounds for 48 h. (H69 cells were used as negative control). MRP1 proteins were detected using goat-anti-rabbit IgG (H+L) (1:1000) and confirmation of equal protein loading detected with anti α-tubulin antibody (1:5000). (B) Protein band densities were quantified using Image Studio Lite (LI-COR Biotechnology, Lincoln, NE) software with uneven sample loading and transfer corrected per individual protein band relative to α-tubulin. Results are representative of at least 2 independent experiments.

3.3.5 Molecular docking analysis of the identified inhibitors with bovine MRP1

The identified MRP1 inhibitors were docked with bovine MRP1 (91% sequence identity with human MRP1 protein) Cryo-EM structure using MK571 as reference inhibitor. The best-scored poses of the compounds are represented in Figures 3.6-3.8. The hydroxyl group at carbon 17 and the triazole moiety in HL 15 formed hydrogen bond interactions with ARG:593:A residues of bovine MRP1 (Figure 3.6B). In addition, HL 15 showed equal consensus score (Table 3.3) with MK571 suggesting that both compounds may have similar binding affinities towards MRP1. Compound HL 21 hydrogen bonded with ASN:1244:A residues in MRP1 however with a higher binding affinity (consensus score: 8) than MK571. Compounds HL 38 and HL 53, all showed lower consensus scores (6) than the reference compound though with no observable hydrogen bond interactions.

Table 3.3 Molecular docking consensus scores, molecular weights and H-bonding interactions for identified MRP1 inhibitors (CIEA analogs). MK571 used as reference drug.

Compound	**Mol. Wt.**	**Consensus score**	**Target H-bonding residues**
MK571	454.602	10	-
HL 15	424.572	10	ARG: 593:A
HL 21	486.605	8	ASN:1244:A
HL 38	538.637	6	-
HL 53	562.701	6	-

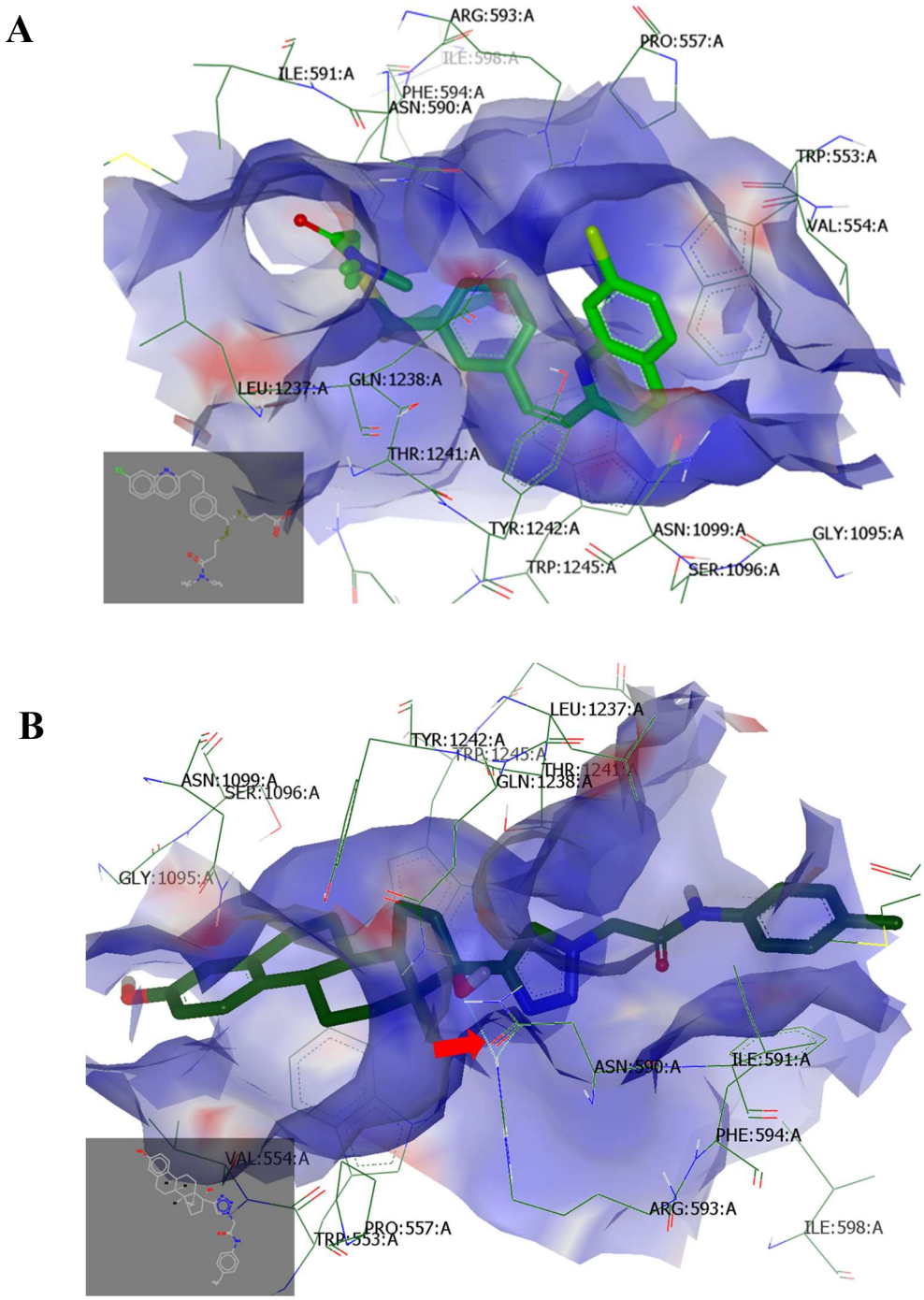

Figure 3.6 Dock poses for (A) MK571 and (B) HL 15 within the binding pocket of bovine MRP1 (PDB ID: 5UJ9). Red arrows indicate location of hydrogen bonds (green dashed lines).

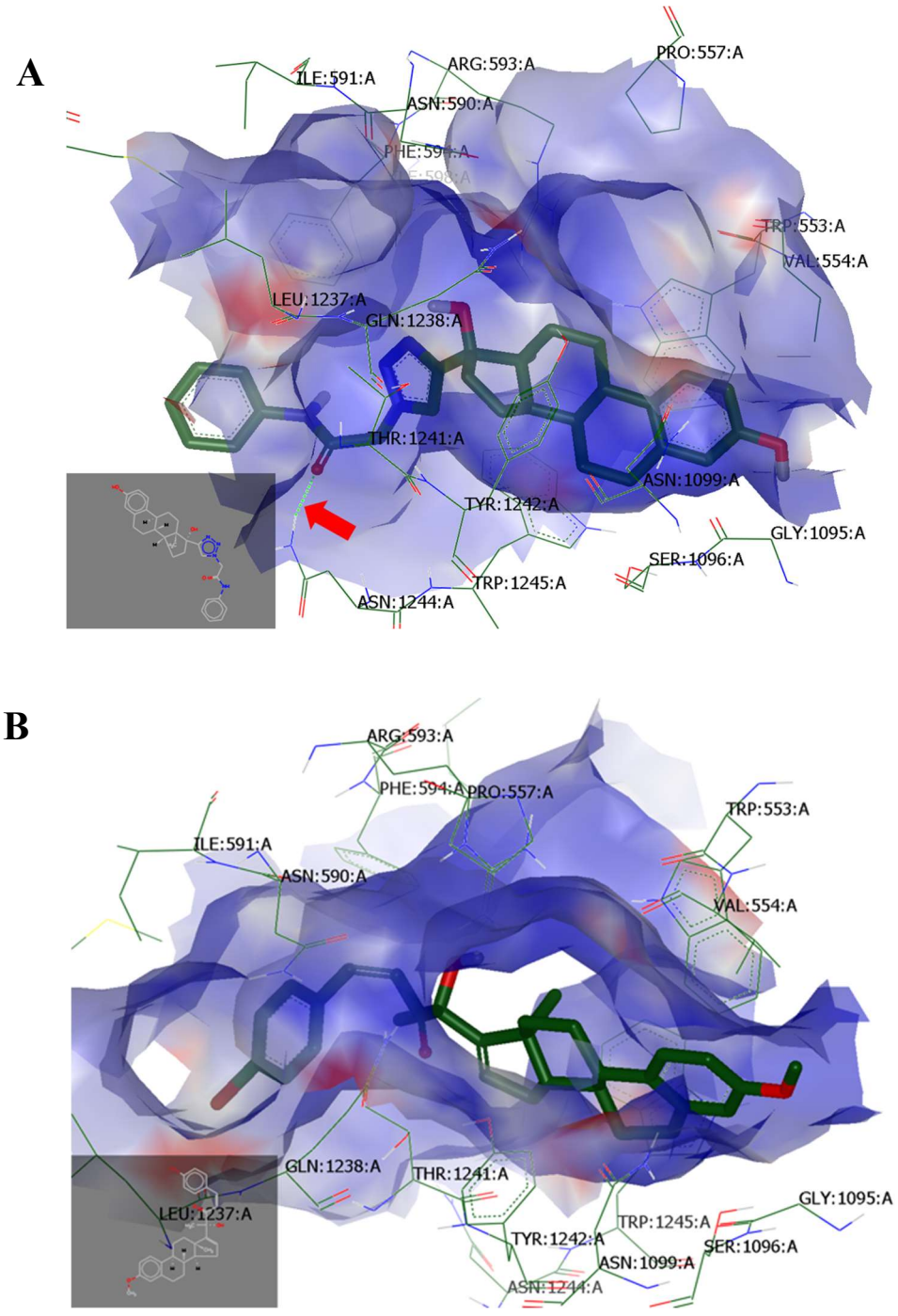

Figure 3.7 Dock poses for (A) HL 21 and (B) HL 38 within the binding pocket of bovine MRP1 (PDB ID: 5UJ9). Red arrows indicate location of hydrogen bonds (green dashed lines).

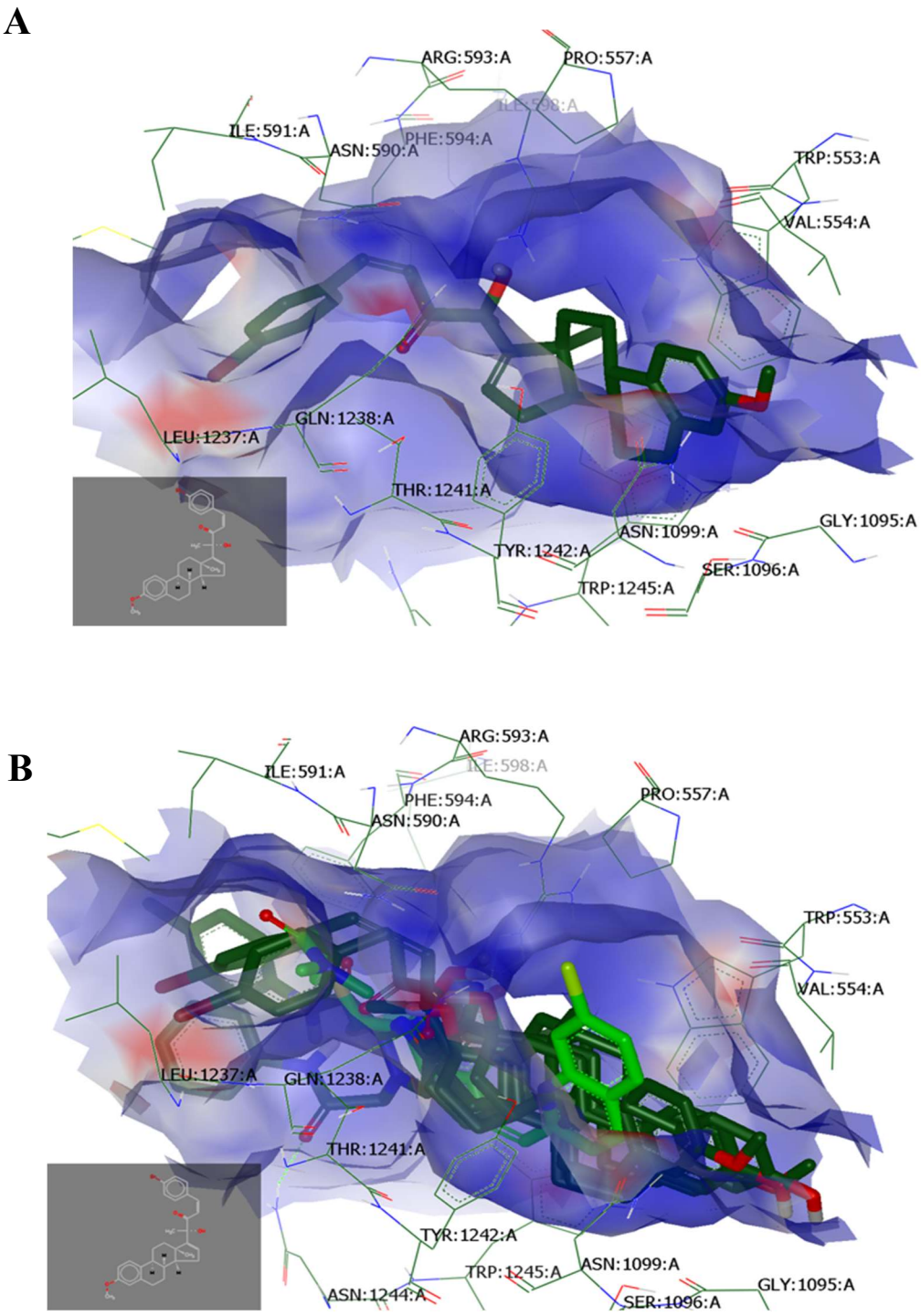

Figure 3.8 Dock poses for (A) HL 53 and (B) Overlay of all compounds along with MK571 (green) within the binding pocket of bovine MRP1 (PDB ID: 5UJ9).

3.3.6 Identified CIEA hits interaction with MRP1

To determine if the identified MRP1 inhibitors directly interact with MRP1 protein, we employed a novel structural-based approach [56]. This approach makes use of two-color recombinant protein engineered from fusing a GFP and RFP with MRP1 protein structure. The two-color MRP1 protein can potentially detect the direct interaction of ligands with MRP1 and determine the intramolecular FRET changes as an indicator of conformational changes as a result of ligand binding to MRP1 [57]. Here, fluorescence spectroscopic analysis was used to measure the changes in FRET efficiency that results upon interaction of the CIEA compounds with the two-color MRP1 protein. MK571 was used as positive control and JNJ-26854165, which our group has previously reported as showing no interaction with MRP1 [58] was used as the negative control. The representative fluorescence spectra of the donor control (containing only GFP) and the apo (ligand-free) and ligand-bound (test compound) with two-color MRP1 is shown in Figure 3.9A. From the FRET results, (Figure 3.9B), all the identified CIEA compounds showed an increase in FRET efficiency relative to the apo condition indicating that they directly interact with MRP1. Among the compounds, HL 15 showed the highest FRET change (~7%) while HL 21 induced the smallest change (~3%). As expected, MK571 interacted with MRP1, while JNJ-26854165 yielded no significant FRET change relative to the apo FRET condition.

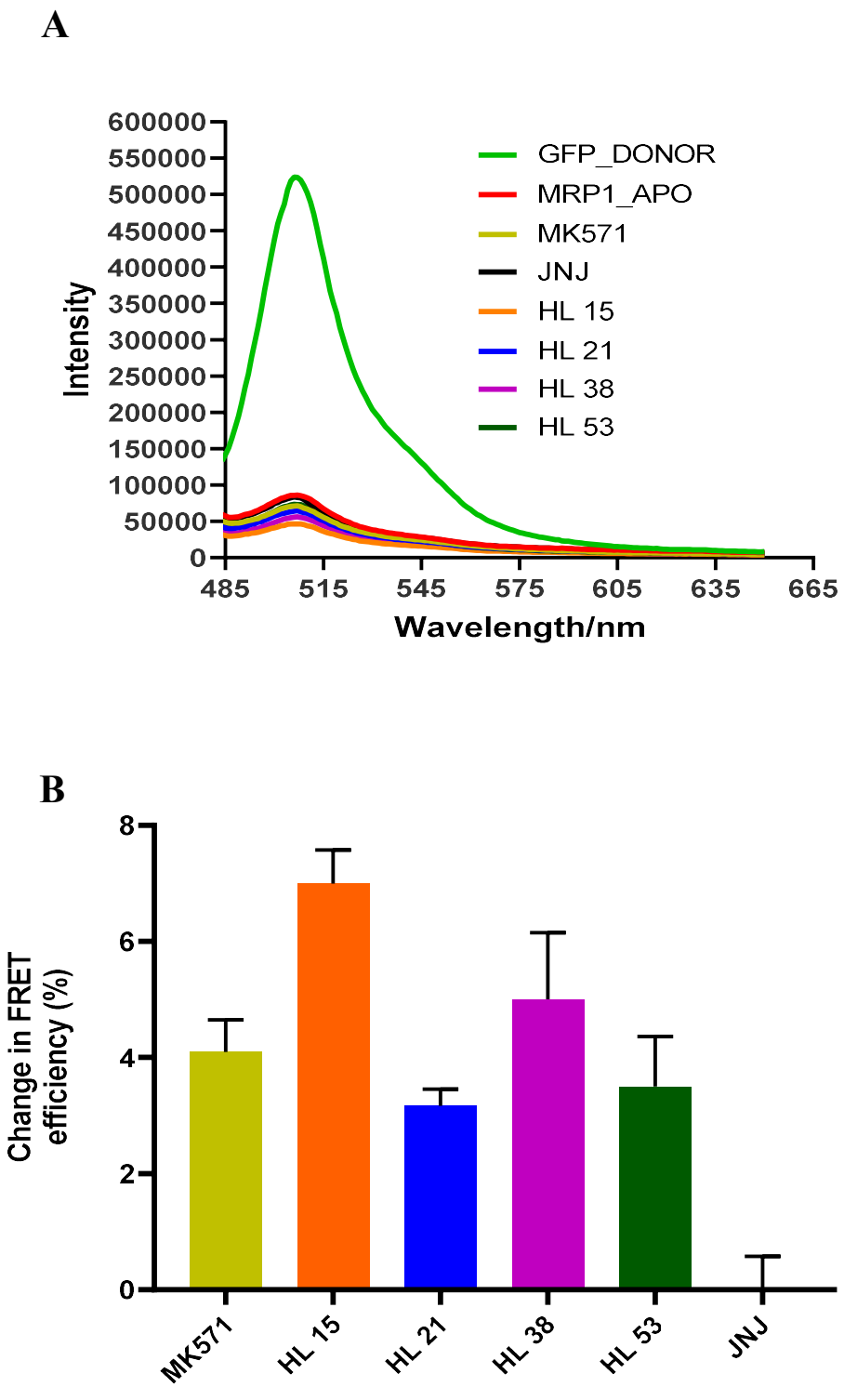

Figure 3.9 Compound induced intramolecular FRET measurements. (A) Representative fluorescence spectra of apo (ligand -free) and ligand –bound (test compounds) of two-

color MRP1 protein (MRP1-GFP was used as donor control). (B) Compound-dependent FRET changes with two-color MRP1 protein. MRP1 membrane vesicles (10 μg) in Tris–sucrose buffer was prepared and incubated with 10 μM of test compounds for 10 min prior to FRET measurements using the fluorometer. GFP and RFP excitations were achieved at 465 nm and 530 nm, respectively. Emissions for both GFP and RFP with integration time of 3 s were recorded at 480–650 nm. Data are representative of two independent experiments performed in triplicates. MK571 and JNJ-26854165 were used as positive and negative controls, respectively. Compound induced FRET efficiencies were normalized with the apo FRET to obtain the percent change in FRET. Results are presented as mean ± SEM.

3.4 Discussion

Evidence have shown the development of MDR by cancer cells against various anticancer agents as the chief obstacle to successful chemotherapy and this has been notably linked to overexpressing of ABC drug efflux transporters. Most cancers that overexpress MRP1 have poor prognosis and patients have low survival rates due to the ability of MRP1 to efflux many first line chemotherapeutic drugs including vincristine, doxorubicin and etoposide. For mostly hematological malignancies such as acute myeloblastic leukemia and acute lymphoblastic leukemia, MRP1 overexpression has been reported as the major determinant limiting the efficacy of chemotherapeutic agents. [25, 30, 59]. Furthermore, studies have indicated that genetic variations in MRP1 such as common polymorphisms may significantly increase drug resistance and disease vulnerability [21, 60, 61]. Therefore the relevance of MRP1 in predicting patience

outcome is a major concern in tumor management in clinical oncology. Clearly, inhibition or downregulation of MRP1 is crucial for favorable therapeutic outcomes in cancer treatment. Notwithstanding, various small molecular entities targeting MRP1 activity have been developed with generally organic anion transport inhibitors (e.g. probenecid, sulfinpyrazone, indomethacin), tyrosine kinase inhibitors (TKIs) and some P-gp inhibitors among the leading MRP1 inhibitors. Although various in vitro evaluations of these inhibitor compounds and their pharmacokinetic assessments with different anticancer agents are still ongoing, motivation for these MRP1 inhibitors to go into clinical trials has almost certainly faded due to setbacks (toxicity and zero clinical benefits) experienced with P-gp inhibitors [62]. Nonetheless, new drug entities are continually being discovered in the hopes of finding the 'one' MRP1 inhibitor.

Presently, there is growing interest in exploiting natural products as new entities for drug design due to the less toxic and potent anticancer properties of natural derivatives. In the present study, we investigated the ability of the natural product-derived cucurbitacin-inspired estrone analogues, CIEAs, as potential MRP1 inhibitors. Firstly, we demonstrated inhibition of MRP1 by these analogue compounds using a high content fluorescence-based transport activity screening assay already developed and validated in our lab. The MRP1 substrates, doxorubicin and calcein red orange were used as fluorescent reporters for detection of MRP1 efflux inhibition by the CIEA compounds in MRP1-overexpressing H69AR cell line. Performing two independent screenings on 81 CIEA compound library, the assay discovered 5 compounds as possible inhibitors of MRP1 against doxorubicin efflux and 3 compounds against calcein red orange efflux. Two compounds, HL 15 and HL 21 were detected as inhibitors for both doxorubicin and

calcein red orange MRP1-mediated efflux suggesting that these two compounds may have common overlapping binding sites for both MRP1 fluorescent substrates. This result is also consistent with several biochemical and structural studies demonstrating MRP1's ability to recognize a broad spectrum of substrates as a result of its multiple distinct substrate binding sites [63]. The assay yielded average Z'-factors of 0.50 and 0.59 for doxorubicin and calcein red orange respectively which prove the fitness and effectiveness of the high content screening assay for detecting cell-permeable, non-toxic and potent inhibitors in live cells.

Subsequently, to understand the mechanism of the CIEA compounds on MRP1-mediated MDR attenuation, and also confirm the high content screening results, we performed drug accumulation studies. Our results indeed showed that the compounds inhibit MRP1 efflux activity by increasing the intracellular concentrations of doxorubicin and calcein red orange in MRP1-overexpressing H69AR cells (Figure 3.3A and 3.3B). Visualization of MRP1-mediated efflux of doxorubicin in live cells using confocal microscopy also validated the inhibitory action of the CIEAs on MRP1 function.

The ability of an MRP1 inhibitor to reverse multidrug resistance in cancer cells could be very valuable in combinatorial treatment of tumors with especially high MRP1 expression levels. In the present study, we were interested to know if the CIEA compounds were able to reverse MRP1-mediated resistance of common chemotherapeutic drugs, vincristine and SN38. However, to prevent the cytotoxic action of the CIEA compounds on the reversal outcome, we first obtained a dose-dependent response of the CIEA compounds on the selected cell line. We then selected the non-cytotoxic compound concentrations for the reversal studies. Three of the CIEA

compounds significantly decreased resistance of H69AR cells towards vincristine. The compounds, HL 15, HL 21 and HL 38 reduced the IC_{50} value of the resistant H69AR cells towards vincristine from 23.4 to 16.7, 15.5 and 18.1 nM, respectively. Moreover, HL 53 did not show any sensitization of H6AR cells towards vincristine. On the other hand, all 4 compounds, HL 15, HL 21, HL 38 and HL 53 reversed resistance of H69AR towards SN38 by reducing IC_{50} value of H69AR resistant cells from 736.4 to 216.2, 631.7, 278.3 and 357.0 nM respectively. Among the 4 compounds, HL 15 was the most potent in reversing resistance of H69AR towards both vincristine and SN38. Interestingly, HL 21 was very effective in decreasing resistance of H69AR against vincristine but was only modest in sensitizing H69AR cells to SN38. The results suggest that substrate selective inhibition of MRP1 by the CIEA compounds may be crucial in considering them for combinatorial therapy with chemotherapeutic agents for MDR reversal. However, to provide a convincing evidence for the purpose stated, it is necessary to investigate their chemosensitization effect in vivo. In addition, it is worth to mention that, the reversal studies of these compounds be replicated with different chemotherapeutic agents or different MRP1 resistant cell lines to deeply elucidate their MDR reversal mechanisms. Further reversal studies could also be performed on other ABC transporter MDR cell lines to ascertain whether the CIEA compounds reversal capabilities are exclusive to MRP1.

The circumvention of MDR mediated by ABC transporters may be as a result from down-regulation at the protein level. We tested this hypothesis by performing Western blotting assay. Our results indicated that, the CIEA compounds have no effect on MRP1 protein expression. The results may also suggest that the mechanisms of

inhibition and reversal of MRP1 MDR by the CIEA compounds are independent of MRP1 protein down-regulation. This could also mean the possibility of the CIEA compounds to inhibit MRP1 activity may be dependent on other protein markers and/or cross talk with other cell signaling pathways, which may directly or indirectly affect the function of MRP1. To better elucidate their mechanism of action, time and concentration-dependent studies should be conducted and also various signaling pathways probed especially those involved in the EGFR route since this receptor is the main pharmacological target of the CIEA compounds.

To confirm our belief that the CIEA compounds are MRP1 inhibitors and also understand their binding interaction with MRP1 protein, we conducted molecular docking studies. The results predicted that the CIEA compounds have better binding affinities towards MRP1 protein than the known MRP1 inhibitor, MK571. To confirm the binding affinities of the CIEA compounds as predicted by the molecular docking studies and also investigate the ability of the compounds to directly interact and induced conformational changes in MRP1 protein structure, we proceeded to use a two-color MRP1 biosensor engineered in our lab coupled with steady-state fluorescence spectroscopy. This biosensor has been used to screen a library of 40-novel clinical anticancer drugs of which 10 of these drugs possibly interacted with MRP1 as substrates [56]. Our FRET-based fluorescence spectroscopy results confirmed that the CIEA compounds directly interact with MRP1 protein which strengthens our conviction that the identified compounds are inhibitors of MRP1.

3.5 Conclusion

In summary, our study demonstrated the competence of the high content screening assay in determining novel inhibitors against MRP1 from a library of cucurbitacin inspired estrone analogues (CIEAs). The study was able to investigate CIEA compounds as potential MRP1 inhibitors. Functional studies with MRP1 confirmed that the CIEA compounds inhibit the function of MRP1 by increasing the accumulation of two MRP1 substrates. We also demonstrated the potential of these CIEA compounds to re-sensitize MRP1 overexpressing cells to anticancer drugs, which is of great benefit to overcome MDR in clinical cancer treatment. Lastly, we also showed that the identified MRP1 inhibitor compounds have direct interaction with MRP1 protein.

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Chapter 4

General Conclusions and Recommendations

In general, we have demonstrated the potential of a novel compound series, CIEA as inhibitors for P-gp and MRP1 mediated multidrug resistance. Using a high content fluorescence-based transport activity assay, we were able to identify hit compounds which were then further validated using established ABC transporter functional assays. The hit compounds were also investigated for their reversal effect on multidrug resistant cell lines towards selected anticancer agents. Protein expression studies were conducted to evaluate the activity of the hit compounds on P-gp and MRP1 protein expression. Finally, in silico molecular docking and FRET-based spectroscopic studies were used to investigate direct interaction of the hit compounds with P-gp or MRP1 proteins.

In the first study, the high content fluorescence-based screening identified 8 CIEA compounds as positive hits for P-gp inhibition. In addition all the hit compounds displayed strong inhibition (in comparison to verapamil) of P-gp in flow cytometry-based calcein efflux and confocal microscopy -based doxorubicin localization assays. The hit compounds also demonstrated various degrees of reversal of resistance and selectivity towards vincristine, paclitaxel and etoposide in P-gp overexpressing multidrug resistant HEK293/P-gp cells. However, all the compounds were very effective in sensitizing MDR P-gp cells to etoposide. Moreover, protein expression studies revealed that the compounds did not alter P-gp expression levels. Thus, the mechanism of P-gp inhibition by the CIEA has no suppression on P-gp protein expression. Molecular docking studies predicted that all the compounds have better binding affinities towards the drug binding pocket of P-gp compared with verapamil, however, HL 20 was very

potent in binding to the P-gp domain. Finally, we were able to confirm that the CIEA compounds are potential ligands of P-gp. We came to the conclusion that the CIEA compounds hold promise for use as agents for inhibiting P-gp mediated multidrug resistance.

In the second study, the high content screening discovered 5 compounds as hits for MRP1-mediated doxorubicin efflux inhibition and 3 compounds as inhibitors of calcein red orange efflux. Two of the compounds (HL 15 and HL 21) identified as calcein red orange inhibitors were also identified to inhibit doxorubicin efflux. The hit compounds that were selected for further studies significantly increased the accumulation of MRP1 substrates, doxorubicin and calcein red orange in functional characterization studies. Drug sensitivity studies also showed selective sensitization of MRP1 overexpressing H69AR cells by the CIEA compounds towards the anticancer drugs, vincristine and SN38, however, HL 15 demonstrated effective sensitization towards both anticancer agents. Similarly as observed in P-gp, the CIEA compounds did not have any effect on MRP1 protein expression. In silico molecular docking and fluorescence spectroscopic studies confirmed inhibitor compounds interaction with MRP1 protein.

It is thought that, this study is the first of its kind to report the therapeutic potential of CIEA with P-gp and MRP1. We anticipate that the results presented herein will provide useful information on cucurbitacin-inspired estrone analogues as sources of new drug discoveries for optimization as therapeutic strategies to overcome MDR in cancer.

It is recommended that further studies are conducted to advance the CIEA compounds to the clinical trials stages. Studies may include elucidating their mechanism of inhibition

such as evaluating their effects on ATPase activity which is an important functional requirement for P-gp and MRP1 mediated transport. In addition, transport studies using membrane vesicles could provide a direct model to understand their mechanism of inhibition. Finally, in vitro 3D tumor or vivo models may be used to assess the ultimate pharmacokinetic impact of these compounds to establish their efficacy, safety and toxicity levels. Depending on their pharmacokinetic profiles, the CIEA compound may be optimized to advance their utilization in clinical studies.