Protein Kinase Inhibitor Effects on P-glycoprotein (P-gp) Activity and Expression in Various Cell Lines

by

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Abstract

Little is known about potential influences of kinase pathway modulation on expression and activity of P-glycoprotein (P-gp). A protein kinase inhibitor (PKI) library was screened, to determine its effects on activity and expression of P-gp, in various cell lines.

Cell lines were incubated with PKI for 24 h. Subsequent P-gp substrate accumulation studies were performed. Changes in P-gp activity and/or expression ≥ 25% compared to control were considered hits. Kinase pathways identified as P-gp activity hits were examined for their ability to modulate permeability.

PKI families GSK-3, Craf1 and VEGFR2 and Tie-2, significantly modulated P-gp activity in the MDCK cell line. PKI families GSK-3, Iκκ and Jnk2/3 significantly modulated P-gp activity in the Caco-2 cell line. Few P-gp activity hits significantly modulated P-gp expression.

PKIs modulate P-gp activity more than P-gp expression in a cell line dependent manner, excluding GSK-3 PKI family, which appears to be cell line independent.

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Table of Contents

List of Tables

List of Figures

Abbreviations

ABC	ATP Binding Cassette
AKT	Beta-Alanyl-Alpha-Ketoglutarate Transaminase
AML	Acute Myeloid Leukemia
AP-1	Activator Protein-1
APC	Adenomatosis Polyposis Coli
ATP	Adenosine Triphosphate
Aβ	Amyloid-Beta
BA	Bile Acids
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
BCRP	Breast Cancer Resistant Protein
bFGF	Basic fibroblast growth factor
BIO	6-bromoindirubin-3'-oxime
BSA	Bovine Serum Albumin
CA	Cholic Acid
Caco-2	Human Caucasian Epithelial Colorectal Adenocarcinoma (Male)
cAMP/PKA	Cyclic Adenosine Monophosphate/Protein Kinase A
CAR	Constitutive Androstane Receptor
CDCA	Chenodeoxycholic Acid
CDK	Cyclin Dependent Kinases
CITCO	6-(4-Chlorophenyl)imidazol[2,1-*b*][1,3]thiazole-

	5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime
CK1γ	Casein Kinase1-γ
CNS	Central Nervous System
cRaf	cRapidly Accelerated Firbrosarcoma
CSBP	Cytokinin Specific Binding Protein
CYP450	Cytochrome P450
ddH20	Double Distilled Water
DKK	Dickkopf
DMEM	Dulbecco's Modified Eagle's Medium
Dsh	Dishevelled
EA	Efflux Activity
EBM-2	Endothelial Cell Growth Media
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ErbB	Erythroblastic Leukemias Viral Oncogene Homolog
ERE	Estrogen Response Element
ERK	Extracellular Signal-regulated Kinase
EVOM	Epithelial Voltohmmeter
Ex Percent	Expression Percent
FGF-2 IIIb	Fibroblast Growth Factor-2 IIIb
FGFR	Fibroblast Growth Factor Receptor
Flk-1	Fetal Liver Kinase Receptor

FXR	Farnesoid-X-Receptor
FXREs	FXR Response Elements
Fz	Frizzled
GLUT-1	Glucose Transporter-1
GSK-3	Glycogen Synthase Kinase-3
GSP	Grape Seed Procyanidin
hCAR	Human CAR
hCMEC/D3	Human Cerebral Microvascular Endothelial/D3
HCT15-79-MT	Human Colon Cancer Cells (lacking NF-κB functional activity)
HCT15-H	Human Colon Cancer Cells (that have a fully functional NF-κB)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	Human Epidermal Growth Factor Receptor 2
hPXR	Human Pregnane X Receptor
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IgG-HRP	Immunoglobulin G-Horse Radish Peroxidase
JNK	c-Jun N-Terminal Kinase
KB-V1	Human Cervix Carcinoma Cell line
kDa	Kilodaltons
L1210/VCR	Mouse Lymphocytic Leukemia, Vincristine Resistant Cells
LCK	Lymphocyte-Specific Protein Tyrosine Kinase
LRP	Lipoprotein Receptor-Related Protein
LS180	Human Caucasian Epithelial Colon Adenocarcinoma (Female)
LXR	Liver X Receptor

MAPK	Mitogen-Activated Protein Kinase
MDCK	Madin Darby Canine Kidney
MDCK-MDR1	Madin Darby Canine Kidney-Multidrug Resistance Protein 1
MDCK-WT	Madin Darby Canine Kidney-Wild Type
MDR	Multidrug Resistance
MRP	Mulitdrug Resistance-Associated Protein
MSK	Mitogen and Stress Activated Protein Kinase
MβCD	Methyl-Beta-Cyclodextrin
NBD	Nucleotide Binding Domain
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NP-40	Nonidet P-40
O/N	Overnight
PBS	Phosphate Buffered Saline Solution
PDGF-B	Platelet-Derived Growth Factor-B
PDGFR	Platelet-Derived Growth Factor Receptor Kinase
PEG	Polyethylene Glycol
P-gp	P-glycoprotein
PI3K/Akt	Phosphoinositide 3-Kinase/ Beta-Alanyl-Alpha-Ketoglutarate Transaminase
PK	Pharmacokinetic
PKA	Protein Kinase A
PKC	Protein Kinase C
PKI	Protein Kinase Inhibitor

PMSF	Phenylmethylsulfonyl Fluoride
PPARs	Peroxisome Proliferator Activated Receptors
PPP(S/T)P	Proline-Proline-Proline-(Serine/Threonine)-Proline
PVDF	Immuno-Blot Polyvinylidene Fluoride
PXR	Pregnane X Receptor
QBC939/5-FU	5-Flourouracil Resistant Human Cholangiocarcinoma Cells
r123	Rhodamine 123
RARs	Retinoid Acid Receptor
Ras	Rat Sarcoma
ROCK1	Rho-Associated Protein Kinase 1
Rp1	Retinitis Pigmentosa-1
RT	Room Temperature
RTK	Cell- Surface Receptor Tyrosine Kinases
RXR	Retinoid X Receptor
SD	Semi-Dry
SEM	Standard Error of the Mean
Ser	Serine
sFRPs	Fz-Related Proteins
SGC7901/VCR	Human Gastric Cancer, Vincristine Resistant
Src Kinase	Sarcoma Kinase
SXR	Steroid and Xenobiotic Receptor
T47D:A18	ER-Positive Breast Cancer Cell Line
TBS-T	Tris-Buffered Saline with Tween

TCF/LEF	T Cell Factor/Lymphoid Enhancer Factor
TEER	Trans Epithelial Electric Resistance
TGF-β	Transforming Growth Factor Beta
Tie-2	Tyrosine-Protein Kinase Receptor-2
TM	Transmembrane
TNFα	Tumor Necrosis Factor Alpha
TR	Thyroid Hormone Receptor
TrkA	Tropomyosin Receptor Kinase A
VDR	Vitamin D Receptor
VEGF	Vascular Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WIF-1	Wnt Inhibitors Factor-1

1.0. Introduction

The adenosine triphosphate binding cassette (ABC) superfamily of transporters are made up of approximately 49 transporters, organized into seven subfamilies, ABCA to ABCG (Shen, Pastan et al. 1998). Members include but are not limited to, P-glycoprotein (P-gp), breast cancer resistant protein (BCRP) and multidrug resistance-associated protein (MRP) subfamilies. These transporters play critical roles in the absorption, distribution and elimination of a broad range of pharmaceuticals and endogenous compounds including ions, heavy metals, steroids, phospholipids, oligopeptides, carbohydrates, antibiotics, and many hydrophobic/hydrophilic drugs (Sauna, Smith et al. 2001; Kuchler 2011). Within a variety of tissues including, the blood-brain barrier (BBB), liver, kidney and intestine these transporters ultimately function to lower the absorption and increase the secretion of their substrates into the surrounding extracellular environment (Sauna, Smith et al. 2001; Kuchler 2011; Deng, Shao et al. 2014) and play important roles in drug detoxification, but become a hindrance in drug therapy. This is accomplished in an ATP driven manner and involves active transport that can occur against concentration gradients.

1.1. Overview of Structure and Mechanism of ABC transporters

Currently, the typical structure of ABC transporters that have been identified includes two separate domains, the ATP binding domain, also known as the nucleotide binding domain (NBD), involved in hydrolyzing ATP and providing the energy required to efflux the substrate and the transmembrane (TM) domain, which forms the channel and possibly binds the substrate to be effluxed (Jones, O'Mara et al. 2009). Structurally, the NBD contains a consensus sequence

made up of 90 to 110 amino acids that is conserved among all members within the ABC transporter family and distinguishes this family from other transporters (Gottesman and Ambudkar 2001). This consensus sequence includes an A walker region, separated from a B walker region by a C linker region. The A walker region is separated from the C linker region by 90 amino acids and the C linker region is separated from the B walker region by 20 amino acids (Jones, O'Mara et al. 2009; Breier, Gibalova et al. 2013). The A walker region is thought to be involved in binding of the β-phosphate of ATP, whereas the B walker region interacts with Mg^{2+} shown to be required for P-gp ATPase activity (Sharom, Liu et al. 1999; Sauna, Smith et al. 2001). Currently, the interaction of the C linker region with ATP is not known, but has been shown to be required for ATP binding and hydrolysis (Leslie, Deeley et al. 2005). Structurally, the TM domains primary sequences are not conserved and are thought to be responsible for the different substrate specificities seen among the ABC transporter family (Gottesman and Ambudkar 2001; Jones, O'Mara et al. 2009).

Among the different members within the ABC transporters, two general transmembrane motifs exist. Full transporters, including P-gp, contain two sets of transmembrane domains (12 TM helices in total) and ATP binding domains. The TM and ATP binding domains in the full transporters are typically homodimeric in nature. Half transporters, including BCRP, contain only one set of TM and ATP binding domains, thus 6 TM helices in total (Gottesman and Ambudkar 2001). The half transporters must come together as either homo- or heterodimers in order to be a functional transporter (Hyde, Emsley et al. 1990; Dean, Hamon et al. 2001).

Understanding the mechanism of substrate transport for ABC transporters is based primarily on studies focusing on P-gp (Borst and Elferink 2002). In P-gp, both NBDs are oriented towards the cytoplasm and both NBDs are essential in allowing drugs to bind to the

drug binding sites (Breier, Gibalova et al. 2013). It was first proposed by Senior, al-Shawi et al. (1995) that both the nucleotide binding domains (NBD) required ATP hydrolysis in sequence in order for P-gp to transport a substrate. It is now known that sequential hydrolysis of ATP within the NBD of P-gp is not required for substrate transport. For instance, inactivation of NBD1 on P-gp, does not affect substrate efflux, whereas inactivation of NBD2 inhibits P-gp mediated substrate efflux (Gao, Cui et al. 2000). In addition, it is also believed that the transmembrane domain and ATP binding sites of P-gp are linked and work together, influencing the adjustment of flexible TM segments in the substrate binding site, allowing P-gp to bind different drug substrates and have a broad substrate specificity (substrate induced fit mechanism) (Loo, Bartlett et al. 2003). While the mechanism of transport is still under investigation, current evidence suggests that P-gp may act as a flippase moving substrate from the inner leaflet to the extracellular space (Higgins and Gottesman 1992). It is also thought that binding of substrate to the high affinity binding site of P-gp results in hydrolysis of ATP and leads to a conformational change, which shifts the substrate to a low affinity site causing it to be released into the extracellular space or outerleaflet of the membrane (Sauna et al. 2001).

1.2. P-gp (ABCB1)

P-glycoprotein was first identified in drug resistant hamster ovary cells by Juliano and Ling (1976). It is the most studied transporter out of the ABC transport family and has been found to have the broadest substrate specificity (Jones, O'Mara et al. 2009). Encoded by the *ABCB1* gene, P-glycoprotein is synthesized as a 140-kDa precursor protein, that is eventually glycosylated to create the fully glycosylated form with a final molecular weight protein of 170 kDa (Greer and Ivey 2007). P-gp is primarily expressed on the apical plasma membrane of epithelial or endothelial cells where it is postulated to have a protective role in preventing toxic

xenobiotics from diffusing across cell membranes that make up these tissues, contributing to xenobiotic efflux, altering xenobiotics distribution, metabolism and absorption, within different tissues of the body where it is expressed (Leslie, Deeley et al. 2005). In addition, P-gp is currently thought of as being the major player in allowing cells to exhibit multidrug resistance (MDR) against numerous drug substrates. For instance, upregulation of P-gp in cancer cells provides increased resistance to a wide variety of chemotherapeutic agents. In saying this, P-gp is also fundamental in regulating endogenous substrate distribution such as certain steroid hormones and phospholipids and may be involved in cell processes including cell differentiation, apoptosis and proliferation (Borst and Elferink 2002; Breier, Gibalova et al. 2013). Some studies have even indicated that the decreased P-gp expression and activity observed in AD patients may be linked to decreased amyloid-beta (Aβ) clearance, considering Aβ is a substrate for P-gp (Hartz, Miller et al. 2010; Qosa, Abuznait et al. 2012). Compounds that are bulky and amphiphatic or exhibit relatively high hydrophobicity with a molecular mass ranging between 300 to 2000 g/mol, tend to be substrates for P-glycoprotein (Sauna, Smith et al. 2001; Leslie, Deeley et al. 2005; Breier, Gibalova et al. 2013). Examples of these types of compounds include vinca alkaloids, calcium channel blockers, anthracyclins, antibiotics, steroid hormones and HIV proteases (Sauna, Smith et al. 2001).

1.3. P-gp Regulation

Multiple mechanisms have been found to modulate P-gp activity and protein expression either directly at the transporter or indirectly through other second messenger cellular pathways. These complex P-gp expression and activity pathways allow the cell to respond effectively to defend against xenobiotic exposure or aid in the transport of endogenous substrates within the cellular and extracellular environment.

P-gp modulation can occur through either direct interactions with the transporter ATP binding sites and/or posttranslational sites, including glycosylation and phosphorylation sites, or indirect modulation through second messengers, affecting P-gp membrane trafficking, fluidity and posttranslational modification. Both types of modulation are discussed below.

1.4. Direct Transporter Modulation of P-gp Activity

P-glycoprotein exists as a large glycosylated protein, heavily N-glycosylated with complex oligosaccharides that can slightly vary depending on the cell type, as well as the tissue it is located in (Schinkel, Kemp et al. 1993). Out of 10 possible glycosylation sites identified on human P-gp based on two-dimensional structural modeling, 3 putative glycosylation sites are only exposed to glycosylation machinery that exists within the lumen of the endoplasmic reticulum (Schinkel, Kemp et al. 1993). These glycosylation sites are located at asparagine residues 91, 94 and 99 within the extracellular loop of P-gp, between transmembrane domains 1 and 2 and have been identified as possible P-gp modulatory sites that seem to play a substantial role in P-gp stability and trafficking to and from the plasma membrane, rather than modulating function of the transporter itself (Schinkel, Kemp et al. 1993; Gribar, Ramachandra et al. 2000; Breier, Gibalova et al. 2013). On the contrary, Seres, Cholujova et al. (2011), demonstrated that known N-glycosylation inhibitor, tunicamycin, had no effect on P-gp membrane localization and functional activity in L1210 P-gp positive cell variants. Therefore, the influence of P-gp glycosylation on P-gp activity and plasma membrane trafficking still remains unclear.

After it was realized that native P-gp was phosphorylated *in vivo*, numerous studies were performed to see if this type of post-translational modification could influence P-gp efflux activity. Structurally, the major phosphorylation site that was identified on human P-gp was

located in the 60 amino acid linker region that connects the two homologous halves of P-gp and was found to contain numerous serine phosphorylation sites for protein kinase A and C including Ser-667, Ser-671, Ser-683 and Ser-661, Ser-667 and Ser-671 for protein kinase A and C, respectively (Chambers, Pohl et al. 1993; Chambers, Pohl et al. 1994). While, site directed mutagenesis of these predicted phosphorylation sites for both protein kinase A and C, appeared to have no effect on P-gp activity (Germann, Chambers et al. 1996), this pathway still remains unclear and may provide another explanation on how P-gp activity is regulated directly by phosphorylation of the transporter.

1.5. Indirect Transporter Modulation of P-gp Activity

Membrane lipid composition has also been hypothesized to affect the activity of P-gp. Within the plasma membrane, P-gp tends to be localized to the apical side and is primarily contained within low-density caveolae filled, signalling micro-domains known as lipid rafts enriched in cholesterol, glycosphingolipids and sphingomyelin (Kamau, Kramer et al. 2005; Yun, Lee et al. 2013). Here, caveolae form a subclass of lipid raft and are distinguished from other lipid rafts because they contain caveolin-1 (Yun, Lee et al. 2013). For instance, it was shown that a reduction in cholesterol levels in lipid rafts by methyl-beta-cyclodextrin (MβCD) in Madin-Darby canine kidney (MDCK) cells, led to a reduction in P-gp substrate rhodamine 123 (r123) efflux in MDCK-MDR1 cells, as well as a shift of P-gp from raft to non-raft fractions, leading to a reduction in P-gp activity (Kamau, Kramer et al. 2005). In addition, ginseng saponin derivative, ginsenoside RP1, which is structurally similar to cholesterol, changed P-gp containing cholesterol rich lipid rafts and also caused a shift of P-gp from lipid raft fractions to non-lipid raft fractions, resulting in decreased MDR resistance and P-gp activity (Yun, Lee et al. 2013). Here, ginsenoside RP1, may compete with cholesterol to be incorporated into the lipid raft

possibly elucidating a mechanism whereby changing the lipid raft content of cholesterol can lead to a reduction in MDR and P-gp activity in MDR cell lines.

1.6. Modulation of P-gp Expression

P-gp expression can also be modulated directly through interactions with transcription factors including orphan nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR) etc.) (Breier, Gibalova et al. 2013). Most studies have focused on this pathway for modulating P-gp expression in different tissues. Orphan nuclear receptors make up part of a gene family that encodes transcription factors that sense endogenous and xenobiotic compounds and regulate gene transcription of gene targets including ABC transporters and cytochrome P450 (CYP450) enzymes. Upon activation by endogenous or xenobiotic ligands, they form heterodimers with members of the retinoid X receptor (RXR) family, and as a complex have been identified as major modulators of P-gp expression, while exhibiting ligand inhibition and activation differences in human, rat and mouse (Chan, Hoque et al. 2011). The members of the RXR family are found in most tissues, including the liver, muscle, kidney and heart, with some members being found more selectively and to a lesser extent throughout the body (Xu, Li et al. 2005). Members of the orphan nuclear receptor family that have been found to induce P-gp expression in different types of tissues when dimerized with RXR include PXR, CAR, thyroid hormone receptor (TR) and vitamin D receptor (VDR). Here, RXR dimerization functions to increase the DNA binding affinity of the RXR complex to P-gp and CYP450 gene promoter elements, increasing their expression and contributing to the interplay between these two types of proteins in limiting substrate influx and increasing substrate metabolism, respectively. Therefore, even though compounds may not be substrates of P-gp, they are still capable of modulating its expression through the interaction with orphan nuclear receptors (Leost, Schultz et al. 2000;

Chan, Hoque et al. 2011; Zhao, Sun et al. 2013). For example, human PXR (hPXR) substrate cisplatin can upregulate P-gp and CYP26 expression, even though it is not a P-gp substrate (Breier, Gibalova et al. 2013).

The PXR family, also known as Steroid and Xenobiotic Receptor (SXR) family (Mani, Huang et al. 2005), have been found to bind a broad range of steroids, xenobiotics and anticancer drugs including anthracyclins, vinca alkaloids and taxanes (Harmsen, Meijerman et al. 2010). In addition, they are highly expressed along with P-gp in barrier tissues including intestine and liver. The PXR family is the most studied of the orphan nuclear receptor family in relation to increasing P-gp expression. For example, it was demonstrated in human caucasian epithelial colon adenocarcinoma, female (LS180) cells co-transfected with hPXR and P-gp reporter construct, that exposure to a variety of anticancer drugs including vincristine, vinblastine and tamoxifen, increased P-gp reporter activity (Harmsen, Meijerman et al. 2010). Furthermore, a reduction in P-gp reporter activity was found upon hPXR knockdown, indicating PXR is involved in drug induced MDR and P-gp induction (Harmsen, Meijerman et al. 2010).

A lesser studied orphan nuclear receptor compared to PXR in relation to inducing P-gp expression is the CAR. The CAR, is highly expressed in liver and to a lesser extent in the intestine and brain, and like PXR, is also activated by a wide range of endogenous and xenobiotic ligands (Xu, Li et al. 2005; Chan, Hoque et al. 2011). For example, 6-(4-chlorophenyl)imidazol[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO), a selective ligand for human CAR (hCAR), increased MDR1 mRNA and protein expression by 40 % and 60 %, respectively in hCMEC/D3 cells, a cell culture model for the BBB (Chan, Hoque et al. 2011).

Orphan nuclear receptors dimerized to RXR that have been found to down-regulate P-gp transcription in co-expressed tissues include, the peroxisome proliferator activated receptors (PPARs) and retinoid acid receptor (RARs).The PPARs family play an important physiological role in the regulation of lipoprotein and fatty acid metabolism (Xu, Li et al. 2005) and members can be found to be expressed in the liver, heart, kidney, intestine, brown adipose tissue, and brain (Xu, Li et al. 2005). For example, PPARγ activation by troglitazone has been shown to decrease P-gp mediated MDR in human gastric cancer, vincristine resistant (SGC7901/VCR) cells at the mRNA and protein levels (Breier, Gibalova et al. 2013). The RAR family, binds all-trans retinoic acid and 9-cis-retinoic acid and was shown to synergistically down-regulate P-gp expression and transport activity with verapamil in L1210/VCR cells (Sulova, Macejova et al. 2008).

Pro-inflammatory modulators including tumor necrosis factor alpha (TNFα) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) have also been found to modulate P-gp activity and expression. For instance, low levels of exposure of TNFα to isolated rat brain capillaries reduced P-gp activity without affecting P-gp expression or brain capillary barrier tight junctions (Hartz, Bauer et al. 2006). In addition, long term exposure of rat brain capillaries to TNFα lead to the activation and translocation of NF-κB into the nucleus, inducing P-gp expression and activity (Bauer, Hartz et al. 2007), thus contributing to the interplay between both these inflammatory modulators. Furthermore, inhibition of NF-κB was found to down regulate *MDR1* mRNA, as well as P-gp protein expression in human colon cancer cells (HCT15-79-MT) lacking NF-κB functional activity compared to HCT15-H control cells that have a fully functional NF-κB (Bentires-Alj, Barbu et al. 2003).

1.7. BCRP (ABCG2)

BCRP is a member of the ABCG subfamily of the ABC superfamily of transporters and exists as a 72-kDa, 655 amino acid protein in its half transporter form (Schinkel and Jonker 2003), and comes together as a homodimer forming a 140-kDa complex (Krishnamurthy, Ross et al. 2004). BCRP is thought to work in conjunction with P-gp and is primarily localized to the apical side of the plasma membrane within cells (Jonker, Smit et al. 2000; Maliepaard, Scheffer et al. 2001). Here, BCRPs predicted physiological function in the mammalian system is playing a protective role, preventing the uptake of toxic xenobiotics from the blood into a variety of mammalian tissues where it is expressed (Maliepaard, Scheffer et al. 2001). Like P-gp, BCRP can substantially impact the pharmacokinetics of substrate drugs in different tissues (Shinkel et al, 2012). BCRP, like P-gp has also been found to be over-expressed in cancer cells and contributes to MDR. For instance, it has been found that an increase in BCRP expression is correlated to drug resistance in acute myeloid leukemia (AML) (Steinbach, Sell et al. 2002). Substrate wise, BCRP transports a narrower range of compounds, but still has substantial substrate overlap with P-gp and other ABC transporters. Drug substrates of BCRP include anthracyclins, mitaxantrone, doxorubicin, and topotecan derivatives. BCRP shares extensive tissue distribution with P-gp and is expressed in numerous tissues including the small intestine, liver, BBB, with the highest levels being found in the placenta (Shinkel et al. 2012).

The regulation of BCRP appears to be quite different compared to P-gp, even though they are usually localized together and are thought to work in conjunction. For instance, it has been shown that BCRP is transcriptionally regulated by the nuclear receptor, liver X receptor (LXR) (Wang, Lan et al. 2004), but is not influenced by PXR or CAR (Handschin and Meyer 2005). In addition, hypoxic conditions have been found to increase BCRP expression (Krishnamurthy,

Ross et al. 2004). Interestingly, sex hormones including testosterone and estradiol have also been found to increase BCRP expression possibly through the androgen or estrogen receptor, respectively. For instance, Ee, Kamalakaran et al. (2004) found a novel estrogen response element (ERE) in the promoter region of the BCRP gene involved in the increase in BCRP expression observed following exposure of ER-positive T47D:A18 breast cancer cells to estradiol. In addition, BCRP mRNA levels were found to decrease in male mouse livers to levels comparable to female mouse livers upon castration (Tanaka, Slitt et al. 2005). Subsequent hormone replacement with 5α-dihydroxytestosterone in the castrated mice, increased BCRP expression in the male liver, suggesting that testosterone plays a role in the regulation of BCRP in the male hepatocyte (Tanaka, Slitt et al. 2005).

1.8. Protein Kinases

Protein kinases are the largest enzymatic family of proteins encoded by the human genome, where as many as 518 putative kinase genes have been identified in the human kinome, where they are involved in controlling many homeostatic and pathological cellular processes (Cohen 2002; Manning, Whyte et al. 2002; Grant 2009). These highly complex and interconnected signalling pathways controlled by the activation status of protein kinases regulate gene transcription, cell growth, cell proliferation, and cell differentiation. Functionally, protein kinases are ATP-dependent phosphotransferases that catalyze the transfer of the terminal phosphate of ATP to tyrosine, serine or threonine residues found on targeted proteins, governing their activation or inhibition and altering cell function through reversible phosphorylation of specific proteins within a signalling cascade (Grant 2009). In addition protein kinases themselves can be phosphorylated by other protein kinases, phosphotransferases or through autophosphorylation events. Structurally, protein kinases are arranged into 12 subdomains that

fold to form an ATP catalytic core, as well as the substrate binding site. Within the catalytic core, the hinge region, hydrophilic channel and conserved activation loop play an important role in the binding and catalysis of ATP (Zhang, Yang et al. 2009). Considering, the crucial role protein kinases play and the numerous protein kinase drug targets available within a signalling cascade, there is no surprise that protein kinases are the second most targeted proteins in drug discovery programs, just behind G-protein coupled receptors (Cohen 2002).

1.9. Protein Kinase Inhibitors

The first protein-kinase or small molecule kinase inhibitors were developed in the early 1980s by Hiroyoshi Hidaka and were ATP competitive, cell permeable compounds (Cohen 2002; Janne, Gray et al. 2009). By targeting the active conformation of the kinase at the highly conserved hinge region located in the ATP binding pocket, these early ATP competitive inhibitors exhibited low potency, were nonspecific and required dosing within the millimolar range to outcompete the high cellular concentrations of ATP (Zhang, Yang et al. 2009; Dar and Shokat 2011; Knapp and Sundstrom 2014). Based on these initial studies, it was thought that generating compounds that specifically inhibited a particular kinase with low to no inhibitory overlap with other protein kinases was nearly impossible and remained a great challenge. This changed when it was realized that specificity can be acquired by targeting areas on a particular kinase that is not conserved among other kinases or targeting conformational differences that become exposed upon binding of ATP (Janne, Gray et al. 2009). In the last 10 years, PKI development has focused on these types of targeted strategies and has allowed PKIs to become highly specific and potent. This evolution in direction has led to the development of approximately three different classes of PKIs based on inhibition mechanism, including the ATP competitive inhibitors, the non-competitive ATP inhibitors and the substrate competitive

inhibitors, which can interact a different sites within the protein kinase, resulting in a non-functional kinase or favouring the inactive conformation (Eldar-Finkelman and Martinez 2011). In fact, in some cases PKIs have been identified to exhibit properties of being both an ATP and substrate competitive inhibitor for a specific kinase (Levitzki 2003). Interestingly, even though ATP competitive inhibitors are non-specific and can inhibit numerous protein kinases, they have been found to be useful in some clinical situations, where a single drug can be used to treat multiple related clinical indications or inhibit multiple pathways involved in contributing to a particular disease (Janne, Gray et al. 2009). Therefore, there is most likely a need for both broad and specific PKI therapies.

Numerous protein kinase targets have been identified in contributing to the proliferation of aberrant signalling pathways identified in diseases such as cancer, neurodegenerative disease, diabetes and inflammatory diseases. The development of specific PKIs for treatment of these diseases allows for a specific approach where aberrant signal transduction pathways involved in these diseases can be specifically targeted without affecting other cellular signalling processes that are important in cell function and maintenance, exhibiting a more favourable side effect profile. For example, instead of using cytotoxic agents that are broad acting and induce apoptosis in cancer cells, as well as normal cells, PKIs targeting aberrant signalling pathways of the cancer cell, can be used to selectively target cancer cell growth and proliferation.

Early on in many different types of malignancies, it was noticed that aberrant protein kinases played a role in tumorigenesis and were considered oncogenic, making them essential for tumor survival. In addition, it was also found that non-mutated protein kinases involved in cell survival, differentiation and proliferation also played a role in tumorigenesis and became up-regulated. For example, over expression of the cell- surface receptor tyrosine kinases (RTK),

epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) in tumorigenesis facilitates the tumor's need for increased nutrient delivery and cell growth by increasing the activation of downstream signalling pathways involved in angiogenesis and cell proliferation. These downstream signalling events occur through the RTKs extracellular domain that selectively binds growth factors epidermal growth factor (EGF) and vascular growth factor (VEGF), respectively. This binding causes the RTK to dimerize, causing activation of the intracellular protein kinase domain of the receptor, which initiates downstream intracellular signalling pathways (Grant 2009).

Currently, some RTK PKIs have made it into the clinic to treat certain types of cancers. For example Iressa (ZD 1839), an EGFR and human epidermal growth factor receptor 2 (HER-2) PKI is used in the treatment of non-small cell lung carcinoma (Levitzki 2003) where it functions to down regulate downstream effector kinases involved in cell survival, including beta-alanyl-alpha-ketoglutarate transaminase (AKT) and extracellular signal-regulated kinase (ERK) (Janne, Gray et al. 2009). Certain PKIs have also been developed to target downstream effector protein kinases of RTK in cancer treatment, including the serine/threonine protein kinases, cyclin dependent kinases (CDK) and members of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/ beta-alanyl-alpha-ketoglutarate transaminase (PI3K/Akt) pathways. Under normal conditions these kinases regulate cell cycle, division and growth, but under cancerous conditions, contribute further to tumor cell growth, migration and proliferation (Levitzki 2003).

1.10. PKIs and their Involvement in P-gp Regulation

Within the literature, few studies have focused on if and how PKIs modulate ABC transport activity and expression. For instance studies looking at PKIs have generally focused on their ability in reducing cell proliferation and migration pathways, especially in cancer type models. However, the regulation of ABC transporter expression and activity could greatly influence tumor cell response to chemotherapeutic agents and the development of MDR. In this regard, much effort has been placed on identification and development of inhibitors/modulators of drug efflux transporters. These agents bind to the transporter and inhibit activity. Interestingly, despite a great deal of effort there are currently no clinically approved MDR reversal agents, due mostly to severe toxicity of the modulating agents. Therefore, using PKIs to modulate ABC transporter activity may provide a novel mechanism in reversing MDR in diseases such as cancer or enhancing P-gp substrate Aβ clearance from the brain at the BBB (Hartz, Miller et al. 2010).

The few studies that do exist in relating PKIs to ABC transport activity and expression have mainly focused on P-gp. Some of the signalling pathways and protein kinases that have been shown to modulate P-gp activity and/or expression include the rat sarcoma (Ras) -mediated pathway, cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, PI3K/Akt pathway and protein kinase C (PKC) (Fine, Patel et al. 1988; Kisucka, Barancik et al. 2001; Ziemann, Riecke et al. 2006; Wang, Qu et al. 2011; Sui, Fan et al. 2012). For example, it was found that using specific MAPK (ERK) inhibitors PD98059 and U0126 towards mouse lymphocytic leukemia, vincristine resistant (L1210/VCR) cells increased their susceptibility towards vincristine in a concentration dependent manner (Kisucka, Barancik et al. 2001). This suggests that the MAPK pathway may be involved in regulating P-gp activity and/or expression or the MAPK inhibitors may have interacted directly with the transporter. In addition, the

exposure of rat hepatocytes to cAMP-dependent protein kinase A (PKA) inhibitor, H89, resulted in a decrease in rat hepatocyte P-gp mRNA levels, indicating that the cAMP/PKA pathway may be involved in P-gp expression regulation (Ziemann, Riecke et al. 2006).

Members of the RTKs have also been found to modulate P-gp activity and/or expression and include the RTKs fibroblast growth factor receptor (FGFR), VEGFR and fibroblast growth factor-2 IIIb (FGF-2 IIIb). For instance, Patel, Tiwari et al. (2013) demonstrated that selective FGFR and small-molecule tyrosine kinase inhibitor, PD173074, used at 2.5 to 5 µM, was able to selectively decrease P-gp activity without affecting P-gp expression in both an ABCB1 transfected human cervix carcinoma cell line, (KB-V1) and a MDR induced cell line, via increased exposure to colchicines, KB-3-1. This decrease in P-gp activity was found to occur through PD173074 directly interacting with the transporter at a site other than the drug binding pocket. Furthermore, owing to PD173074 being a selective ABCB1 inhibitor, exposure of this compound to cell lines over-expressing BCRP or MRP1, did not reverse BCRP nor MRP1 activity (Patel, Tiwari et al. 2013). In addition, in a study performed by Saksena, Priyamvada et al. (2013), FGF10, a growth and migration factor secreted by fibroblasts and endothelial cells, was found to cause up-regulation of P-gp in Caco-2 cells by interacting with the FGF-2 IIIb RTK family. It was also demonstrated that when selective FGFR PKI, PD-161570 was exposed to Caco-2 cells co-incubated with FGF10, P-gp activity decreased. Since FGFR signalling has been shown to involve Erk1/2 MAPK pathway, they also exposed specific Erk1/2 MAPK inhibitor, PD-98059, to FGF10 P-gp induced Caco-2 cells and showed a reduction in P-gp activity. This indicates that ErK1/2 MAPK may be involved in the regulation of P-gp activity in Caco-2 cells (Saksena, Priyamvada et al. 2013). Lastly, VEGF has also been found to regulate P-gp in a concentration dependent manner. For instance, when VEGF was exposed to isolated rat brain

capillaries, it led to a concentration dependent reduction in P-gp activity (Hawkins, Sykes et al. 2010). Interestingly, this effect was found to be fully reversible, did not change the total amount of P-gp protein expression and involved VEGF acting through the fetal liver kinase receptor (Flk-1) and sarcoma (Src) kinase to reduce P-gp activity.

A protein kinase inhibitor found to modulate both P-gp and BCRP is lapatinib, a potent inhibitor of the intracellular tyrosine kinase domains of oncogenic receptors Her-2 and EGFR. In a study performed by Dai, Tiwari et al. (2008), they found that lapatinib reversed the activity of both P-gp and BCRP in ABCB1 and ABCG2 over-expressing MDR cells, towards substrate chemotherapeutics, respectively. Mechanistically though, it was found that lapatinib reversed MDR of both P-gp and BCRP through direct interaction with the transporter, not through inhibition of HER-2 and EGFR.

1.11. Wnt/β-catenin and Regulation of P-gp Expression and Activity

Numerous pathways have been elucidated in the regulation of MDR, where the members of the ABC transporters, especially P-gp, have been found to play a major role. Yet the underlying mechanisms of how P-gp expression and activity are increased in MDR are not clear and largely unknown. Yamada, Takaoka et al. (2000) identified that the promoter region of *MDR1* gene contained T cell factor/lymphoid enhancer factor (TCF/LEF)/β-catenin response elements and through cDNA microarray analysis identifying that the *MDR1* gene was indeed a target for the TCF/LEF/β-catenin transcription complex. Since this observation, studies have focused on if and how the canonical wnt/β-catenin pathway is involved in regulation of P-gp expression, activity and MDR. For example, one such study conducted by Shen, Zhang et al. (2013), showed that 5-flourouracil resistant human cholangiocarcinoma cells (QBC939/5-FU),

had increased β-catenin levels and P-gp expression compared to 5-FU susceptible controls. Furthermore, it was shown that the exposure of QBC939/5-FU resistant and QBC939 control cells to wnt/β-catenin pathway activator wnt3a, resulted in increased P-gp and β-catenin expression, enhancing their 5-FU resistance. Furthermore, QBC939/5-FU resistant cells exposed to siRNA targeted towards β-catenin (siβ-catenin), restored their susceptibility towards chemotherapeutics, providing evidence that the wnt/β-catenin pathway is involved in P-gp mediated MDR. In addition, Lim, Kania et al. (2008), demonstrated that enhanced β-catenin signalling caused by GSK-3β inhibitors, as well as wnt receptor agonists, lead to increased expression of P-gp as well as other ABC transports including MRP4 (ABCC4) and BCRP (ABCG2) in rat primary and human immortalized brain endothelial cells. Furthermore, the addition of transcriptional β-catenin signalling antagonist resulted in a decrease in P-gp expression (Lim, Kania et al. 2008). Therefore, there is mounting evidence that the canonical wnt/β-catenin pathway is important in regulating P-gp expression and activity.

The wnt signalling pathway can be divided into two types, one involving β-catenin, known as the canonical wnt/β-catenin signalling pathway and the other independent of β-catenin, known as the non-canonical wnt signalling pathway. Since most studies, though few, have focused on looking at the effects of wnt/β-catenin signalling on P-gp mediated MDR, the focus of this section will be on the canonical wnt/β-catenin signalling pathway.

The wnt/β-catenin signalling pathway has been found to play an important role in regulating and promoting cell proliferation, tissue expansion, and cell fate in a wide variety of tissues including brain, bone, gut and blood (Nelson and Nusse 2004; Liu, Wan et al. 2014). For instance, in the gut the canonical wnt/β-catenin signalling pathway has been found to be involved in the constant epithelium turn-over in the small intestine where progenitor cells within the villi

and crypt of the small intestine differentiate into epithelia (Clevers 2006). In addition, wnt/β-catenin signalling has been found to be important in maintaining barrier properties and barrier tight junctions in the BBB through the regulation of cadherin-3 and P-gp protein expression, as well as controlling angiogenesis in the central nervous system (Liebner, Corada et al. 2008; Daneman, Agalliu et al. 2009; Liu, Wan et al. 2014). Considering the canonical wnt/β-catenin signalling pathways physiological role in a variety of tissues, it isn't surprising that under aberrant conditions the wnt/β catenin pathway has been linked to diseases such as cancer and Alzheimer's disease (Clevers 2006).

Within the mammalian genome, approximately 20 secreted wnt proteins have been identified that are divided into 12 conserved wnt subfamilies (Clevers 2006). These wnt proteins, formed in the Golgi, are targeted towards the cell surface. Once the cell surface is reached, the wnt proteins become secreted into the extracellular space, where they can interact and bind with cell surface receptors on neighbouring cells known as frizzled (Fz) receptor proteins. In vertebrates, once a wnt protein binds to a Fz cell surface receptor, it causes a conformational change that allows interaction with lipoprotein receptor-related protein (LRP) 5 or -6 to form a co-receptor complex (Pinson, Brennan et al. 2000; Tamai, Semenov et al. 2000). Upon formation of the Fz/LRP complex once a wnt protein is bound, dishevelled (Dsh), a cytoplasmic protein, becomes phosphorylated, but its function in the wnt signalling pathway is currently unknown (Clevers 2006). What has been shown to be important in the activation and proliferation of downstream cellular signalling from the Fz(LRP5/6) co-receptor is five proline-proline-proline-(serine/threonine)-proline (PPP(S/T)P) motifs. Here it has been identified that both GSK-3β and casein kinase1-γ (CK1γ) are involved in carrying on the downstream wnt signalling pathway by phosphorylating sites within the PPP(S/T)P motifs and sites that flank these regions, however,

which kinase(s) controls wnt stimulation is still under debate (Davidson, Wu et al. 2005; Zeng, Tamai et al. 2005). Once phosphorylation of these sites on the LRP portion of the Fz(LRP5/6) co-receptor has occurred, wnt suppressor and β-catenin destruction complex scaffolding protein axin, docks onto the phosphorylated Fz(LRP5/6) co-receptor, preventing the formation of the β-catenin destruction complex and resulting in the prevention of β-catenin ubiquitination (Clevers 2006; Liu, Wan et al. 2014). Therefore, activation of the canonical wnt/β-catenin pathway stabilizes and increases the cell pool of β-catenin allowing it to enter the nucleus. Upon entering the nucleus, β-catenin acts as a transcription co-factor where it displaces groucho from the TCF/LEF transcription repressor complex and then binds TCF/LEF forming a transcription activator complex (Behrens, von Kries et al. 1996; Nelson and Nusse 2004; Daniels and Weis 2005). The newly formed β-catenin(TCF/LEF) transcription activator complex can then bind to its promoter element and increase the transcription of wnt genes favouring cell proliferation and tissue growth. For example, wnt/β-catenin pathway regulated genes known to increase in transcription in endothelial cells include claudin-3, glucose transporter-1 (GLUT-1), platelet-derived growth factor-B (PDGF-B), as well as P-gp (Lim, Kania et al. 2008; Reis, Czupalla et al. 2012). β-catenin also is known to play a second role in maintaining tight junctions and cell to cell adhesion, where it acts to link cadherins to the actin cytoskeleton in cadherin junctions (Behrens, von Kries et al. 1996; Clevers 2006).

Conversely, if Fz is not bound by a wnt protein, the canonical wnt/β-catenin pathway is not activated. Interestingly, GSK-3β also plays a role in antagonizing β-catenin production, whereas during wnt activation, GSK-3β acts as a β-catenin production agonist. Currently it is theorized that membrane associated GSK-3β is involved in activation of the wnt/β-catenin pathway, whereas cytosolic GSK-3β is thought to be involved in inhibiting the wnt/β-catenin

pathway (Zeng, Tamai et al. 2005). Here, CK1γ phosphorylates β-catenin at conserved Ser/Thr residues (Clevers 2006), and is followed by GSK-3β phosphorylation, unlike the stimulatory pathway, where GSK-3β can phosphorylate LRP5/6 in a non-CK1γ dependent manner. This phosphorylation event is done by the β-catenin destruction complex, which consists of GSK-3β, CK1γ, adenomatosis polyposis coli (APC) and axin, which acts as a scaffolding protein in holding the complex together. Once phosphorylated by this complex, β-catenin becomes recognized by the proteasome and is degraded (Nelson and Nusse 2004). Therefore, at the nucleus level, β-catenin translocation becomes absent and the groucho/TCF/LEF complex remains bound to its response element, acting as a transcriptional repressor of wnt genes.

2.0. Objectives

The objectives of these studies were to screen a PKI library and identify PKI families and compounds that influence ABC transporter expression and activity in different human representative cell lines including kidney, intestine and brain. Focus was put on the ABC transporter P-gp, as this transporter is found to be highly involved in altering the pharmacokinetics (PK) of numerous drug substrates and is the primary force behind MDR. Furthermore, we screened a canonical wnt/β-catenin library using a human BBB representative cell line to identify whether this pathway is important in regulating P-gp expression and/or activity within the BBB, where P-gp forms one of the major barrier properties in limiting cytotoxic agents from entering the central nervous system (CNS). In addition, we also screened known P-gp inducer, FXR ligand CDCA, as a positive control in our P-gp activity and expression experimental models to validate our experimental models for the identification of screened compounds that significantly modulated P-gp activity and/or expression. Lastly, we

wanted to relate the impact of any identified PKI or wnt/β-catenin inhibitor found to significantly modulate P-gp expression and/or activity on permeability of a P-gp substrate across a monolayer.

3.0. Materials and Methods

3.1. Materials/Reagents

PKI library was provided by GlaxoSmithKline in Research Park, NC. Wnt/β-catenin activator and inhibitory pathway library was provided by Enzo Life Sciences (Brockville, ON, Canada). IRdye 800CW polyethylene glycol (PEG) and In-Cell Western kit were purchased from Licor (Omaha, NE, USA). elacridar (GF120918) was purchased from Toronto Research Chemical Inc. (North York, ON, Canada). Mouse anti-human *ABCG1* antibody (NBP1-42581) and rabbit anti-mouse IgG antibody (NB720-H) were purchased from Novus Biologicals (Littleton, CO, USA). Dulbecco's modified eagle's medium, standard fetal bovine serum, non-essential amino acids solution and 1X trypsin were obtained from Hyclone (Logan, UT, USA). Penicillin-streptomycin was purchased from MP Biomedicals (Solon, OH, USA). Endothelial cell growth media (EBM-2) was purchased from Lonza (Basel, Switzerland) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), basic fibroblast growth factor (bFGF) recombinant human protein and chemically defined lipid concentrate were purchased from Life Technologies (Carlsbad, CA, USA). 96 black-well and clear-well plates, 24-well plates and 6-well trans-well polycarbonate membrane inserts (0.4 μm pore size; 24 mm diameter) were purchased from Corning Incorporated (Corning, NY, USA). COmplete, Mini, ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablet was obtained from Roche Applied Sciences (Indianapolis, IL, USA) and the Pierce bicinchoninic acid (BCA)

protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Immuno-blot polyvinylidene fluoride (PVDF) membrane, Bio-Rad trans-blot semi-dry (SD) electrophoretic transfer cell, clarity western blot substrate and bio-safe coomassie blue were purchased from (Bio-Rad, Mississauga, ON, Canada). Re-blot plus strong solution was purchased from Chemicon International (Billerica, MA, USA). All other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and R&D systems (Minneapolis, MN, USA).

3.2. Cell Culture

Madine-Darby canine kidney epithelial cells-WT (MDCK-WT) were purchased from American Type Tissue Culture Collection (Manassas, VA, USA) and MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1) were provided by M. Gottesman, National Cancer Institute (Bethesda, MD, USA). Human caucasian colon adenocarcinoma-2 (Caco-2) cells were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Both MDCK-WT and Caco-2 cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin and 1% (v/v) non-essential amino acids at pH 7.4. P-gp expression was maintained in MDCK-MDR1 cells via the addition of 80 ng/ml colchicine to the DMEM media. Immortalized human cerebral microvascular endothelial (hCMEC/D3) cells were obtained from Professor Pierre Couraud, INSERM, France. The hCMEC/D3 were cultured on collagen coated plates and grown in EBM-2 media supplemented with 5 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin, 1.4 µM hydrocortisone, 5 µg/ml ascorbic acid, 10 mM HEPES, 1 ng/ml bFGF and 1 % (v/v) chemically defined lipid concentrate at pH 7.4. Cells were cultured in 75 cm^2 culture flasks and seeded onto 96-black-well tissue culture plates at a density of 15,625 cells/cm^2 or 24-well culture plates at a seeding density of 26,316 cells/cm^2 or 6-trans-well polycarbonate (0.4 µM) inserts at a density of

535,332 and 642,398 cells/cm^2 for MDCK-WT and MDCK-MDR1, respectively. Cells were grown in a humidified environment at 37 °C with 5 % CO_2 with media replacement every other day until confluent and every day upon reaching confluency.

3.3. P-gp and BCRP Transporter Activity Assays (Complete Screening of GSK PKI Library for Modulation of P-gp Activity in MDCK Cell Line was performed by Ruth Habte)

Cells were cultured to 80 % confluency on 96-black-well plates and pre-treated with various PKIs, or wnt/β-catenin activators or inhibitors at 2 μM or 0.2 μM (if significant cytotoxicity occurred at 2 μM treatment) in media over a 24 h period. For the MDCK-MDR1 cell line, 80 ng/ml colchicine was not added to the incubation media during this 24 h period. Control groups received only cultured media. After the 24 h period, cells were pre-treated for 30-min at 37 °C with 100 μl assay buffer/well (AB, containing in g/L; 7.10 NaCl, 0.22 KCl, 0.21 $CaCl_2$, 0.30 $MgSO_4$, 2.10 $NaHCO_3$, 2.38 HEPES, 1.80 glucose, and 0.07 K_2HPO_4 at a pH of 7.4) with or without 5 μM GF120918 (potent P-gp inhibitor) for the PKI and wnt library screens. For the concentration dependent activity assays instead of 30 min pre-treatment with AB, DMEM (+) media was used with or without 5 μM GF120918. Cells were then incubated with 3.2 μM r123, a P-gp probe substrate or 3.2 μM mitoxantrone, a BCRP probe substrate, with or without 5 μM GF120918 in AB at 37 °C, over a 90 min period. Following this, solutions were aspirated, and the cells were washed three times with 100 μl/well of ice-cold phosphate buffered saline solution ((PBS) containing in g/L; 8.5 NaCl, 0.46 NaH_2PO_4, and 0.95 Na_2HPO_4). R123 fluorescence was measured in the cells using Synergy HT plate reader at 488 nm excitation wavelength and 520 nm emission wavelength, whereas mitoxantrone fluorescence was measured using the Odyssey near infrared imaging system (LI-COR, Biosciences, Lincoln, NE) at the 700 nm channel.

3.4. Determination of P-gp and BCRP Activity using Efflux Activity (EA)

To determine P-gp and BCRP activity per PKI treatment, an efflux activity (EA) was calculated using the formula shown below. In the MDCK cell lines EA was determined by taking the normalized fluorescence values obtained in MDCK-WT cells and dividing that by the corresponding normalized fluorescence values obtained in MDCK-MDR1 cells. As there are no parental or wild-type cells for Caco-2 and hCMEC/D3 cell lines, EA was determined as the ratio of fluorescence values under GF 120918 treated conditions divided by fluorescence values under non-GF control conditions. GF120918 treated conditions represented the MDCK-WT cell line and non-GF treated conditions represented the MDCK-MDR1 cell line to allow comparisons across cell lines for Caco-2 and hCMEC/D3. EA values were obtained for the both PKI treated and control (no PKI) groups.

$$EA = \frac{MDCKWT\ normalized\ fluorescence\ values}{MDCKMDR1\ normalized\ fluorescence\ values}\ or\ \frac{GF\ 120918\ treated\ conditions}{non\ GF120918\ treated\ conditions}$$

To determine how much transporter activity was altered per PKI treatment compared to control, an EA Percent was determined based on the following equation:

$$EA\ Percent = \left(\frac{(EA\ PKI - EA\ control\ )}{EA\ control}\right) x\ 100$$

PKIs that yielded EA percents ≥ 25 % compared to controls (no PKI) were considered to significantly alter transporter activity and were termed, "P-gp activity hits". P-gp activity hits were followed up to see if they also altered P-gp or BCRP expression using the In-Cell Western assay described below.

3.5. Concentration Dependent Accumulation Studies, P-gp

MDCK-WT and -MDR1 cells were grown to confluency in 24-well and 96-black-well plates and MDCK-MDR1 cells were treated with varying concentrations of PKI and positive control CDCA ranging from 1 nM to 100 µM at 1 % (v/v) DMSO, for 24 h in DMEM(+) media. During this 24 h incubation period, 80 ng/ml colchicine was not provided to the MDCK-MDR1 cells. MDCK-WT did not receive PKI treatment and was strictly used as a non-P-gp transfected cell line control. Following PKI or positive control treatment, cells were then pre-treated with 0.5 ml DMEM(+) media for 30 min at 37 °C with or without 5 µM GF120918. Immediately following pre-treatment, solutions in wells were aspirated and incubated with DMEM(+) media containing 3.2 µM r123 with or without 5 µM GF120918 for 2 h at 37 °C. Following incubation, cells were washed three times with 0.5 ml/well of ice cold PBS and solubilised with 0.5 ml/well of 1 % (v/v) triton-X 100 overnight (O/N). R123 fluorescence was read using the Synergy HT plate reader at 488 nm excitation wavelength and 520 nm emission wavelength and normalized to cellular protein. Cellular protein was measured at an absorbance at 562 nm, using the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, Illinois), as described previously.

3.6. Bi-Directional Equilibrium Permeability Studies, P-gp

To determine active transcellular mediated transport by P-gp, directional equilibrium permeability studies were performed using the MDCK-WT, MDCK-MDR1 and Caco-2 cell lines using 6-trans-well polycarbonate (0.4 µm pore size, 24 mm diameter) inserts. Monolayer progression was monitored by measuring trans epithelial electric resistance (TEER) using the epithelial voltohmmeter (EVOM) World Precision Instruments Inc., Stevenage, United Kingdom) every day up until the experiment was performed. Monolayers were allowed to grow

for 9 and 14 days until TEER readings were ≥ 165/119 Ωcm^{2}and ≥ 726 Ωcm^2 for MDCK-MDR1/WT and Caco-2 cell lines, respectively. On the day of the experiment media was removed and replaced with DMEM(+) media with or without activity modulating agents and GF120918, in addition to 3.2 µM r123 at the same concentration on both the apical (1.5 ml) and basolateral (2.5 ml) sides. IRDye 800CW PEG was used as a paracellular maker and was added only to the apical side at 0.1 µM. The cells were incubated with these solutions at 37 °C for 24 h. During this 24 h period colchicine was not added to the MDCK-MDR1 cells. At the end of the 24 h period, 100 µl samples were taken from the apical and basolateral compartment of each monolayer and were transferred to a 96-black-well plate. IRDye 800CW PEG fluorescence was measured using the Odyssey near infrared imaging system (LI-COR, Biosciences, Lincoln, Nebraska) at the 800 nm channel and r123 fluorescence was measured using the Synergy HT plate reader at 488 nm excitation wavelength and 520 nm emission wavelength. A percent flux was calculated for IRDye 800CW PEG to give an idea on how tight the cell monolayer was at the 24 h time point. A percent flux ≥ 20 % was considered leaky. P-gp active transcellular mediated transport was determined by calculating the apical to basolateral ratio (a/b) of r123 concentration, as described previously (Gaillard et al. 2000).

3.7. Immunofluorescence Assay and Quantification of P-gp Expression (Complete Screening of GSK PKI P-gp Activity Hits for Alterations in P-gp Expression in the MDCK Cell Line was performed by Ruth Habte)

PKIs that were classified as hits (altered P-gp activity greater than 25 % compared to control (no PKI)), were analysed for potential changes in *ABCG1* (P-gp) expression using the In-Cell Western assay protocols developed for the Odyssey infrared imaging system by LI-COR Biosciences (Lincoln, NE). Briefly, cells were fixed and permeabilized using 4 % (v/v)

formaldehyde and 1 % (v/v) triton-X, respectively. Cells were then blocked with blocking buffer, and incubated with primary antibody overnight (O/N). Mouse anti-human *ABCG1* (NBP1-42581, 1:100, Novus Biologicals, USA) and mouse anti-human *ABCG2* (NBP1-59749, 1:100, Novus Biologicals, USA) was used for P-gp and BCRP, respectively. Cells were then washed and incubated for 1 h at room temperature with secondary immunofluorescent antibody, IRDye 800CW-infrared fluorescent dye-conjugated goat anti-mouse antibody (1:500, LI-COR Biosciences, NE, USA). Cells were washed and IRDye 800CW fluorescence was measured using the Odyssey near infrared imaging system (LI-COR, Biosciences, Lincoln, NE) at the 800 nm channel. Background secondary antibody staining controls were performed with the omission of the primary antibody to determine non-specific binding potential of the secondary antibody. P-gp protein expression levels were normalized for cell number via draq5 and sapphire700 DNA stains and were measured at the 700 nm channel of the Odyssey near infrared imaging system (LI-COR, Biosciences, Lincoln, NE) and compared to expression levels in cells receiving no PKI treatment.

To determine how much transporter expression was altered per PKI treatment compared to control an Expression Percent (Ex Percent) was calculated from the IRDye 800CW fluorescence values obtained from the In-Cell Western assay, using the following equation:

$$Ex\ Percent = \left(\frac{(normalized\ Ex\ PKI - normalized\ Ex\ control)}{normalized\ Ex\ control}\right) 100$$

PKIs that yielded Ex percents ≥ 25 % compared to controls (no PKI) were considered to significantly alter transporter expression and were termed, “PKI expression hits”.

3.8. Western blot Analysis and Quantification of P-gp Expression

MDCK-MDR1 and –WT, Caco-2 and hCMEC/D3 cells were grown to 80 % confluence in T-75 flasks. T-75 flasks containing MDCK-MDR1 and –WT cells were treated by adding 100 µM CDCA dissolved in DMSO, LiCl (10-100 mM) dissolved in double distilled water (ddH_20), to DMEM (+) media for 24 h. After the 24 h treatment period, cell media was aspirated and cells washed with 5 ml PBS buffer per T-75 flask. Then 200 µl to 300 µl of ice cold nonidet P-40 (NP-40) lysis buffer was added directly to the T-75 flask and cells were harvested. Total cell lysate was then agitated for 30 min at 4 °C, followed by centrifugation at 20,000 rpm for 20 min at 4 °C. Here, the total cell protein supernatant was collected and the pellet was discarded. Protein concentration was measured using the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA. Briefly, two types of reagents, A (containing sodium carbonate, sodium bicarbonate, BCA and sodium tartate in 0.1 M sodium hydroxide) and B (containing 4 % (w/v) cupric sulphate) are combined in a 50:1, Reagent A:B ratio and added to wells containing cell lysate. Upon addition of reagent solution to cell lysate, Cu^{+2} is reduced to Cu^{+1} in a protein concentration dependent manner. Resulting Cu^{+1} combines with two molecules of BCA, forming a purple-coloured reaction product that increases linearly with increasing protein concentration and can be measured at a maximum absorbance at 562 nm. Total protein in each sample is then quantified in mg/ml based on a bovine serum albumin (BSA) standard.

Total cell protein samples were prepared for electrophoresis by adding laemmli loading buffer (Sigma-Aldrich, St. Louis, MO) to 60 µg of total cell protein of MDCK-MDR1 and –WT CDCA or LiCl treated and solvent matched controls. For Caco-2 and hCMEC/D3 cell lines, 80 µg of total cell protein was used. Subsequently, total cell protein samples were denatured by heating for 15 min at 42 °C before loading onto an 8 % polyacrylamide gel and run at 150 V.

Subsequently, separated protein samples were then transferred to an immuno-blot PVDF membrane (Bio-Rad Laboratories, Mississauga, Ontario), using Bio-Rad trans-blot semi-dry (SD) electrophoretic transfer cell (Bio-Rad, Mississauga, Ontario). Total transfer time was 2 h, maintained at 15 V.

Immediately following transfer, gel electrophoresis was evaluated by staining the gel with approximately 20 ml Bio-Safe coomassie blue (Bio-Rad, Mississauga, Ontario) for one hour at RT. Additionally, the membrane was incubated in 10 ml blocking buffer consisting of 5 % (w/v) skim milk in tris-buffered saline with tween (TBS-T) (50 mM tris-HCL, 150 mM NaCl pH 7.5, 0.05 % (v/v) Tween-20), under constant agitation for 60 min at RT. Subsequently, membranes were incubated with mouse anti-human *ABCG1* primary antibody (NBP1-42581), diluted to 1:50 (2 µg/ml) in blocking buffer, under constant agitation at 4 °C, O/N. The next day, membranes were washed three times at RT for 10 min each in TBS-T, followed by incubation of the membranes with rabbit anti-mouse immunoglobulin G-horse radish peroxidase (IgG-HRP) secondary antibody (NB720-H), diluted to 1:20,000 (0.05 µg/ml) in blocking buffer at RT, for 60 min.

After incubation with secondary antibody, membranes were washed with TBS-T, as described previously and bands were visualized by incubating membranes in clarity western blot substrate (Bio-Rad, Mississauga, Ontario) for 60 sec. Membranes were immediately visualized and quantified, following clarity blot substrate removal, using the Bio-Rad ChemiDoc MP imaging system and ImageLab 5.1 Beta Build 1 software.

Actin was used to determine the consistency in the total amount of protein loaded per lane. The membranes were stripped by incubating them in re-blot plus strong solution (Chemicon

International, Billerica, MA, USA) at RT for 15 min, under constant agitation. The membrane was then treated, following the similar procedure as described above. After incubating in blocking buffer, the membrane was removed and actin antibody (A1978) from Sigma-Aldrich Chemical Company (St. Louis, MO), was added at 1:10,000 at RT, for 1 h under constant agitation. Afterward, membranes were washed three times in TBS-T buffer followed by incubation of rabbit anti-mouse IgG-HRP secondary antibody (NB720-H), diluted to 1:20,000 (0.05 µg/ml) in blocking buffer at RT, for 60 min under constant agitation. Subsequently, excess secondary antibody was removed and the membrane was washed three times in TBS-T at RT, for 10 min each with constant shaking, which was followed by visualization and quantification of actin as described above.

P-gp expression in each lane was quantified by taking the volume (intensity) of the band corresponding to P-gp (170-kDa) and dividing that by the volume (intensity) of actin found in the same lane, using the Bio-Rad ChemiDoc MP imaging system and ImageLab 5.1 Beta Build 1 software.

3.9. Statistical Analyses

All data were expressed as mean ± standard error of the mean (SEM). Comparison among the groups of data were evaluated by either one-way ANOVA or two-way ANOVA in conjunction with either the Dunnett's multiple comparison of the means test or Tukey's multiple comparison of the mean test, where seen fit. The Dunnett's multiple comparison test was used when comparing the means of multiple treatments to a non-treated control, whereas the Tukeys multiple comparisons test was used to compare each mean to each other mean within a particular

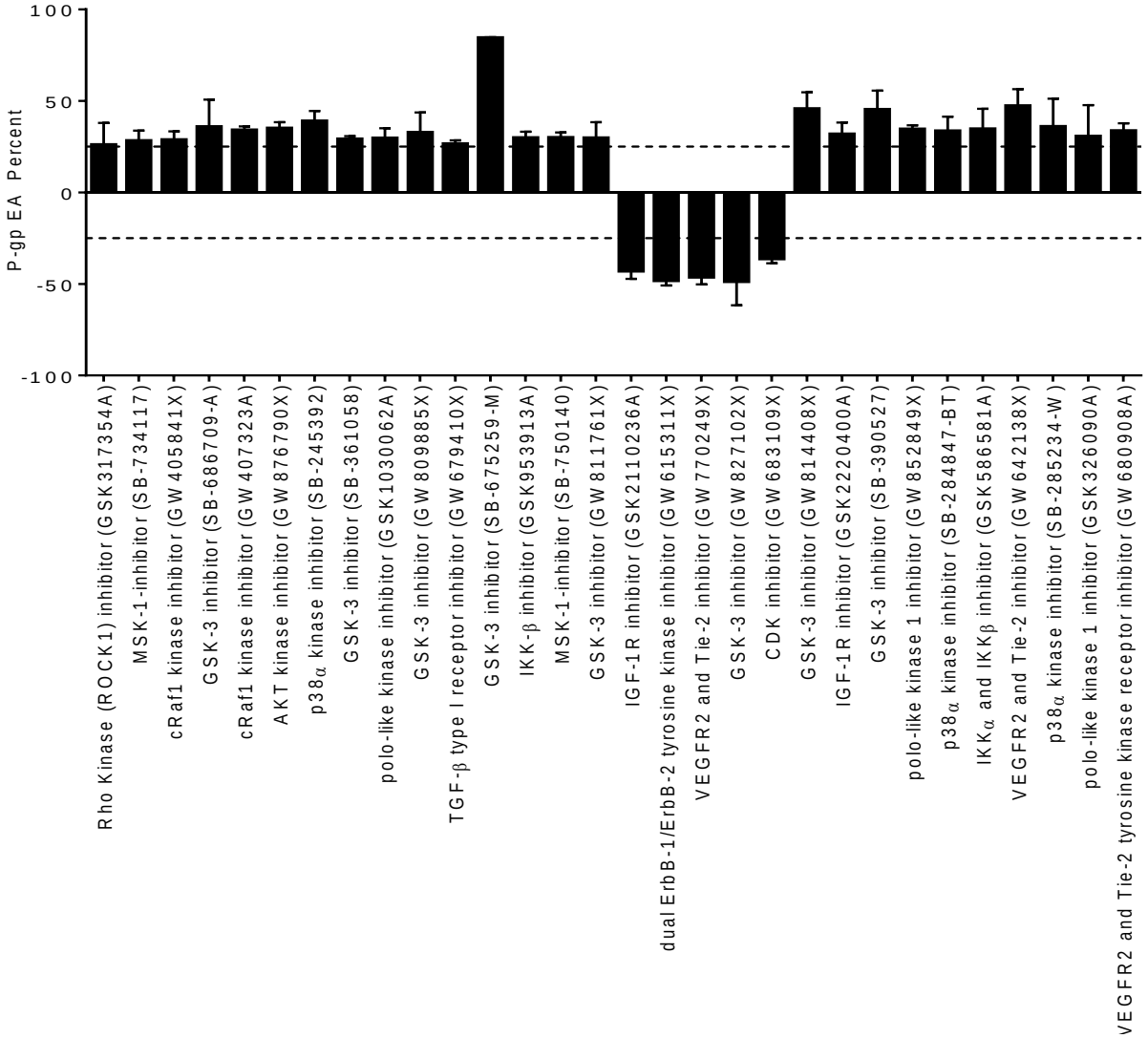
data set. The tests were performed using GraphPad Prism, version 6 software (San Diego, CA, USA) where *p* values less than 0.05 were considered significant, unless otherwise noted.

4.0. Results

4.1. P-gp and BCRP Transporter Activity Assays

To examine whether PKIs from a library consisting of 360 compounds, impacted on P-gp activity, transporter activity assays were performed using P-gp fluorescent markers, after chronic exposure (24 h) of a particular PKI in different cell lines. Of the 360 PKIs examined in the MDCK cell line, 30 were found to be hits, altering EA by ≥ 25% compared to controls (no PKI) (Figure 1). Most of the hits (25 PKIs) resulted in increased P-gp activity, although 5 PKIs produced a decrease in P-gp activity (Figure 1). PKIs that targeted the GSK-3 family of protein kinases gave the most hits (8 hits). The, PKI, SB-675259-M, targeting GSK-3 yielded the greatest increase in P-gp activity compared to the rest of the 30 hits with an overall increase in P-gp EA percent of 84.5 ± 0.2 %, compared to control. The PKI GW827102X targeting the GSK-3 protein kinase family was found to exhibit the greatest decrease in P-gp activity by 48.7 ± 13.0 % compared to control, in the MDCK cell line (Figure 1).

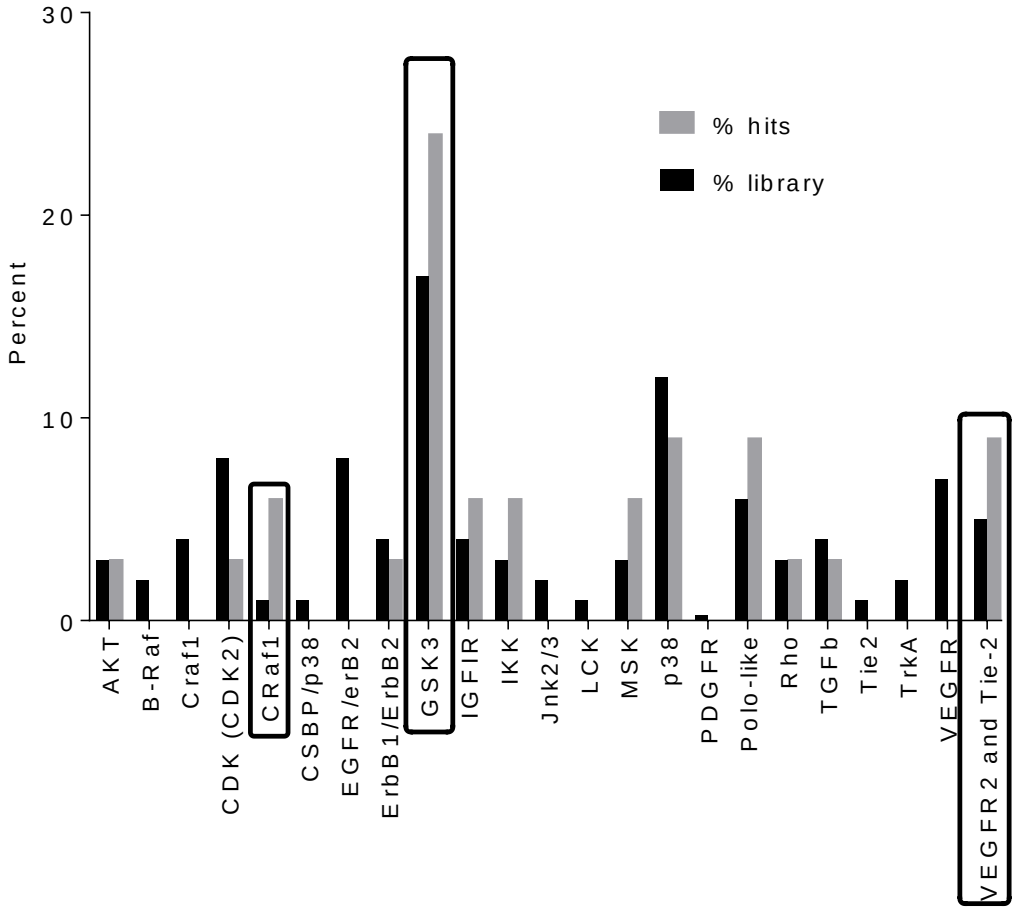
Figure 1: P-gp activity hits identified from the GlaxoSmithKline PKI library screen in the MDCK cell line. Values represent the mean ± SEM of n= 3 wells per PKI. Alterations in P-gp EA of ≥ 25 % compared to non-treated control were considered hits and are represented by the horizontal dashed lines. PKIs are identified as the protein kinase they inhibit, in addition to GlaxoSmithKline library compound identification in brackets.



To compare and identify particular protein kinase families that maybe important in regulating P-gp activity, the percent hits for each PKI family was calculated and plotted in

relation to the particular PKIs contribution to the chemical library. In the MDCK cell line, PKIs that targeted GSK-3, Craf1 and VEGFR/Tie-2 protein kinase families were identified as having a greater number of hits than would be expected based on the distribution within the PKI library (Figure 2).

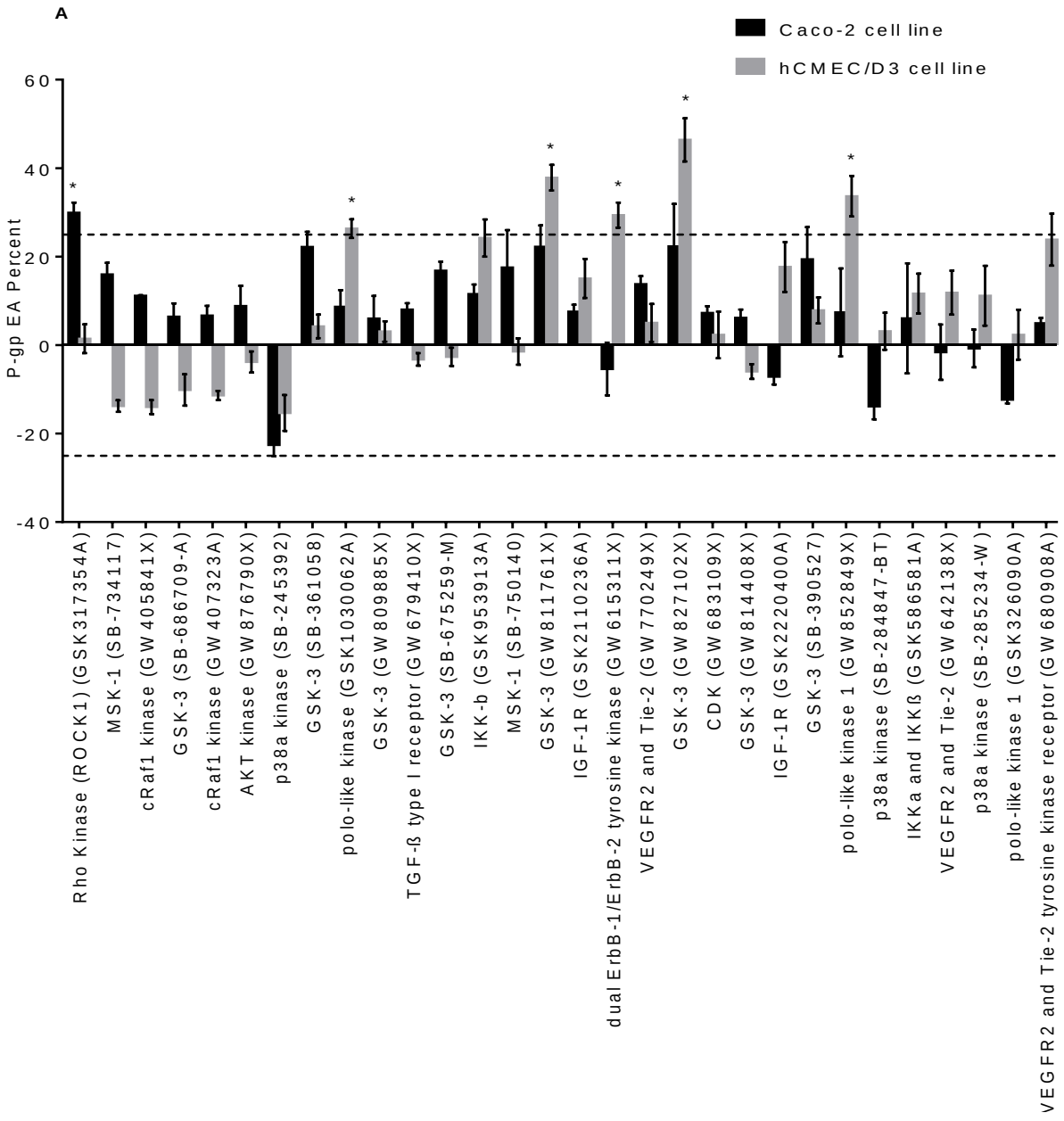
Figure 2: Percent distribution of PKI families in the GlaxoSmithKline library and corresponding percent hits from P-gp activity assay in the MDCK cell line. Outlined boxes highlight PKI families that appear to significantly modulate P-gp activity in the MDCK cell line compared to the PKI family composition of the GlaxoSmithKline library. Percent library and percent PKI hits were calculated by taking the PKIs per PKI family divided by the total PKIs in the library and the amount of hits per PKI family divided by the total amount of hits identified in the MDCK cell line, respectively.

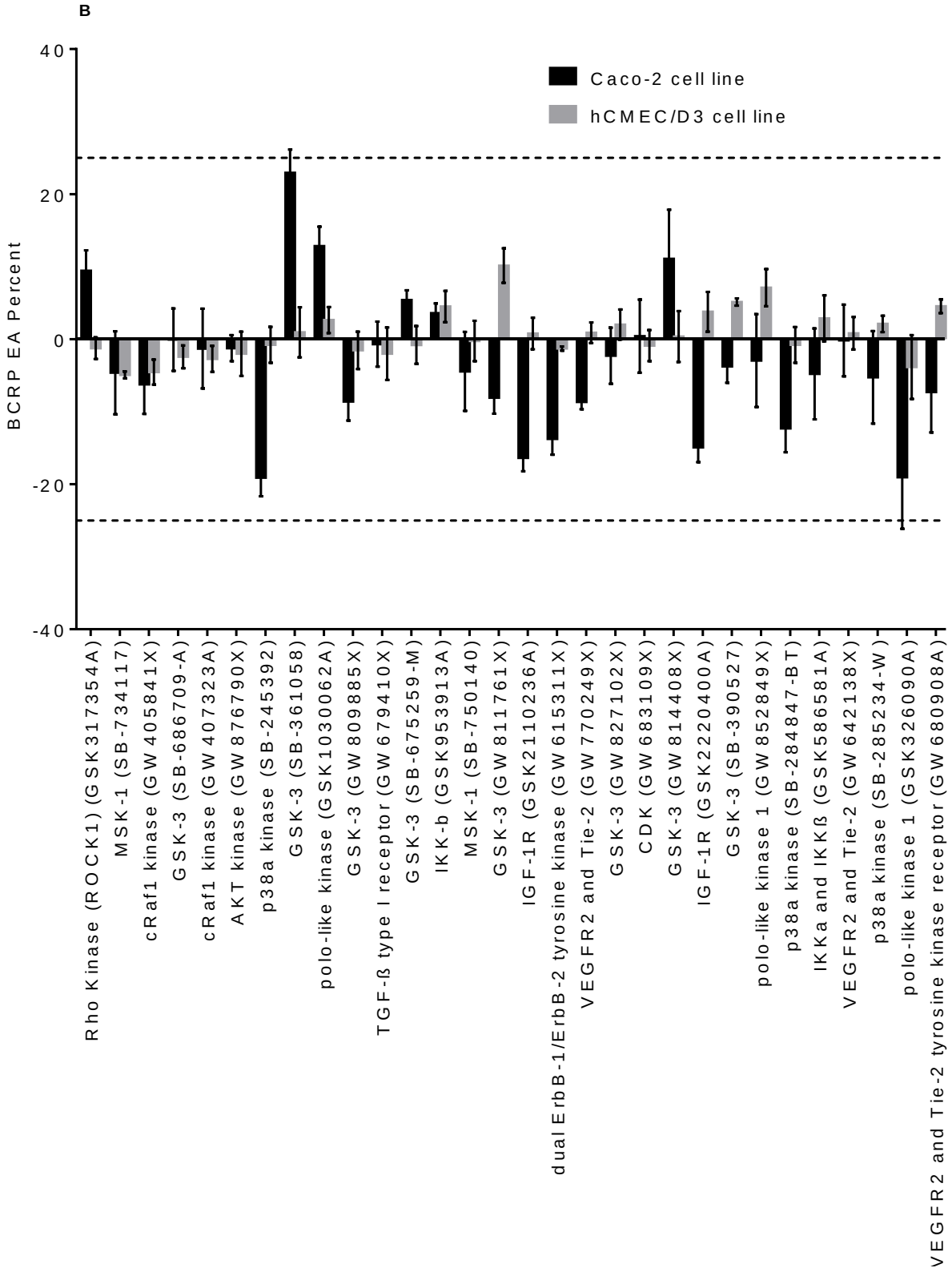


After identifying that PKIs modulate P-gp activity in the MDCK cell line, the 30 PKI hits identified were then screened and analyzed in both Caco-2 and hCMEC/D3 cell lines to see

whether or not modulation of P-gp activity by PKIs was cell line dependent (Figure 3A). In addition, PKIs effect on BCRP was also analyzed (Figure 3B). These studies were performed using transporter activity assays, as described previously. Examination of the 30 PKI hits identified in the MDCK screen in both Caco-2 and hCMEC/D3 cell lines resulted in a total of six "P-gp activity hits", that all lead to an increase in P-gp activity (Figure 3A). Rho-associated protein kinase (ROCK1) (GSK317354A) was the only compound of the 30 MDCK hits that was identified as a hit in Caco-2 and increased P-gp activity by 29.9 ± 2.3 % compared to control. Five out of the previous 30 hits were identified in hCMEC/D3, including two GSK3 inhibitors (GW811761X and GW827102X), two polo-like kinase inhibitors (GSK1030062A and GW852849X) and a dual ErbB-1/ErbB-2 inhibitor (GW615311X) which increased P-gp expression by 37.8 ± 2.9 %, 46.3 ± 4.9 %, 26.3 ± 2.1 %, 33.7 ± 4.6 % and 29.4 ± 2.8 %, respectively compared to control (Figure 3A). When these 30 PKI hits were screened in both Caco-2 and hCMEC/D3 for modulation of BCRP activity, none were found to be hits in modulating BCRP activity in these cell lines (Figure 3B).

Figure 3: Screen of PKI "hits" from MDCK cell line in Caco-2 and hCMEC/D3 cell lines. The PKIs were assayed for alterations in (A) P-gp activity and (B) BCRP activity. Values represent the mean ± SEM of n=3 samples per PKI. * EA percent values ≥ 25 % compared to non-treated matched controls were considered hits. PKIs identified based on protein kinase they inhibit, in addition to GlaxoSmithKline library PKI identification in brackets.




B
40
20
0
-20
-40
BCRP EA Percent
Caco-2 cell line
hCMEC/D3 cell line
Rho Kinase (ROCK1) (GSK317354A)
MSK-1 (SB-734117)
cRaf1 kinase (GW405841X)
GSK-3 (SB-686709-A)
cRaf1 kinase (GW407323A)
AKT kinase (GW876790X)
p38a kinase (SB-245392)
GSK-3 (SB-361058)
polo-like kinase (GSK1030062A)
GSK-3 (GW809885X)
TGF-ß type I receptor (GW679410X)
GSK-3 (SB-675259-M)
IKK-b (GSK953913A)
MSK-1 (SB-750140)
GSK-3 (GW811761X)
IGF-1R (GSK2110236A)
dual ErbB-1/ErbB-2 tyrosine kinase (GW615311X)
VEGFR2 and Tie-2 (GW770249X)
GSK-3 (GW827102X)
CDK (GW683109X)
GSK-3 (GW814408X)
IGF-1R (GSK2220400A)
GSK-3 (SB-390527)
polo-like kinase 1 (GW852849X)
p38a kinase (SB-284847-BT)
IKKa and IKKß (GSK586581A)
VEGFR2 and Tie-2 (GW642138X)
p38a kinase (SB-285234-W)
polo-like kinase 1 (GSK326090A)
VEGFR2 and Tie-2 tyrosine kinase receptor (GW680908A)

After establishing that P-gp activity tends to be more influenced by PKIs compared to BCRP and that this effect is cell line dependent, a complete screen of the PKI library was performed in the Caco-2 cell line, excluding the 30 PKI compounds that were screened in the Caco-2 cell line previously. Of the 330 PKIs examined, 18 were found to be hits (Figure 4). As in the MDCK cells, the PKIs targeting GSK-3 gave the most hits (4 hits). PKI GW276655X, targeting CDK2 led to the greatest increase in P-gp activity of 48.8 ± 21.2 % compared to control, whereas PKI SB-400868-A, targeting TGF-β decreased P-gp activity significantly by 35.0 ± 8.9 % compared to control (Figure 4). In the Caco-2 cell line PKIs targeting GSK-3, Iκκ and Jnk2/3 appear to have a greater number of hits than would be expected based on the distribution within the PKI library (Figure 5).

Figure 4: P-gp activity hits identified from 330 PKI GlaxoSmithKline PKI library screening assay in the Caco-2 cell line. Dashed lines indicate the 25% change assay set points. Values represent the Mean ± SEM of 3 samples per PKI.

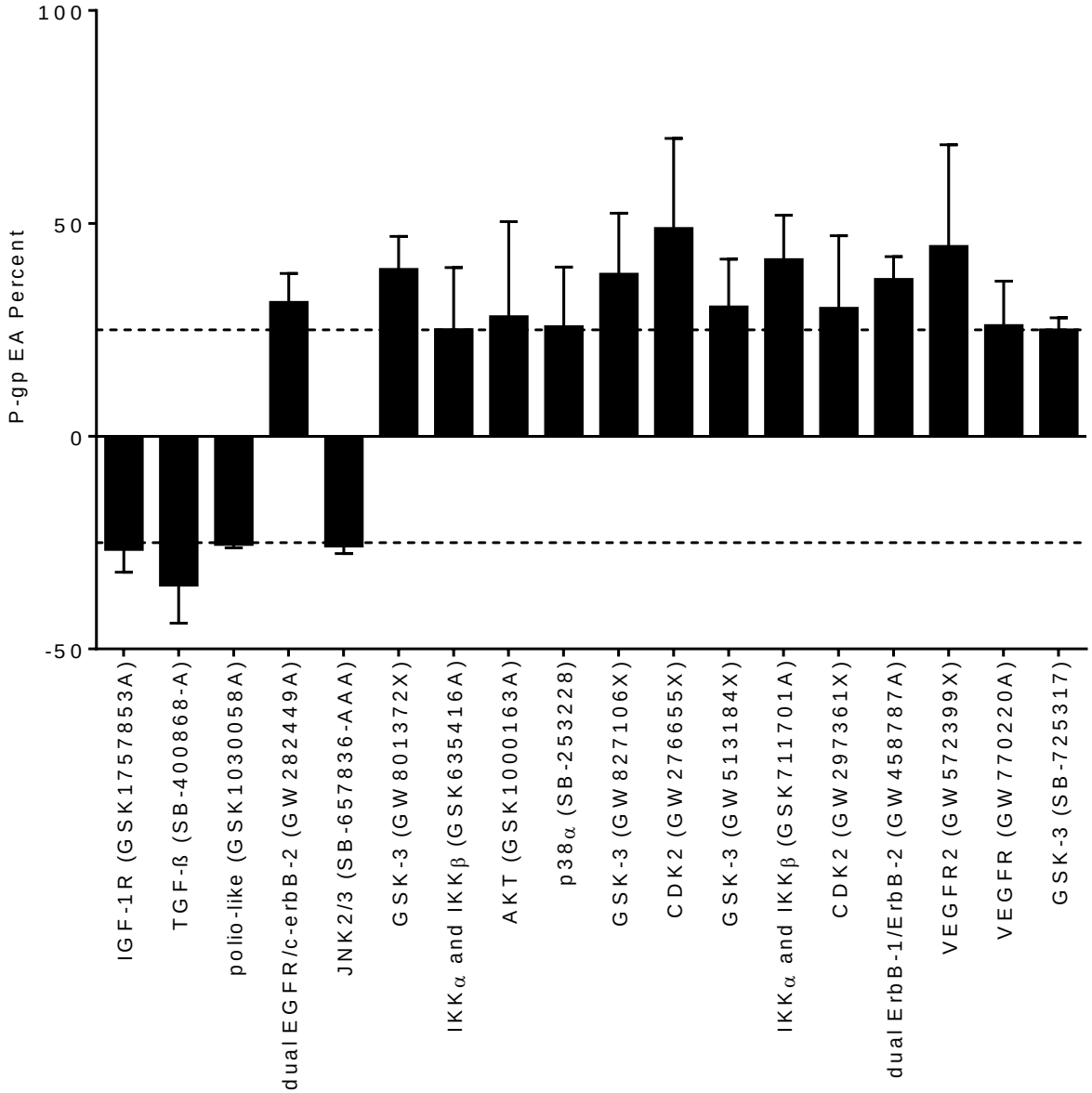
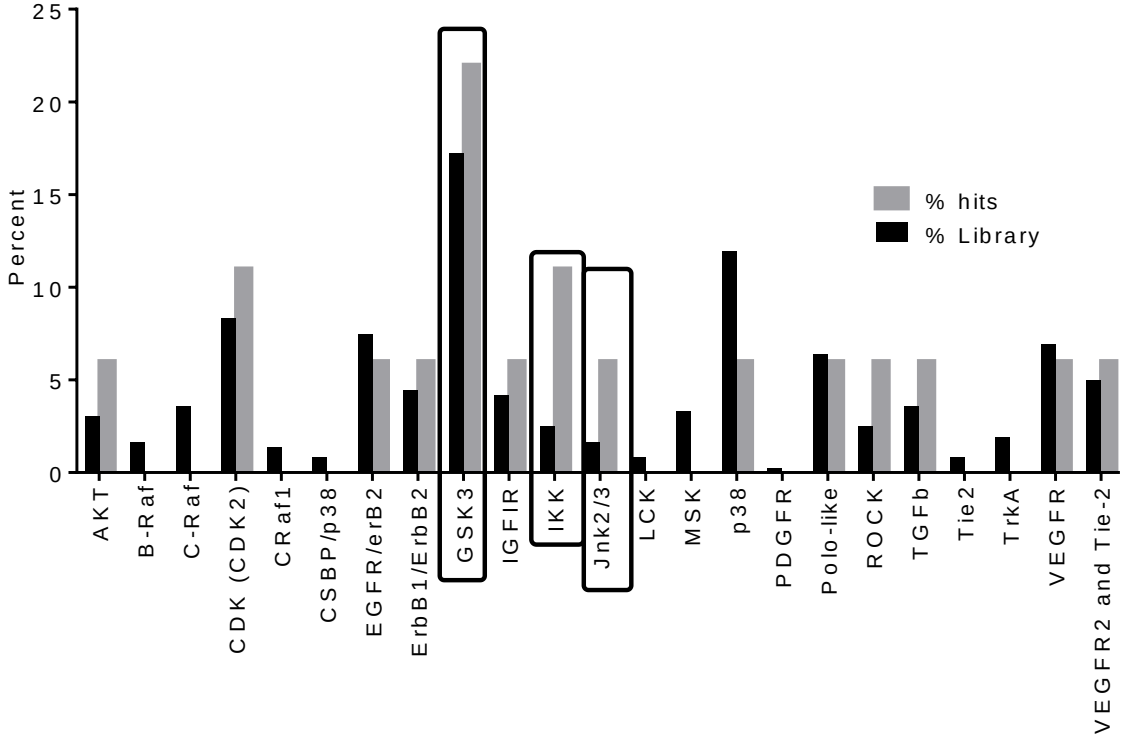


Figure 5: Percent distribution of GlaxoSmithKline library PKI families and corresponding percent hits within complete chronic (24 h) screen in Caco-2 cell line. Outlined boxes highlight PKI families that appear to have greater activity than would be anticipated based on the profile of the GlaxoSmithKline library. Percent library values represent the frequency of occurrence within the GlaxoSmithKline library of PKIs. Percent hits represent the frequency of occurrence within the total hits identified in the Caco-2 cell line.



The initial screening of PKIs indicated the GSK-3 protein kinase family as potentially important for modulating P-gp activity. As GSK-3 protein kinase activity is known to influence the wnt/β-catenin pathway in brain endothelial cells, additional screening assays were performed in hCMEC/D3 cells with a wnt/β-catenin library containing activators and inhibitors of this pathway (Table 1).

Table 1: Wnt/β-catenin pathway library of activator and inhibitory compounds list with corresponding target and mode of action. Adapted from Enzo Life Sciences (Brockville, ON, Canada) wnt/β-catenin library compound list BML-2838-0100 Version 1.0, with permission (obtained on Nov. 18, 2014).

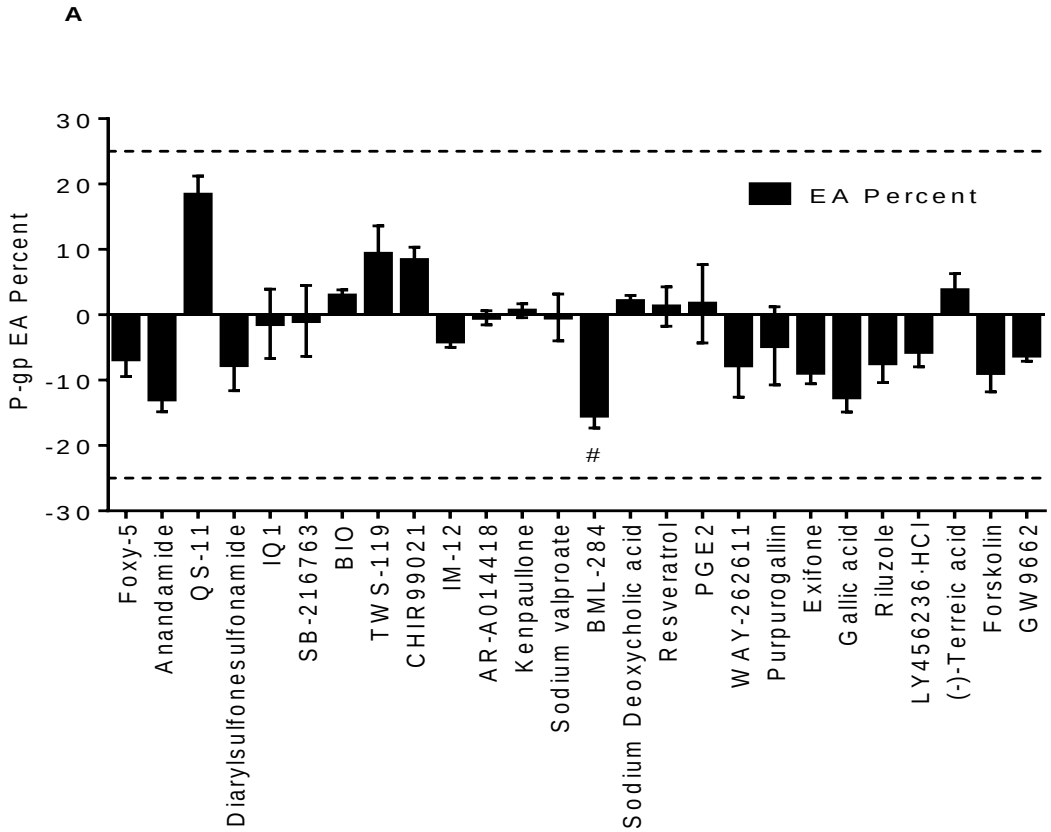
Wnt/β-catenin Pathway Activators		
Compound	**Target**	**Mode of action**
Foxy-5	Wnt5a	Agonist
Anandamide	Wnt5a	Activator
QS-11	ARFGAP1	Inhibitor
Diarylsulfonesulfonamide	sFRP-1	Inhibitor
IQ1	PP2A	Activator
SB-216763	GSK-3b	Inhibitor
BIO	GSK-3b	Inhibitor
TWS-119	GSK-3b	Inhibitor
CHIR99021	GSK-3b	Inhibitor
IM-12	GSK-3b	Inhibitor
AR-A014418	GSK-3b	Inhibitor
Kenpaullone	GSK-3b	Inhibitor
Sodium valproate	GSK-3b	Inhibitor
BML-284	GSK-3b independent	Unknown
Sodium Deoxycholic acid	β-catenin	Unknown
Resveratrol	Ak t& Erk / GSK-3b	Increase / Decrease
PGE2	cAMP/PKA	Unknown
WAY-262611	Dkk	Inhibitor
Purpurogallin	Dkk1	Inhibitor
Exifone	Dkk1	Inhibitor
Gallic acid	Dkk1	Inhibitor
Riluzole	GRM1	Inhibitor
LY456236·HCl	GRM1	Inhibitor
(-)-Terreic acid	BTK	Inhibitor
Forskolin	Adenylyl cyclase	Activator
GW9662	PPARγ	Inhibitor
Wnt/β-catenin Pathway Inhibitors		
Box5	Wnt5a	Antagonist
TNP-470	Wnt PCP pathway	Inhibitor
IWR-1	Axin	Stabilizer
XAV939	Axin / tankyrase	Stabilizer / Inhibitor
IWP-2	Porcupine	Inhibitor
Bafilomycin A1	V-ATPase	Inhibitor
Sulindac Sulfide	COX-2	Suppression Wnt target gene
Celecoxib	COX-2	Suppression Wnt target gene
Diclofenac·Na	COX-2	Suppression Wnt target gene
Niclosamide	Frizzled / Dishevelled	Internalization / Downregulation
ICG-001	CREB-binding protein	Inhibitor
Harmine·HCl	Wnt-dependent gene expression	Inhibitor
Curcumin	TCf4/β-catenin interaction	Inhibitor
PNU-74654	TCf4/β-catenin interaction	Inhibitor
Quercetin	TCf4/β-catenin interaction	Inhibitor
BML285 (Diaminoquinazoline)	TCf4/β-catenin interaction	Inhibitor

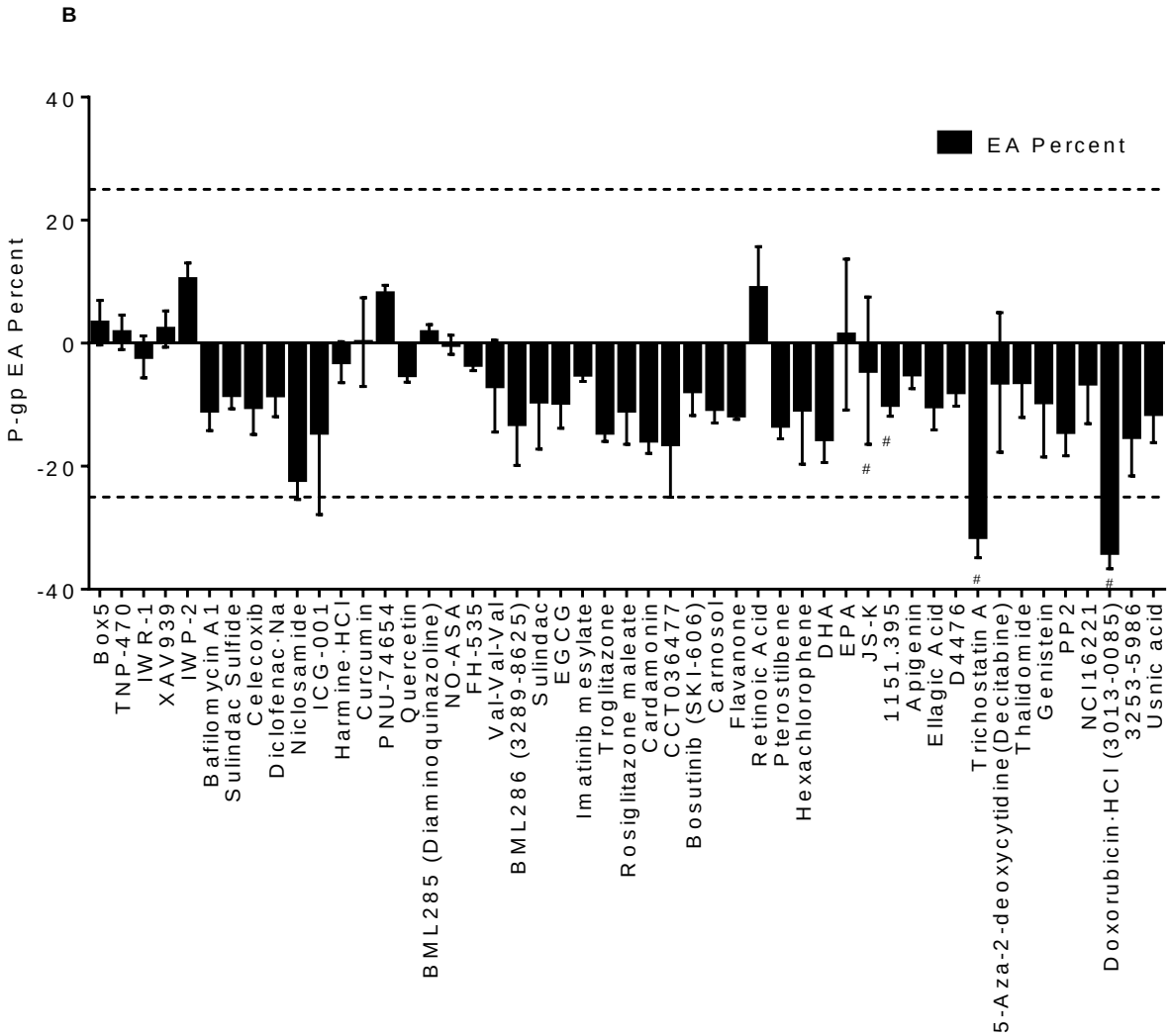
NO-ASA	TCf4/β-catenin interaction	Inhibitor
FH-535	TCf4/β-catenin interaction	Inhibitor
Val-Val-Val	Dishevelled/PDZ protein interaction	Inhibitor
BML286 (3289-8625)	Dishevelled/PDZ domain	Inhibitor
Sulindac	Dishevelled/PDZ protein interaction	Inhibitor
EGCG	HBP1 transcriptional repressor	Induction
Imatinib mesylate	β-catenin signaling	Inhibitor
Troglitazone	PPARγ	Agonist / Inhibition of β-catenin
Rosiglitazone maleate	PPARγ	Agonist / Inhibition of β-catenin
Cardamonin	Down-regulation β-catenin	Inhibitor
CCT036477	β-catenin transcription	Inhibitor
Bosutinib (SKI-606)	c-Src-dependent β-catenin phosphorylation	Inhibitor
Carnosol	β-catenin phosphorylation	Inhibitor
Flavanone	Transcription of β-catenin/Tcf responsive genes	Inhibitor
Retinoic Acid	Transcription of β-catenin/Tcf responsive genes	Inhibitor
Pterostilbene	β-catenin and cyclin D levels	Decrease
Hexachlorophene	Siah-1 / Cyclin d	Activation / Repression
DHA	β-catenin degradation	Induction
EPA	β-catenin degradation	Induction
JS-K	β-catenin	Degradation via NO
1151.395	CK1a	Activator
Apigenin	CK2	Inhibitor
Ellagic Acid	CK2	Inhibitor
D4476	CK1δ/ε	Inhibitor
Trichostatin A	Dkk1	Reversal epigenetic silencing
5-Aza-2-deoxycytidine(Decitabine)	Dkk1	Reversal epigenetic silencing
Thalidomide	Dkk1/Bmp target genes	Upregulation
Genistein	Tyrosine kinase	Inhibitor
PP2	Src kinase	Inhibitor
NCI16221	LRP5/6	Unknown
Doxorubicin·HCl (3013-0085)	LRP5/6	Unknown
3253-5986	LRP5/6	Unknown
Usnic acid	LRP5/6	Unknown

Screening wnt activator and inhibitor compounds at a concentration of 2 µM in the hCMEC/D3 cells was chosen as the initial screening concentration based on the previous GlaxoSmithKline PKI library screen and resulted in no hits. Therefore, none were considered significant in modulating P-gp activity (Figure 6A and B). Wnt pathway inhibitor compounds trichostatin A (targeting Dkk1) and doxorubicin-HCL (3013-0085) (targeting LRP5/6) reduced P-gp activity by -31.5 ± 3.4% and -34.0 ± 2.6%, respectively (Figure 6B). Cell cytotoxicity was quantified by using draq5 DNA stain for cell number at the 2 µM concentration for both the wnt

pathway activators and inhibitors in the hCMEC/D3 cell line. Treatment with wnt pathway activator compound BML-284 and wnt pathway inhibitor compounds JS-K, 1151.395, trichostatin A and doxorubicin-HCL (3013-0085) at the 2 µM concentration were found to significantly reduce cell number compared to control via draq5 staining (Figure 6A and B). These compounds were therefore screened for alterations in P-gp activity in the hCMEC/D3 cells at a reduced concentration (0.2 µM), where none were found to be hits and significantly affect P-gp expression (Figure 7).

Figure 6: Wnt library screen of wnt pathway (A) activators and (B) inhibitors at 2 µM concentration and their impacts on P-gp activity in the hCMEC/D3 cell line. P-gp EA Percent was calculated and represented as mean ± SEM (n=3) per wnt activator and inhibitor compound. None of the wnt pathway activators or inhibitors screened were considered hits. "#" indicates compound caused significant cell death as compared to control via draq5 DNA staining.




B
EA Percent
P-gp EA Percent
40
20
0
-20
-40
Box5
TNP-470
IWR-1
XAV939
IWP-2
Bafilomycin A1
Sulindac Sulfide
Celecoxib
Diclofenac Na
Niclosamide
ICG-001
Harmine HCl
Curcumin
PNU-74654
Quercetin
BML285 (Diaminoquinazoline)
NO-ASA
FH-535
Val-Val-Val
BML286 (3289-8625)
Sulindac
EGCG
Imatinib mesylate
Troglitazone
Rosiglitazone maleate
Cardamonin
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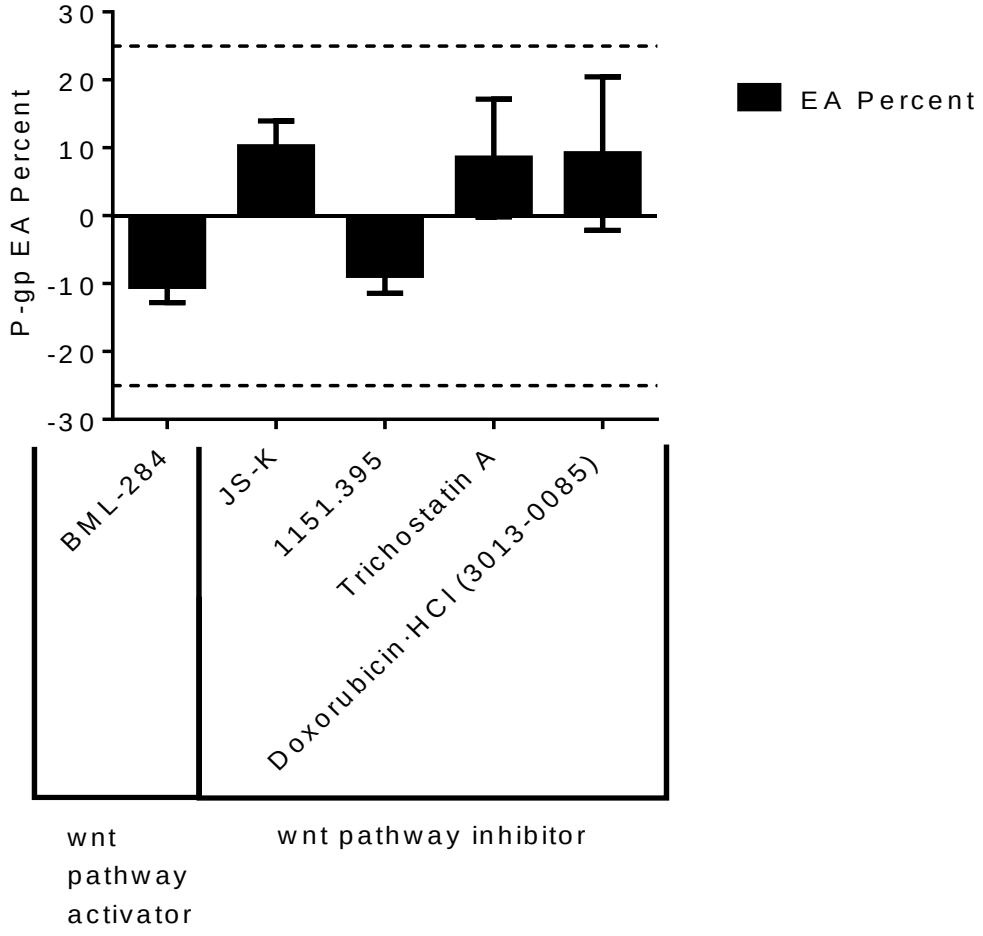
Bosutinib (SKI-606)
Carnosol
Flavanone
Retinoic Acid
Pterostilbene
Hexachlorophene
DHA
EPA
JS-K
1151.395
Apigenin
Ellagic Acid
D4476
Trichostatin A
5-Aza-2-deoxycytidine(Decitabine)
Thalidomide
Genistein
PP2
NCI16221
Doxorubicin HCl (3013-0085)
3253-5986
Usnic acid

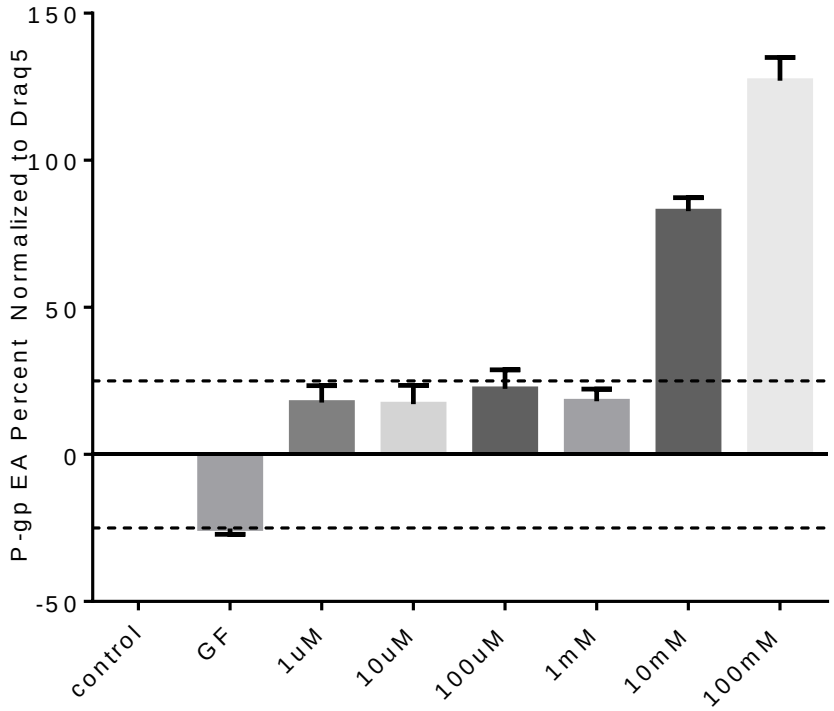
Figure 7: Wnt pathway activators and inhibitors that significantly caused cell death detected by draq5 DNA staining in the hCMEC/D3 cell line were screened at 0.2 μM. P-gp EA percent mean ± SEM (n=3) per wnt pathway activator or inhibitor compound. None of these wnt pathway activators or inhibitors screened at this concentration were considered as P-gp activity hits.



Since GSK-3 PKIs were found to significantly modulate P-gp activity in the MDCK cell line, known GSK-3 inhibitor, LiCl was analyzed for its modulatory capability of P-gp activity. Upon exposing both MDCK-MDR1 and MDCK-WT cells to LiCl in a concentration dependent manner for a 24 h period, LiCl at 100 mM and 10 mM were considered P-gp activity hits, as they

significantly increased P-gp activity when normalized to draq5 DNA stain at 127 ± 11.2 and 82.7 ± 6.5 % compared to control, respectively (Figure 8).

Figure 8: GSK-3 inhibitor, LiCl concentration dependent uptake screen in MDCK cell line for its effects on P-gp activity. R123 fluorescent values used to calculate P-gp EA percent were normalized to draq5 DNA stain, where P-gp EA percent was represented as the mean ± SEM (n=3) per concentration of LiCl. Concentrations that modulated P-gp activity ≥ 25% compared to non-treated control were considered P-gp activity hits. Dashed lines represent ± 25 % P-gp EA percent normalized to draq5 values.

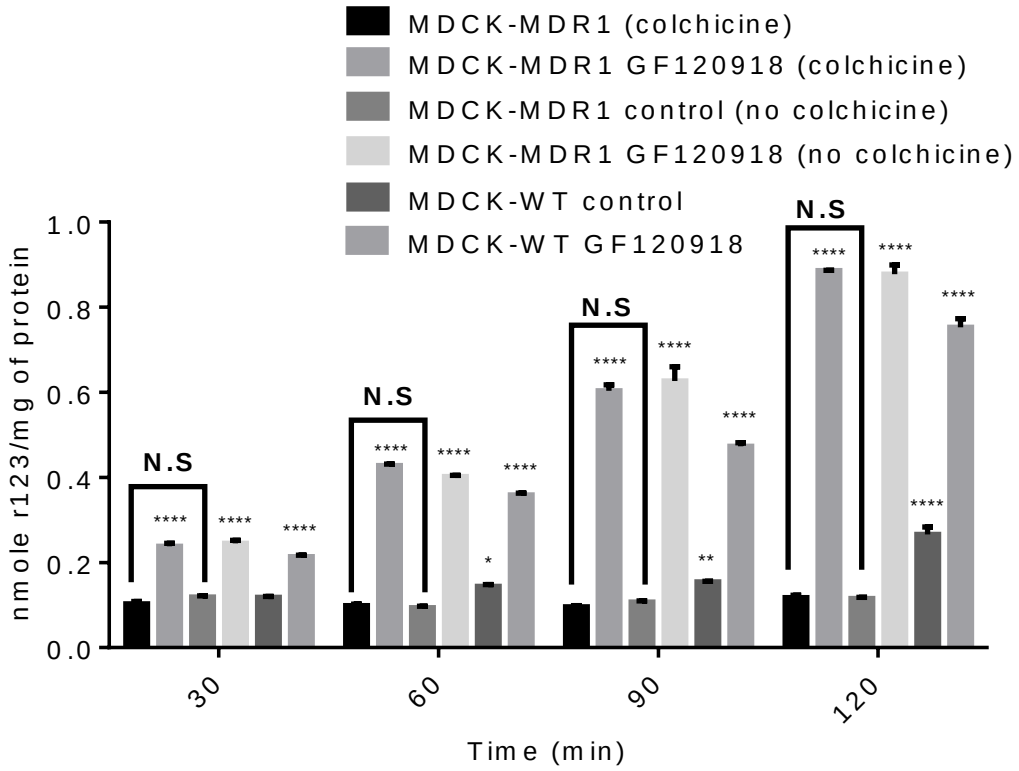


4.2. Concentration Dependent Accumulation Studies, P-gp

PKI families that modulated P-gp expression and activity significantly in the MDCK and Caco-2 cell line were then analyzed using concentration dependent accumulation studies to

determine the optimal dose that yielded the greatest efficacy in modulating P-gp expression and activity. Considering the MDCK-MDR1 cell line requires 80 ng/ml colchicine added to the incubation media during cell culturing to maintain P-gp over-expression, and was not added during the PKI or wnt compound incubation over the 24 h period, we needed to see whether or not this was a confounding variable on P-gp activity in the MDCK cell line during the P-gp transporter activity assays, concentration dependent accumulation and bi-directional equilibrium permeability studies. No significant difference in r123 accumulation was found between the 24 h colchicine treated and non-treated MDCK-MDR1 cells during the concentration dependent accumulation study (Figure 9).

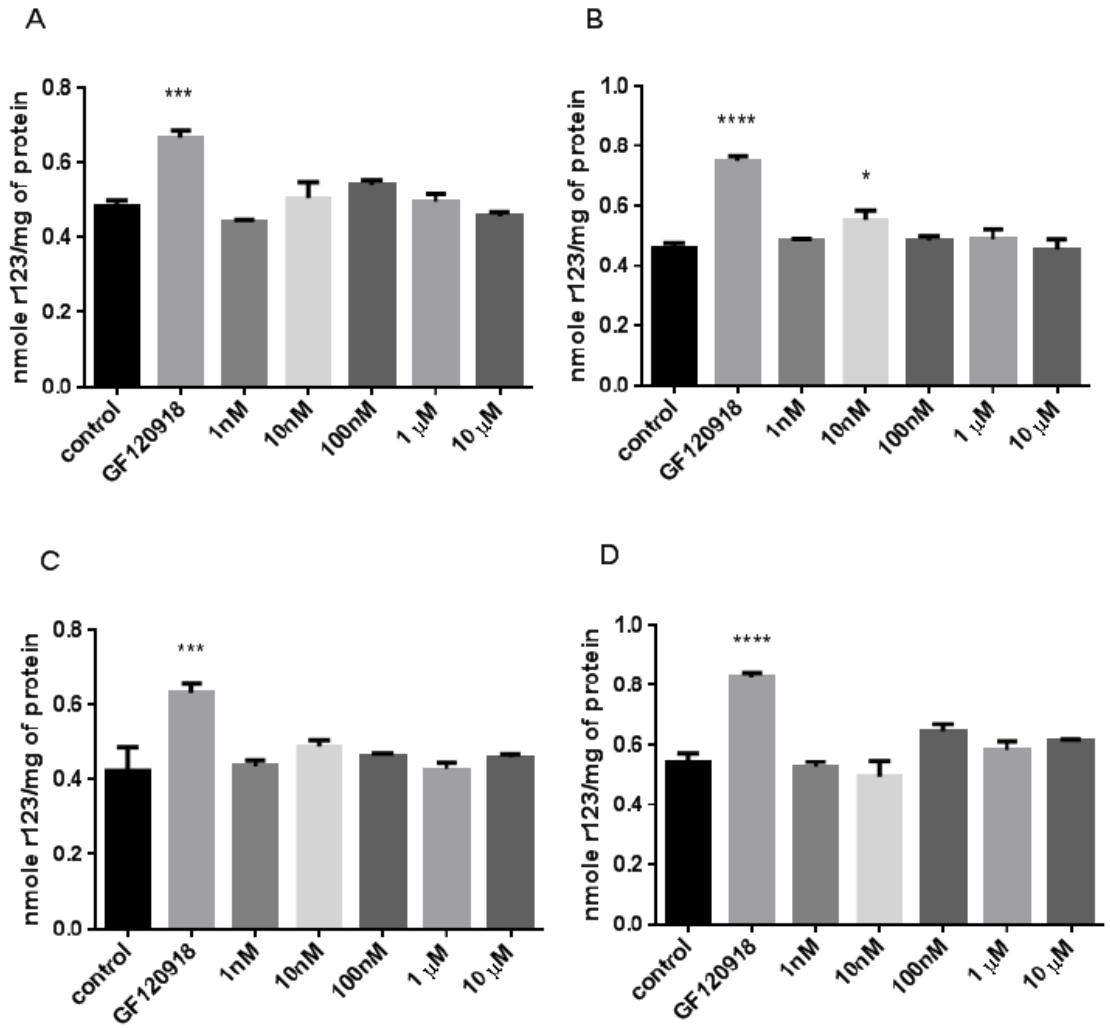
Figure 9: Chronic (24 h) concentration dependent uptake study performed in the presence and absence of 80 ng/ml colchicine in the MDCK-MDR1 cell line, using the 24 well format. MDCK-WT was used as a non-transfected control and potent P-gp inhibitor, GF120918 was used as positive control. Two-way ANOVA was performed and the Dunnett's multiple comparison test was utilized to compare each mean ± SEM (n=3) to MDCK-MDR1 (colchicine treated) per time point, where ****p<0.0001, **p<0.01, *p<0.05. No significant difference (N.S.)



Since PKIs used in the previous screens were no longer available from GlaxoSmithKline, commercially available PKIs were ordered that inhibited similar targets as the PKI P-gp hits identified in the MDCK, Caco-2 and hCMEC/D3 screens. Concentration dependent accumulation studies were performed in the Caco-2 cell line with commercially available PKIs including azakenpaullone (GSK-3 inhibitor), roscovitine (CDK1 inhibitor), GW788388 (TGF-β,

ALK5 inhibitor) and GSK429286 (ROCK1 inhibitor) and were incubated at varying concentrations to determine the efficacious dose in modulating P-gp activity over a 24 h period (Figure 10). In the Caco-2 cell line, azakenpaullone, GW788388 and GSK 429286 appeared to have no effect on p-gp activity at the various concentrations used (Figure 10A, C and D), whereas roscovitine decreased P-gp activity significantly ($p<0.05$) compared to control of 0.556 ± 0.030 nmole r123/mg of protein and 0.460 ± 0.017 at 10 nM (Figure 10B).

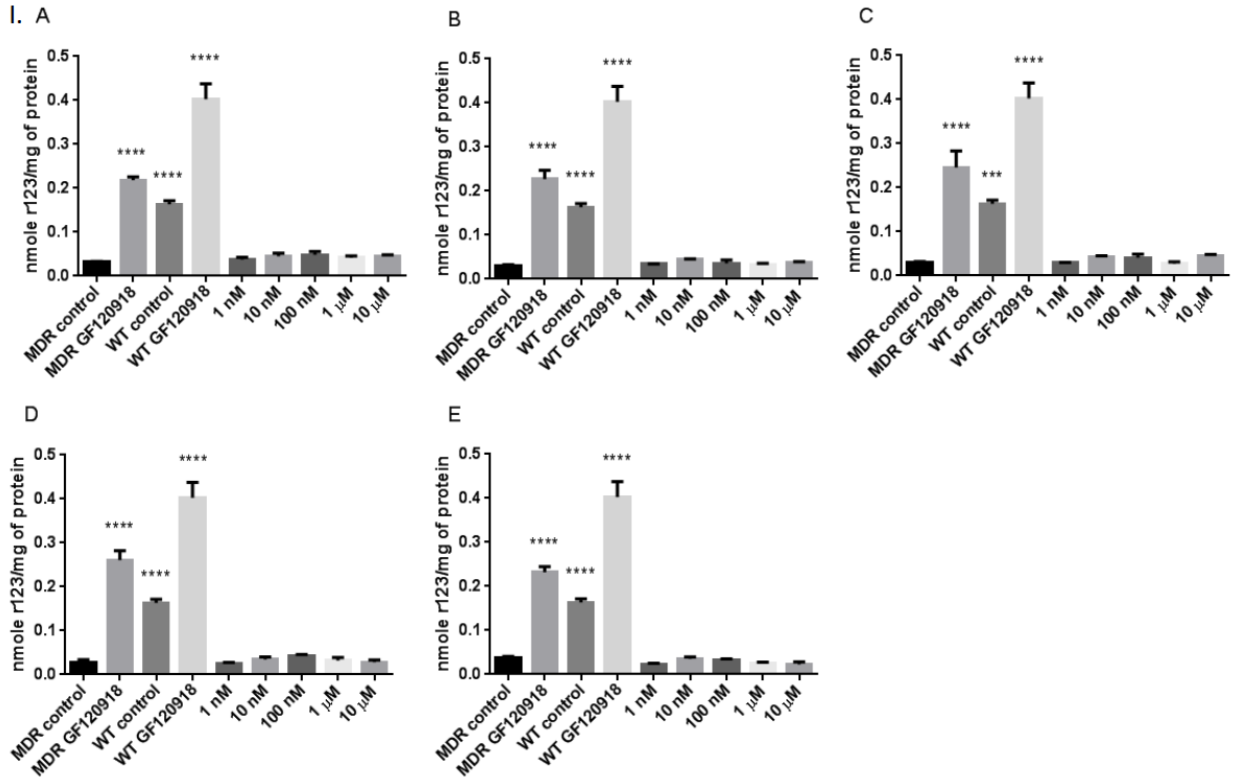
Figure 10: Chronic concentration dependent uptake studies of commercially available PKIs (A) azakenpaullone (GSK-3 inhibitor), (B) roscovitine (CDK1 inhibitor), (C) GW788388 (TGF-β, ALK5 inhibitor), (D) GSK 429286 (ROCK1 inhibitor) in the Caco-2 cell line, analyzed at varying concentrations for their effects on P-gp activity, using the 24 well format. The Mean ± SEM (n=3) of r123 accumulation was normalized to protein and a potent P-gp inhibitor, GF120918 was used as positive control.

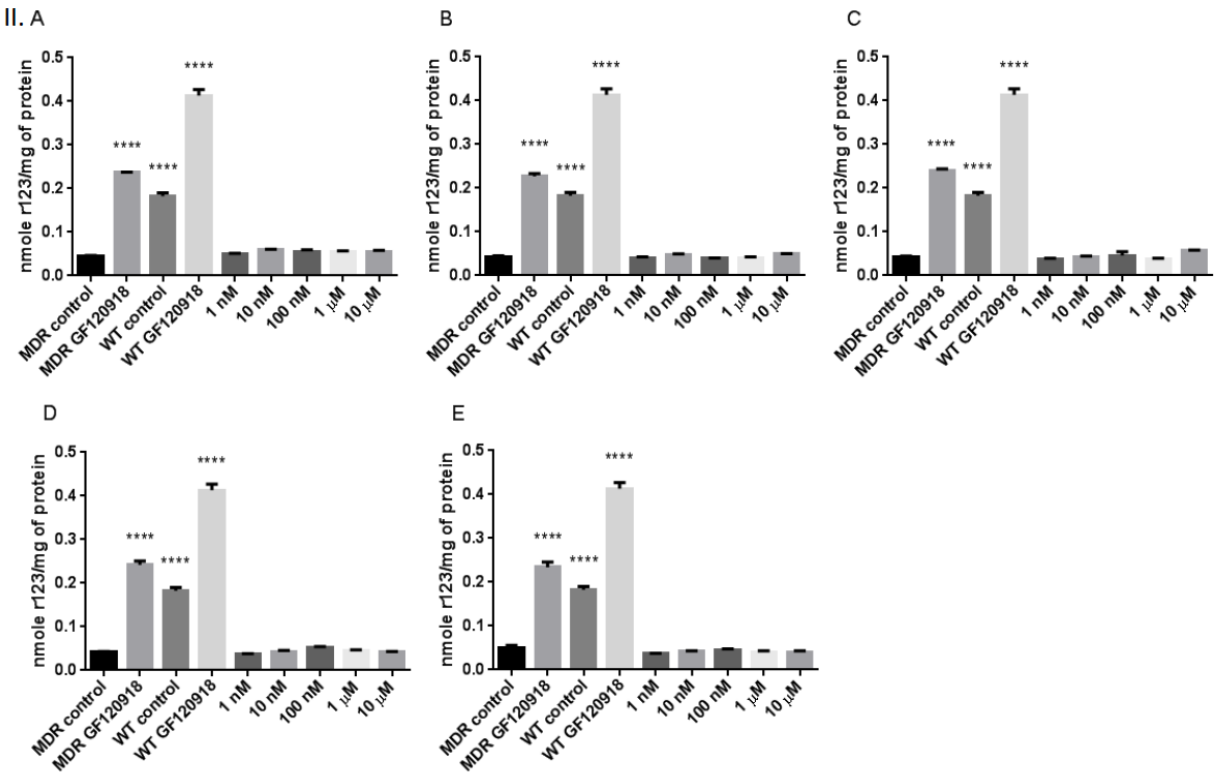


Considering GSK-3 PKIs appeared to significantly modulate P-gp activity in the MDCK-MDR1 cell line, commercially available PKIs targeting GSK-3 were analyzed for their effects on

P-gp activity in the MDCK-MDR1 cell line (Figure 11). Each GSK-3 inhibitor used, inhibited GSK-3 by a different mechanism of action. The compounds used included tideglusib (ATP non competitive GSK-3 inhibitor, brain permeable), indirubin-3-oxime (ATP competitive inhibitor more selective towards GSK-3 compared to CDK), kenpaullone (ATP competitive GSK-3 and CDK inhibitor), TDZD-8 (ATP non competitive GSK-3 inhibitor) and L803-mts (Substrate competitive GSK-3 inhibitor). Chronic concentration dependent accumulation studies in the MDCK-MDR1 cell line demonstrated that none of these compounds significantly modulated P-gp activity at the 1 nm to 10 µM concentration ranges used in neither the 24 well (Figure 11-I) or 96 black-well format (figure 11-II).

Figure 11: Chronic concentration dependent uptake studies of commercially available GSK-3 inhibitors exhibiting different mechanisms of action and their effects on P-gp activity in the MDCK-MDR1 cell line, using the 24- and 96-well format. I. (A) tideglusib, (B) indirubin-3-oxime, (C) kenpaullone, (D) TDZD-8, (E) L803-mts using the 24-well format. II. (A) tideglusib, (B) indirubin-3-oxime, (C) kenpaullone, (D) TDZD-8, (E) L803-mts using the 96-black-well format. MDCK-WT cells were used as a non-P-gp transfected control. The Mean ± SEM (n=3) of r123 was normalized to protein and a potent P-gp inhibitor, GF120918 was used as a positive control. One-way ANOVA was performed and the Dunnett's multiple comparison test was utilised to compare each mean to MDCK-MDR1 control, where ****p<0.001, ***p<0.01.



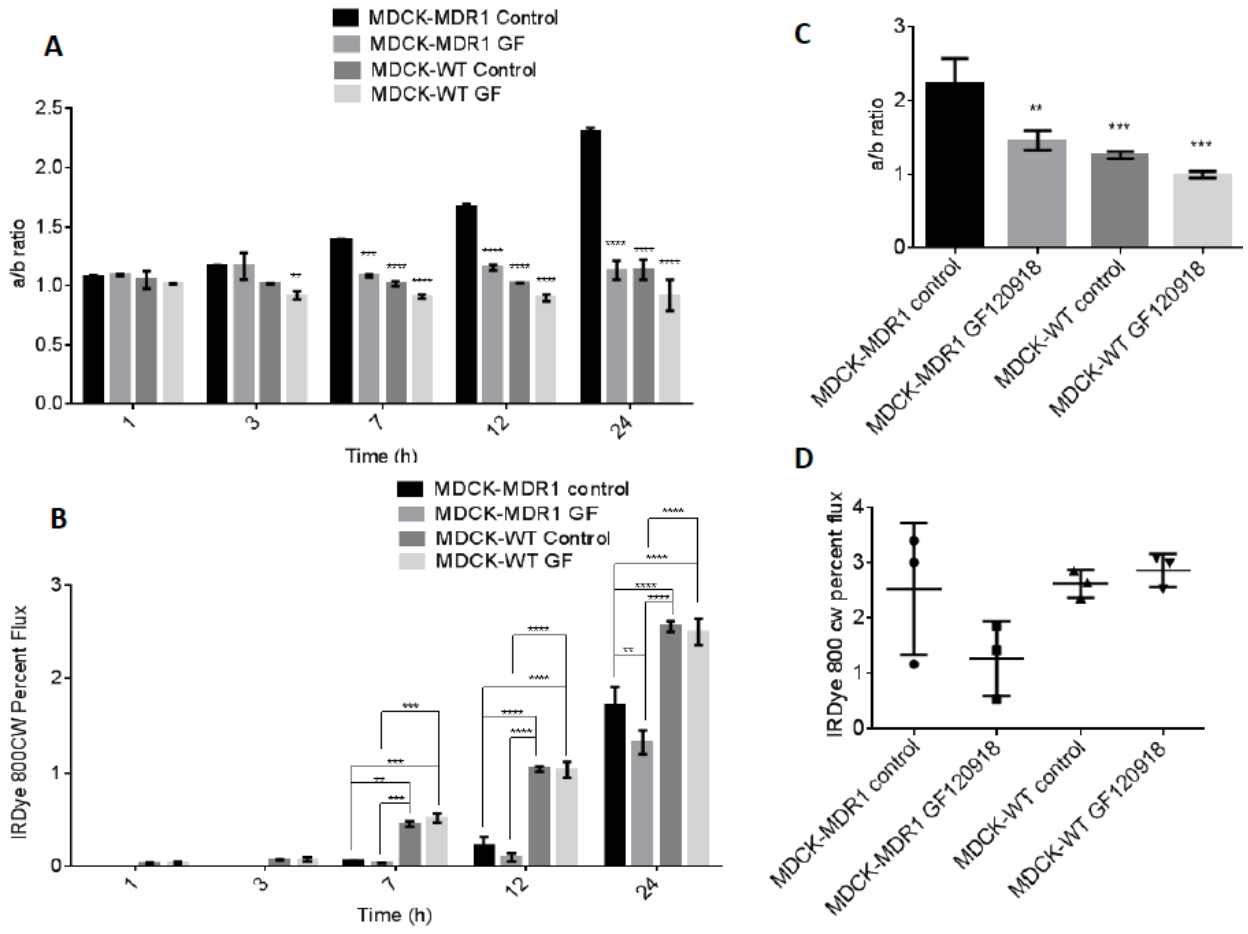


4.3. Bi-Directional Equilibrium Permeability Studies, P-gp

Chronic 24 h Bi-directional equilibrium studies focused on the MDCK cell line to see whether or not any of the compounds that modulated P-gp activity and/or expression in the previous studies significantly impacted on the permeability of P-gp substrate r123 across a monolayer. A time dependent bi-directional equilibrium permeability study was performed up to 24 h in the MDCK cell line (Figure 12). The 24 h time point showed r123 equilibrium conditions were accomplished under the MDCK-MDR1 and –WT control and GF120918 treated conditions (Figure 12A). Tight junction paracellular marker IRDye 800CW PEG mean percent flux remained under 3 % up to the 24 h time point, indicating a tight and good quality monolayer (Figure 12B). In addition, a 24 h bi-directional equilibrium permeability study was performed in the MDCK cell line (Figure 12C and D), again demonstrating r123 equilibrium conditions were

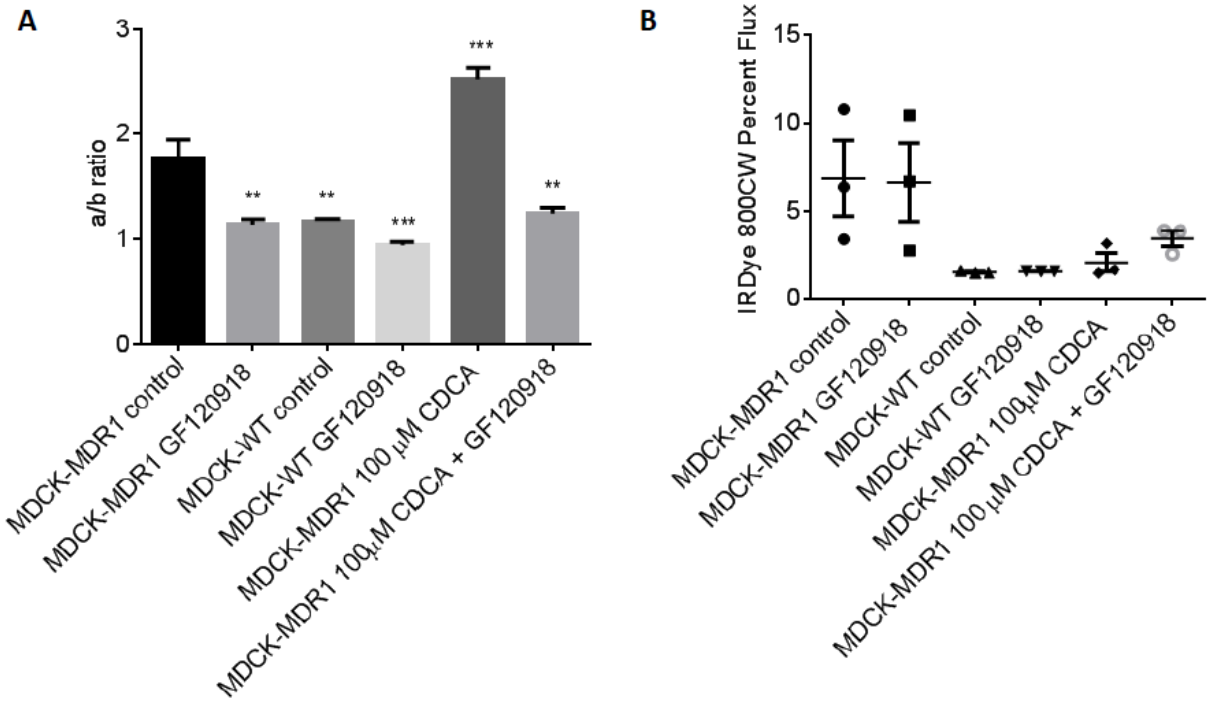
met at the 24 h time point (Figure 12C) and a good quality monolayer was achieved, with IRDye 800CW PEG mean percent flux remaining below 3 % (Figure 12D).

Figure 12: Time dependent 24 h P-gp bi-directional equilibrium permeability study in MDCK cell line. (A and C) A/b ratio of P-gp substrate r123 permeability and (B and D) paracellular marker IRDye 800CW PEG percent flux in MDCK-MDR1 and –WT cell lines with or without P-gp inhibitor GF120918 treatment, (A and B) over various time points up to 24 h (C and D) and 24 h. (A and B) Two-way ANOVA and (C and D) One-way ANOVA were performed and the (A and C) Dunnett's multiple comparison test was utilized to compare each Mean ± SEM (n=3) to MDCK-MDR1 control and Tukey's multiple comparisons test to compare each mean ± SEM (n=3) (B) to each other, per time point and (D) to each other, where ****p<0.0001, ***p<0.001, **p<0.01.



CDCA dosed at 100 µM was used as a proof of concept to demonstrate that our bi-directional equilibrium permeability studies are appropriate for determining the modulatory effects of screened compounds on the permeability across a cell monolayer (Figure 13). 100 µM CDCA treatment was found to increase the a/b ratio (P-gp activity) compared to control of 2.52 ± 0.11 and 1.78 ± 0.17, respectively (figure 13A). Paracellular marker IRDye 800CW PEG mean percent flux remained below 10 % for the 24 h period, indicating a good quality monolayer (Figure 13B).

Figure 13: 100 µM CDCA treatment and its effect on 24 h P-gp bi-directional equilibrium permeability in the MDCK cell line. (A) A/b ratio of P-gp substrate r123 permeability and (B) paracellular marker IRDye 800CW percent flux, where MDCK-MDR1 was treated with or without 100 µM CDCA and MDCK-MDR1 and –WT were treated with or without P-gp inhibitor GF120918. (A and B) One-way ANOVA was performed and the (A) Dunnett's multiple comparison test was utilized to compare each mean ± SEM (n=3) to MDCK-MDR1 control and (B) Tukey's multiple comparisons test to compare each mean ± SEM (n=3) to each other, where ***p<0.001, **p<0.01.

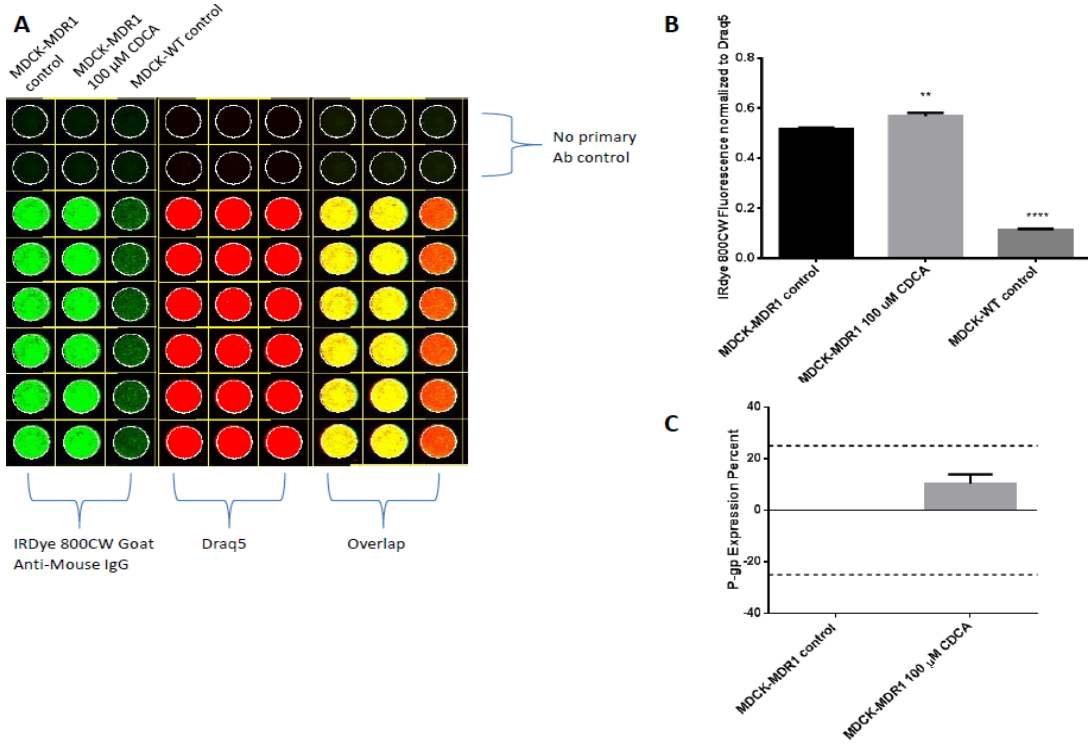


4.4. Immunofluorescence Assay and Quantification of P-gp and BCRP Expression

As a proof of concept, CDCA, a known P-gp inducer in the MDCK cell line was analyzed to demonstrate that the P-gp immunofluorescence In-Cell Western model was appropriate for identifying compounds that modulated P-gp activity and/or expression. CDCA

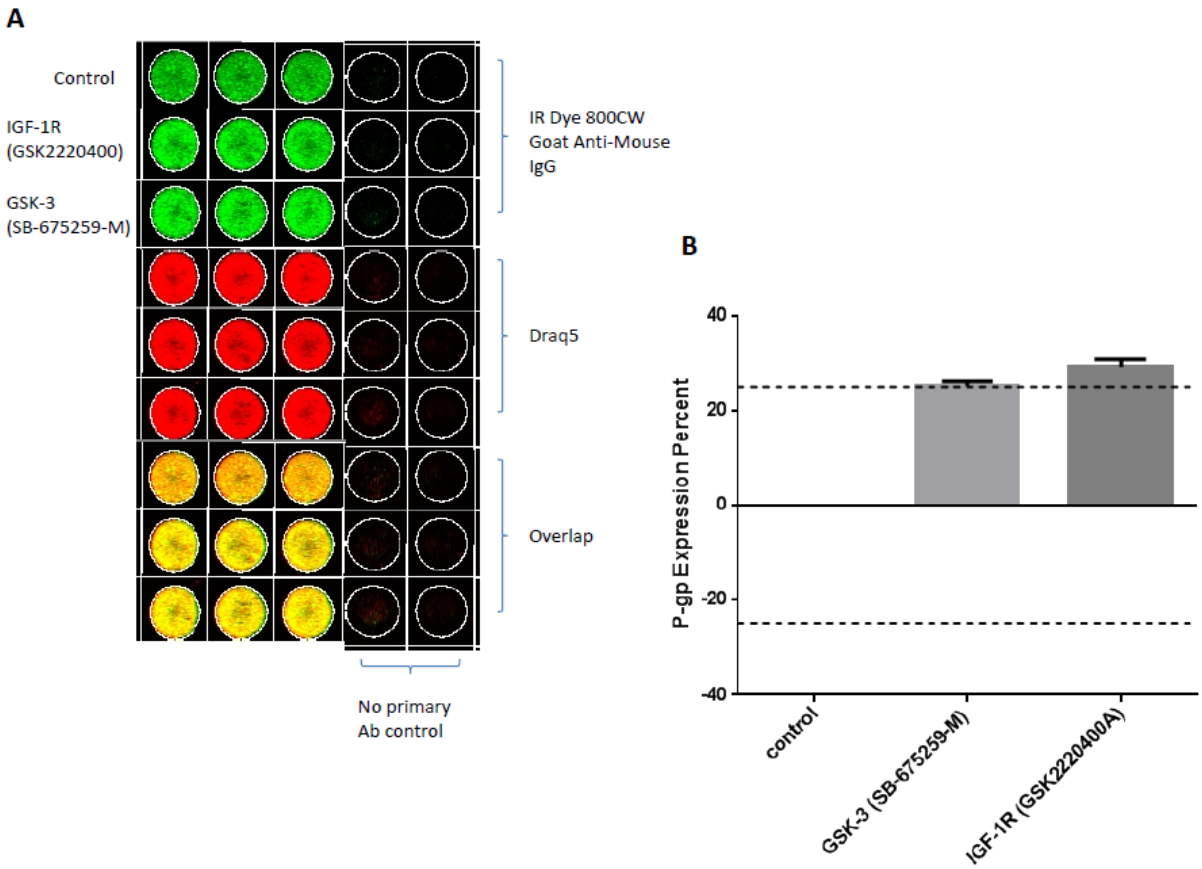
was dosed at 100 µM in the MDCK-MDR1 for 24 h and was analyzed for modulation of P-gp expression compared to non-treated MDCK-MDR1 control and non-P-gp transfected and PKI treated MDCK –WT control. CDCA was found to increase P-gp expression as shown by immunofluorescence (Figure 14A) compared to control. Quantification of P-gp expression demonstrated a 10 % increase in P-gp activity in the MDCK-MDR1 cells compared to control of 0.567 ± 0.014 and 0.514 ± 0.008, respectively (Figure 14B). When MDCK-MDR1 100 µM CDCA treated and MDCK-MDR1 non-treated matched control are represented as P-gp expression percent values, MDCK-MDR1 100 µM CDCA treated is not considered a P-gp expression hit (Figure 14C).

Figure 14: In-Cell Western of CDCAs chronic effect on P-gp expression in the MDCK cell line. MDCK cells were chronically treated with or without 100 µM CDCA, using MDCK-WT as a non-transfected control. (A) Near infrared images (n=6) of secondary antibody (IRDye 800CW PEG goat anti-mouse IgG) at the 800 nm wavelength and draq5 DNA stain at the 700 nm wavelength at focus offset 4 mm, intensity 7 using the Odyssey near infrared imager for MDCK-MDR1 control and 100 µM CDCA treated, and MDCK-WT control wells. Overlap of secondary antibody and draq5 shown on the right side. (B) P-gp expression quantified by taking the IRDye 800CW PEG fluorescence and normalizing it to draq5. One-way ANOVA was performed and the Dunnett's multiple comparisons test was utilized to compare each mean ± SEM (n=6) to MDCK-MDR1 control, where ****p<0.0001 and **p<0.01. (C) P-gp expression percent of MDCK-MDR1 100 µM CDCA treated and control. Dashed lines represent P-gp expression percent value of ± 25%.



Hits that were identified in the chronic 24 h accumulation studies in MDCK, Caco-2 and hCMEC/D3 lines were then analyzed for their effects on P-gp expression using the In-Cell Western technique. In the MDCK-MDR1 cell line, two PKIs out of the 30 hits were found to be P-gp expression hits. Both, GSK-3 PKIs (SB-675259-M) and insulin-like growth factor 1 receptor (IGF-1R) PKI (GSK2220400A) increased P-gp expression by 25.2 ± 1.0 % and 29.3 ± 1.5 %, respectively (Figure 15) .

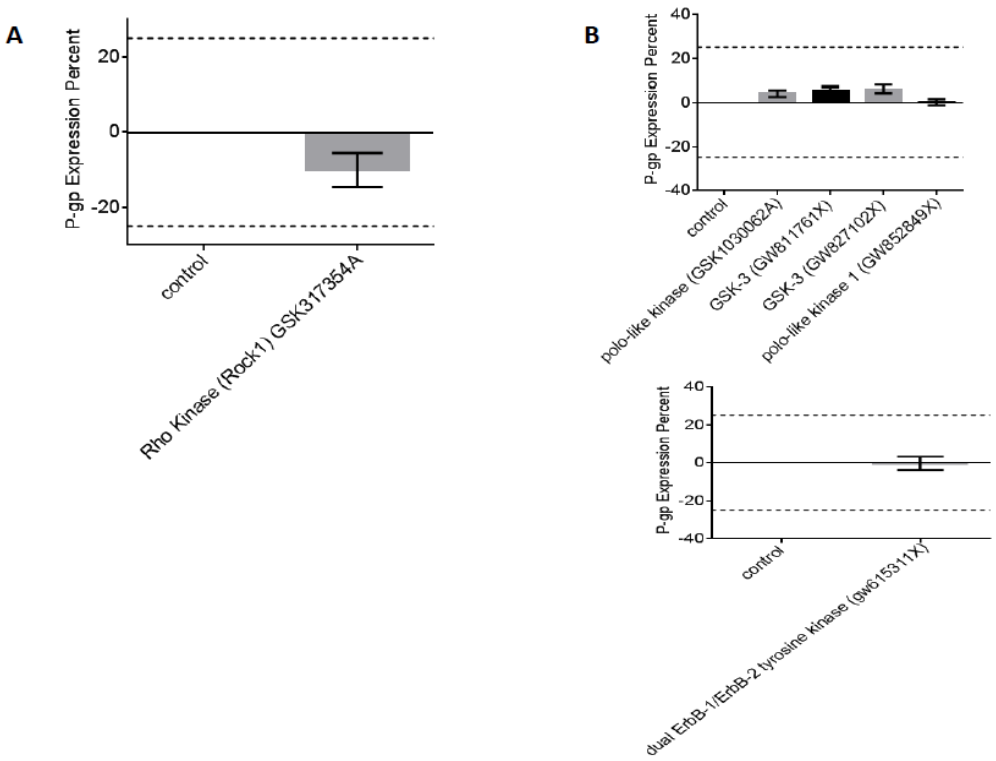
Figure 15: In-Cell Western of MDCK-MDR1 P-gp activity hits (GSK-3 (SB-675259-M) and IGF-1R (GSK2220400A)), identified as the only P-gp expression hits, in the MDCK cell line. (A) Near infrared images (n=3) of secondary antibody (IRDye 800CW Goat Anti-mouse IgG) at the 800 nm wavelength and draq5 DNA stain at the 700 nm wavelength at focus offset 4 mm, intensity 6 using the Odyssey near infrared imager. Overlap of secondary antibody and draq5 shown at bottom. (B) P-gp expression quantified and calculated as P-gp expression percent (n=3). Dashed lines represent P-gp expression percent ± 25.



Six of the 30 MDCK PKI P-gp activity hits that were identified as P-gp activity hits in the Caco-2 and hCMEC/D3 cell lines were analyzed for their effects on P-gp expression. None

of the six PKIs were identified as being P-gp expression hits in the Caco-2 or hCMEC/D3 cell lines (Figure 16).

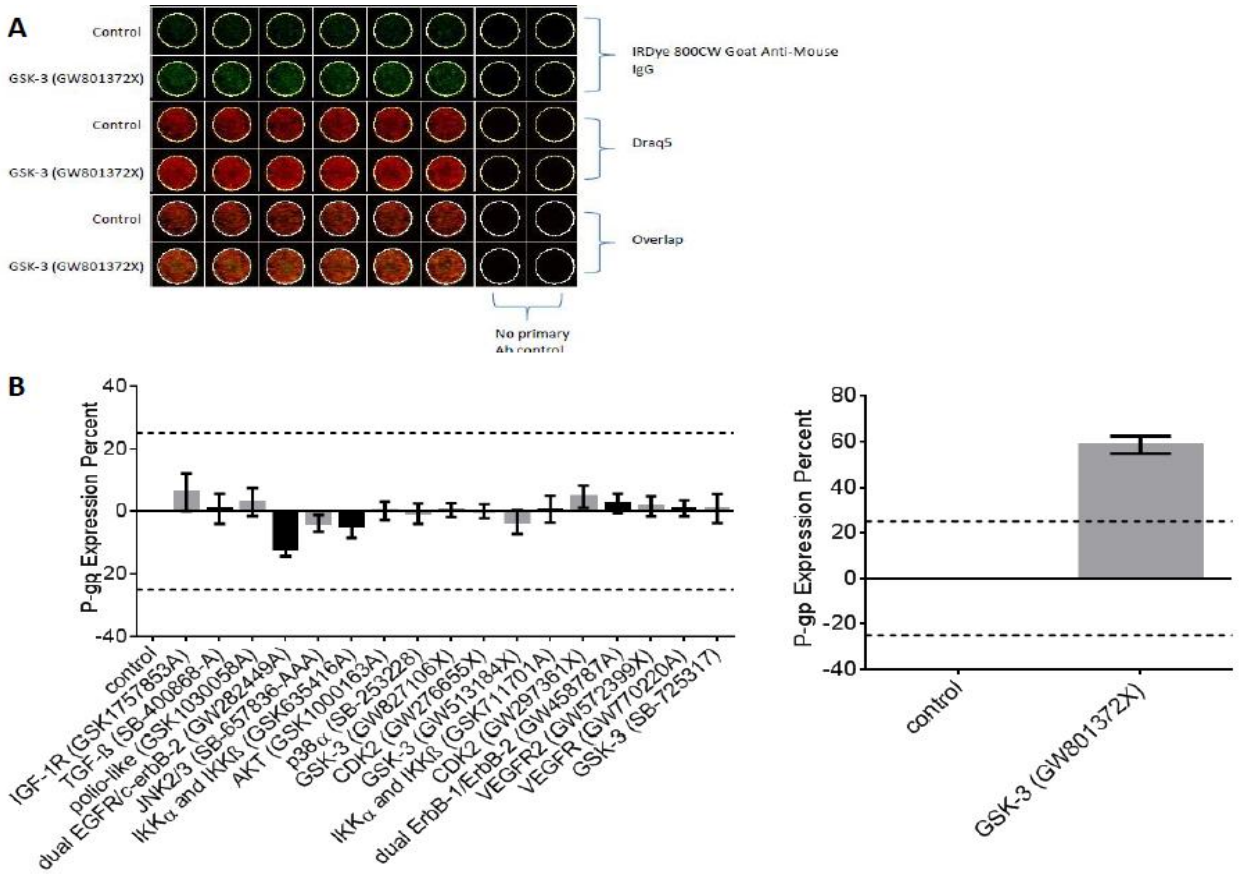
Figure 16: In-Cell Western quantification of P-gp expression after chronic exposure of the 30 MDCK P-gp activity hits screened in the Caco-2 and hCMEC/D3 cell lines. P-gp expression was quantified using near infrared images (n=6) of secondary antibody (IRDye 800CW goat anti-mouse IgG) at the 800 nm wavelength normalized to draq5 DNA stain at the 700 nm wavelength at focus offset 4 mm (A) intensity 5 and (B) intensity 5.5. (A) P-gp activity hit rho kinase (ROCK1) GSK317354A PKI effect on P-gp expression in Caco-2 cell line and (B) P-gp activity hits, polo-like kinase (GSK1030062A), GSK-3 (GW811761X and GW827102X), polo-like kinase 1 (GW852849X) and dual ErbB-1/ErbB-2 tyrosine kinase (GW615311X) and their effects on P-gp expression in hCMEC/D3 cell line. None were identified as being P-gp expression hits.



The 18 PKI P-gp activity hits from the 330 PKI library screen in Caco-2 were also analyzed using the In-Cell Western technique to see if they altered P-gp expression. Demonstrated by immunofluorescence staining, only one PKI was considered a P-gp expression

hit in the Caco-2 cell line out of the 18 P-gp activity hits screened, PKI GSK-3 (GW801372X) (Figure 17A). After quantification, PKI GSK-3 (GW801372X) was shown to significantly increase P-gp expression by 58.5 ± 3.8 % compared to non-treated matched control (Figure 17B).

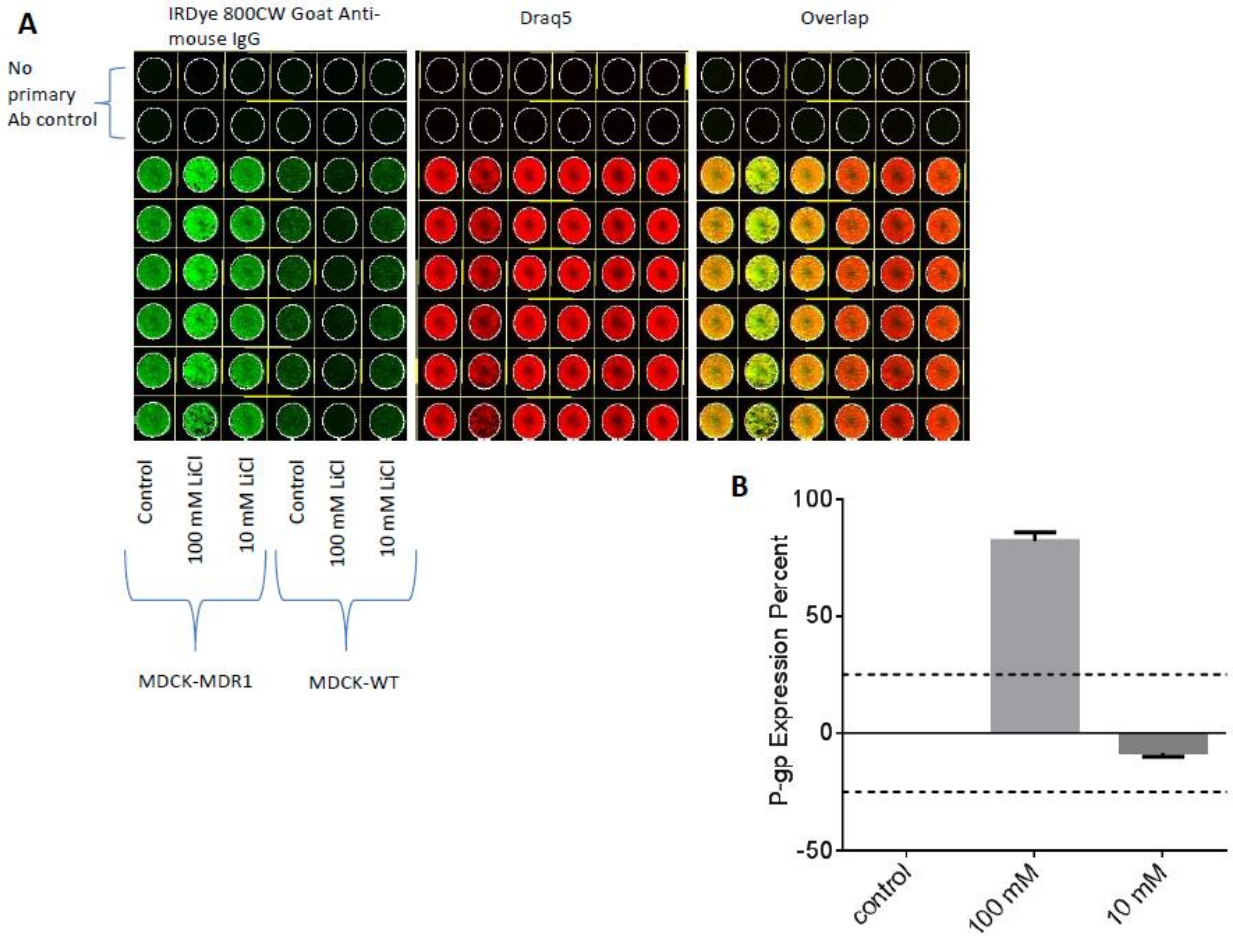
Figure 17: In-Cell Western of PKI P-gp activity hits identified in the 330 PKI complete library screen in Caco-2 and their effects on P-gp expression. (A) Near infrared images of control and PKI treatment wells (n=6) of secondary antibody (IRDye 800CW goat anti-mouse IgG) at the 800 nm wavelength and draq5 DNA stain at the 700 nm wavelength at focus offset 4 mm, intensity 6 using the Odyssey near infrared imager. Overlap of secondary antibody and draq5 shown at bottom. (B) P-gp expression percent expressed as mean ± SEM (n=3, n=6 for GSK-3 (GW801372X)). PKI GSK-3 (GW801372X) was considered a P-gp expression hit.



LiCl was also analysed in MDCK cells to see if the 10 mM and 100 mM doses found to increase P-gp activity, also modulated P-gp expression over a 24 h incubation period (Figure 18). It was found that the 100 mM LiCl treatment, significantly modulated P-gp expression (Figure

18A) and led to an increase in P-gp expression of 81.8 ± 3.9 % compared to non-treated matched control (Figure 18B).

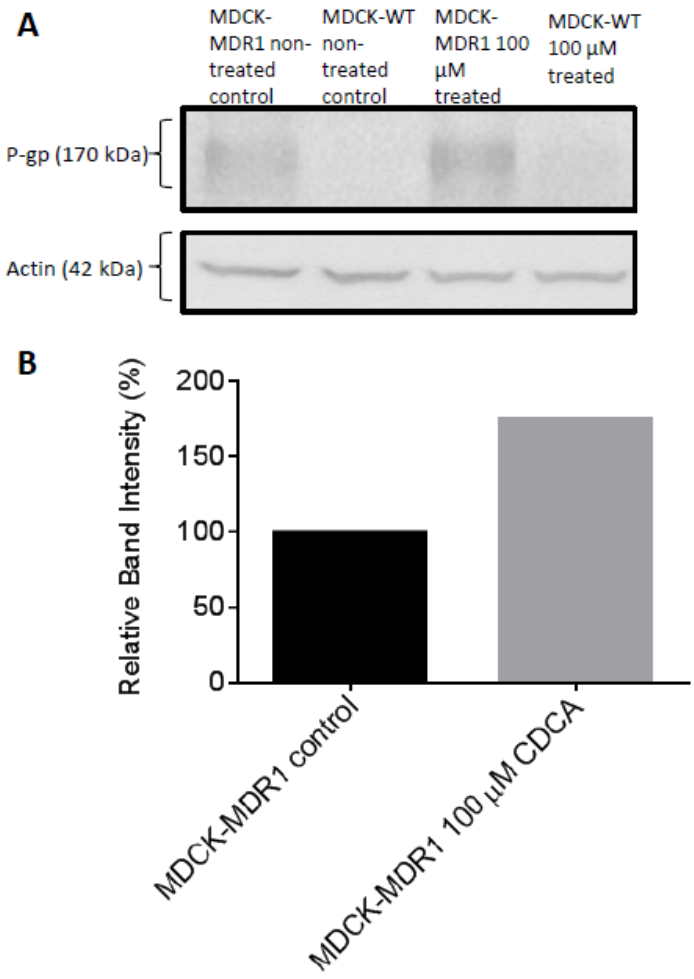
Figure 18: In-Cell Western of the chronic treatment effects of LiCl on P-gp expression in the MDCK cell line. MDCK cells were chronically exposed to 100 mM and 10 mM LiCl. (A) Near infrared images of control and LiCl treatment wells (n=6) of secondary antibody (IRDye 800CW goat anti-mouse IgG) at the 800 nm wavelength and draq5 DNA stain at the 700 nm wavelength at focus offset 4 mm, intensity 6, using the Odyssey near infrared imager. Overlap of secondary antibody and draq5 shown at far right. (B) P-gp expression percent expressed as mean ± SEM (n=6), where 100 mM LiCl treatment was considered a P-gp expression hit.



4.5. Western Blot Analysis and Quantification of P-gp Expression

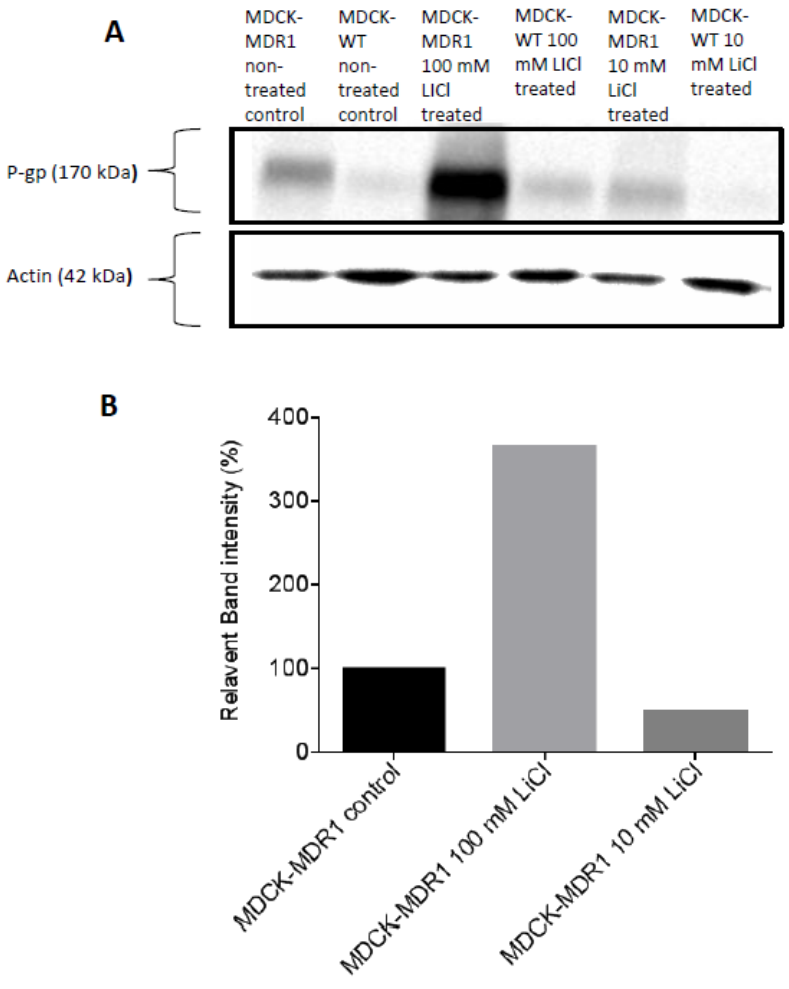
Western blot analysis was performed using the 100 µM CDCA treatment in both MDCK-MDR1 and MDCK-WT cell line (Figure 19). Using primary antibody mouse anti-human *ABCG1* (NBP1-42581) and secondary antibody rabbit anti-mouse IgG-HRP (NB720-H) in the western blot analysis, human P-gp band (170 kDa) was identified in both the MDCK-MDR1 non-treated matched control and MDCK-MDR1 100 µM CDCA treated (Figure 19A). On the contrary, no human P-gp band was identified in the MDCK-WT non-treated matched control and MDCK-WT 100 µM CDCA treated (Figure 19A). Analysis of MDCK-MDR1 100 µM treated control showed a relative band intensity percent of 175 % compared to MDCK-MDR1 non-treated matched control set at 100%, when normalized to actin (Figure 19B).

Figure 19: Western blot analysis of human P-gp expression in MDCK cell line chronically treated with 100 µM CDCA, using non-treated matched controls for 24 h. (A) Western blot using primary antibody, mouse anti-human *ABCG1* (NBP1-42581) and secondary antibody, rabbit anti-mouse IgG-HRP (NB720-1). Membrane bound human P-gp (170 kDa) was identified in both MDCK-MDR1 non-treated matched control and MDCK-MDR1 100 µM CDCA treated. (B) Relative band intensity (%) of human P-gp (170 kDa) normalized to actin within both MDCK-MDR1 non-treated matched control and MDCK-MDR1 100 µM treated. All samples analyzed were n=1, where MDCK-MDR1 control was set at 100% relative band intensity and all treatments were calculated relative to control.



Western blot analysis was also performed on 100 mM and 10 mM LiCl MDCK-MDR1 and MDCK-WT treated with non-treated matched controls (Figure 20). Human P-gp (170 kDa) bands were identified in MDCK-MDR1 100 mM and 10 mM LiCl treated, as well as non-treated matched control (Figure 20A). Relative band intensity for 100 mM and 10 mM LiCl treated MDCK-MDR1 indicated an increase of 365 % and reduction of 48.0 % P-gp expression, respectively (Figure 20B).

Figure 20: Western blot analysis of human P-gp expression in MDCK-MDR1 and MDCK-WT cells chronically treated with 100 mM and 10 mM LiCl, using non-treated matched controls. (A) Western blot using primary antibody, mouse anti-human *ABCG1* (NBP1-42581) and secondary rabbit anti-mouse IgG-HRP (NB720-H). Membrane bound human P-gp (170 kDa) was identified in the MDCK-MDR1 non-treated matched control, MDCK-MDR1 100 mM and MDCK-MDR1 10 mM LiCl treated. (B) Relative band intensity (%) of human P-gp (170 kDa) normalized to actin for MDCK-MDR1 non-treated control, MDCK-MDR1 100 mM and MDCK-MDR1 10 mM LiCl treated. All samples were n=1, where MDCK-MDR1 control was set at 100% relative band intensity and all treatments were calculated relative to control.



5.0. Discussion

PKIs have been highly studied and used therapeutically to block specific protein kinase mediated signalling cascades involved in highly proliferative diseases including cancer and chronic inflammation (Cohen 2002; Zhang, Yang et al. 2009; Malemud and Blumenthal 2014). However, few studies have examined the extent to which alterations in protein kinase pathways can influence ABC transporter function and expression in the cell. Considering ABC transporters play a significant role in drug tissue distribution and MDR (Gottesman and Ambudkar 2001; Aszalos 2007; Breier, Gibalova et al. 2013), looking at modulation of these transporters through blocking selected protein kinases, may identify new methods by which to diminish MDR and modulate therapeutic or endogenous substrate tissue distribution. Considering P-gp alters the tissue distribution of a broad range of drugs and is the largest contributing factor to MDR of all the ABC transporters (Binkhathlan and Lavasanifar 2013), the present studies focused on modulation of P-gp activity with PKIs.

To determine potential cell-dependent effects of the PKIs, the assessment of P-gp function and expression was examined in multiple cell lines through the screening of a PKI library. Cell lines that were chosen included the MDCK cell line (representative of human kidney proximal tubule), Caco-2 cell line (representative of human intestinal epithelial) and hCMEC/D3 cell line (representative of human brain capillary endothelial). The cell lines chosen in the present study are representative of the BBB, kidney and intestine, involved in absorption, distribution, and elimination of drugs and xenobiotics and accordingly, are highly important from a PK perspective. The PKI library provided by GlaxoSmithKline contained 360 compounds targeting over 25 different protein kinase families including both receptor tyrosine kinase

families VEGFR and ErbB, in addition to serine/threonine kinase families, Craf1, JNK and GSK-3.

5.1. PKIs Effect on P-gp Activity in MDCK, Caco-2 and hCMEC/D3 Cell Lines

A screen of the 360 PKI GlaxoSmithKline library in MDCK cells identified the GSK-3 protein kinase as a potential pathway of interest for regulating P-gp function. The importance of the GSK-3 pathway is indicated by the 30 P-gp activity hits identified in the MDCK cell line screening assay. Most of the GSK-3 PKI hits appeared to result in an increase in P-gp activity in the MDCK-MDR1 cell line. Paradoxically, the PKIs producing the greatest increase and decrease in P-gp activity in the MDCK cell line were both GSK-3 family PKIs, SB-675259-M and GW827102X, respectively. Potential reasons for this could involve off-target effects on other protein kinases. For example, previous studies conducted by Leost, Schultz et al. (2000) demonstrated that alsterpaullone, an ATP competitive CDK protein kinase inhibitor, also potently inhibited GSK-3β protein kinase, indicating high cross inhibition between these two enzymes. In addition, the library we obtained from GlaxoSmithKline contained competitive PKIs of the ATP binding site of the various protein kinases. It is known that PKIs that inhibit protein kinases through an ATP competitive process are non-specific, considering this site is conserved and shared among protein kinases (Zhang, Yang et al. 2009; Dar and Shokat 2011; Knapp and Sundstrom 2014). Thus, compounds identified as GSK-3 inhibitors could have activity on other protein kinase systems that impact on P-gp function and activity. Alternatively, as P-gp itself has two ATP binding sites, there is the possibility that selected PKIs may inhibit transporter activity through non-kinase mechanisms such as competitive interference with the fluorescent transport probe or direct inhibition of ATP hydrolysis. Such interactions could produce a false positive for PKIs that were identified as inhibitors of P-gp activity. While this could potentially explain how

the GSK-3 inhibitor produced the best reductions in P-gp activity, the potential for this occurring in the present study was minimized in the assay design by examining the effects of PKIs following 24 h treatment. After the 24 h treatment the cells are washed and the PKIs removed for the P-gp functional tests.

When comparing the percent PKI family composition of the library to the percent P-gp hits caused by a particular PKI family in the MDCK cell line, it was identified that Craf1, GSK-3 and VEGFR2 and Tie2 PKI families are important in modulating P-gp activity. Although, no previous studies have looked at the modulatory effects of Craf1, VEGFR2, or Tie2 PKIs on P-gp activity in the MDCK cell line, studies conducted by Zhao, Sun et al. (2013) showed that grape seed procyanidin (GSP) reversed P-gp mediated MDR to paclitaxel in paclitaxel resistant A2780/T cells through the inhibition of the MAPK/ERK pathway by decreasing the phosphorylation of ERK1/2. Considering, Craf1 and VEGFR2 and Tie2 protein kinases are found upstream of MAPKs ERK1/2 and are involved in their activation, our findings that Craf1 or VEGFR2 and Tie2 PKI families appear to be important in modulating P-gp activity in the MDCK cell line, are consistent with these previous studies.

The PKIs identified as hits in the MDCK cells, were also analyzed for their effects on P-gp activity in the Caco-2 and hCMEC/D3 cell lines. Out of the 30 MDCK hits, only one (rho kinase (ROCK1) GSK317354A PKI) was found to be a P-gp hit in the Caco-2 cell line increasing P-gp activity above 25% threshold. The increased P-gp activity observed with the ROCK1 inhibitor in Caco-2 contradicts previous studies conducted by Kobori, Harada et al. (2013) where it was shown that activation of rho kinase (ROCK1) leads to increased P-gp activity and expression in isolated intestinal ileal mucosa from mice. As there was only one ROCK1 inhibitor in the screening library it is difficult to know whether this is indicative of the

ROCK1 inhibitor class or perhaps an effect attributable to the ROCK1 inhibitor used, which may have off-target effects that we are unaware of. However, as the commercially available agents that inhibit ROCK1 did not have any activity in the Caco-2 cells, it may be more of a function of the specific inhibitor (GSK317354A) than ROCK1 inhibitors in general.

In the hCMEC/D3 cell line, five hits from the original MDCK screen, two polio like (GSK1030062A and GW852849X), two GSK-3 (GW811761X and GW827102X) and one dual ErbB-1/ErbB-2 PKI (GW615311X)) were found to be P-gp hits. Consistent with our findings of ErbB-1/-2 inhibitors increasing P-gp activity in hCMEC/D3 cell line, are studies conducted by Harmsen, Meijerman et al. (2013), which demonstrated ErbB-1 (also known as epidermal growth factor receptor) inhibitor, gefitinib, induced P-gp activity in LS180 cells. It should be noted that the effects reported with getiftinib were attributed to increased expression of P-gp through activation of the orphan nuclear receptor, PXR (Harmsen, Meijerman et al. 2010). In addition, noting only 6 P-gp hits were identified in the Caco-2 and hCMEC/D3 cell lines out of the 30 P-gp hits identified in the MDCK cell line, suggests that PKIs may modulate P-gp activity in a cell line dependent manner.

The 30 PKIs identified as hits in the P-gp activity assay in MDCK cells were also screened in both the Caco-2 and hCMEC/D3 cell lines for effects on BCRP activity. However, none of the agents examined had a significant effect on mitoxantrone accumulation in the cells. As mitoxantrone is a known BCRP substrate (Tan, Killeen et al. 2014) our findings suggest that the PKIs examined did not modulate BCRP activity in the Caco-2 or hCMEC/D3 cell lines. These findings are somewhat surprising, considering studies have shown GSK-3 inhibition results in increased activity and expression of both P-gp and BCRP through the activation of the wnt/β-catenin pathway in rat brains (Harati, Benech et al. 2013). In addition it has been shown

that BCRP works in conjunction with P-gp to limit the influx of xenobiotic and endogenous substrates (Jonker, Smit et al. 2000; Maliepaard, Scheffer et al. 2001). Considering that PKIs may be more potent in modulating P-gp activity, compared to BCRP activity, increasing the PKI treatment concentration in our BCRP activity assays may have resulted in a significant effect on BCRP activity. In addition, the mitoxantrone concentration that was utilized in our BCRP activity assays may have been saturating the transporter, masking the modulatory effect of the PKI on BCRP activity.

Screening of the complete GlaxoSmithKline PKI library was also done in Caco-2 cells. When comparing the percent PKI family composition of the library to the percent P-gp activity from the complete Caco-2 GlaxoSmithKline PKI library screen, it was identified that GSK-3, Iκκ and Jnk2/3 PKI families contained a greater number of hits than would be expected based on the distribution of the PKI families within the library alone. The PKI family that led to the most P-gp activity hits was the GSK-3 PKI family. Since GSK-3 PKI family led to the most hits in both the Caco-2 and MDCK cell line, one might expect this protein kinase to be important in modulating P-gp activity in a cell line independent manner. One such pathway that supports this idea is the ubiquitously expressed wnt/β-catenin pathway. Upon GSK-3β inhibition, free β-catenin can complex and form a transcription complex upon entering the nucleus, that up regulates P-gp activity and expression (Lim, Kania et al. 2008). In addition, CDK2 (GW276655X) PKI led to the greatest increase in P-gp activity, whereas TGF-β (SB-400868A) led to the greatest decrease in P-gp activity in the Caco-2 cell line. As the PKIs important in modulating P-gp activity in the MDCK cell line are dissimilar to the Caco-2 cell line, excluding the GSK-3 PKI family, the findings of the present study suggests that protein kinase inhibitors may modulate P-gp activity in a cell line dependent manner.

In support of our findings that Iκκ pathway is important in modulating P-gp activity in the Caco-2 cell line, are studies performed by Aszalos (2007). Here, Aszalos (2007) demonstrated that dioscin increased the intracellular concentration of methotrexate in Caco-2 cells by decreasing P-gp activity via the inhibition of proteasomal Iκβ degradation and subsequent reduction in P-gp inducer transcription factor, Nf-κβ. Briefly, Iκκ PKIs can achieve a similar effect as dioscin by means of inhibiting the Iκκ complex (Iκκα and Iκκβ), which is responsible for phosphorylating Iκβ (NF-κβ inhibitor) and tagging it for ubiquitination by the proteasome. Therefore, Ikβ remains bound to NF-κβ and subsequently prevents NF-κβ from entering the nucleus, which can lead to a reduction in P-gp expression and activity (Tan, Killeen et al. 2014). Contradictory to Aszalos (2007), is that all of the Iκκ PKIs in the Caco-2 cell line resulted in an increase in P-gp activity. Therefore it is quite possible that these PKIs are directly interacting with the P-gp transporter to increase activity or they are involved in modulating an additional signalling pathway, involved in up-regulating P-gp activity.

JNK2/3 PKIs were also found to be important P-gp modulators in the Caco-2 cell line and resulted in a significant decrease in P-gp activity. Studies by Qosa, Abuznait et al. (2012) support our findings, where they have demonstrated that knockdown of JNK2 by microRNA-200c in MDR colorectal cells attenuated P-gp activity and expression, increasing the sensitivity of MDR colorectal cells to chemotherapeutic drugs, inducing apoptosis. In addition, Chen, Bian et al. (2014) showed that the use of c-jun (downstream of JNK2/3) inhibitor SP600125 decreased MDR1 mRNA and increased susceptibility of Caco-2 vinblastin resistant cells to P-gp substrate vinblastin. In addition, Wang, Kathawala et al. (2014) found that vinblastin resistant Caco-2 cells transfected with plasmid containing an AP-1 site attached to a luciferase reporter gene had increased AP-1 site luciferase activity, where it is known that both vinblastin and c-jun interact

to increase gene transcription. Considering that the MDR1 gene also contains an AP-1 site, connects our findings that JNK2/3 PKIs can modulate P-gp activity in Caco-2 cells.

5.2. Canonical Wnt/β-catenin Activator and Inhibitor Compound Library Effect on P-gp Activity in the hCMEC/D3 Cell Line

The canonical wnt/β-catenin pathway has been found to regulate MDR1 gene expression, through the activities of GSK-3β (Lim, Kania et al. 2008). In addition, the canonical wnt/β-catenin pathway plays a role in the formation and regulation of BBB components involved in maintaining its restrictive properties, including tight junctional complexes and ABC transporters (Liu, Wan et al. 2014). Therefore, modulation of P-gp activity was analyzed by screening a canonical wnt/β-catenin activator and inhibitor library in a human BBB representative cell line, hCMEC/D3.

When treating hCMEC/D3 cells at 2 µM or 0.2 µM for a 24 h period (if compound caused significant cell death at 2 µM) per wnt library activator and inhibitor compound, none were found to modulate P-gp activity significantly. Studies conducted by Lim, Kania et al. (2008) treated hCMEC/D3 cells with increasing concentrations of GSK-3β inhibitor, 6-bromoindirubin-3'-oxime (BIO), up to 1 µM for 24 h and subsequently performed P-gp activity assays using calcein-AM as their P-gp substrate marker for 30 min and analyzed the fluorescent content using flow cytometry. Conversely to our studies, Lim, Kania et al. (2008) demonstrated that P-gp activity increased significantly using the above mentioned methods. Since our GSK-3 inhibitors, including BIO, did not show any significant impact on P-gp activity, may be attributable to the fact that our methods encompassed fluorescent detection instruments that are not as sensitive as flow cytometry and may have not been able to detect small changes in P-gp activity. In addition our methods involved exposing hCMEC/D3 cells over a 90 min period with

r123, which may have saturated the P-gp transporter at this time point and therefore masked small effects of wnt library GSK-3β inhibitors on P-gp activity in this cell line.

When looking at quercetin, TCF/β-catenin inhibitor, Lim, Kania et al. (2008) found a decrease in P-gp expression, in the hCMEC/D3 cells. Here, hCMEC/D3 cells were treated for 5 days with 5 µM quercetin. While, Lim, Kania et al. (2008) did not look directly at quercetin's effects on P-gp activity, our studies found no effect on P-gp activity when treated with 2 µM quercetin for 24 h in the hCMEC/D3 cells. Therefore, either quercetin's effects on P-gp expression are not enough to detect a change in P-gp activity in our studies or assuming a decrease in P-gp expression also results in a decrease in P-gp activity, increasing the duration of exposure or treatment concentration of quercetin in our studies, may have resulted in a decrease in P-gp activity.

5.3. LiCl Effect on P-gp Activity in the MDCK Cell Line

Studies done by King and Jope (2005) and Binkhathlan and Lavasanifar (2013) showed that LiCl potently (IC_{50} 2 mM for GSK-3β) inhibits GSK-3β. In addition, LiCl was found to increase β-catenin levels through the inhibition of GSK-3β (Lim, Kania et al. 2008). Therefore, following our previous studies, where GSK-3 PKIs were found to modulate P-gp activity in both Caco-2 and MDCK cell line, LiCl was analyzed in the MDCK cell line for modulation of P-gp activity. Incubation of LiCl in the MDCK cell line increased P-gp activity in a concentration dependent manner over a 24 h period, up to 127 ± 11 % at 100 mM, compared to control. Our findings were confirmed by Lim, Kania et al. (2008) where they demonstrated that LiCl inhibition of GSK-3β, increased P-gp activity in the hCMEC/D3 cell line.

5.4. Commercially Available PKIs of P-gp Hit PKI Families, Screened in the Caco-2 Cell Line and MDCK Cell line

At this point, PKIs from the GlaxoSmithKline PKI library became depleted and our focus shifted to screening commercially available compounds using concentration dependent accumulation studies, that targeted similar protein kinases as the PKI P-gp activity and expression hits.

In the Caco-2 cell line commercially available compounds that were screened over a 24 h period, included azakenpaullone (GSK-3 inhibitor), roscovitine (CDK1 inhibitor), GW788388 (TGF-β, ALK5 inhibitor) and GSK429286 (Rho kinase (ROCK1) inhibitor). Interestingly, only roscovitine affected P-gp activity and led to a significant decrease in P-gp activity (increase in r123 accumulation) at the 10 nM treatment, but not at the other concentrations up to 10 µM. roscovitine's effect on P-gp activity is contradictory, to what we have seen in previous screens using CDK inhibitors, where P-gp activity was increased in both the Caco-2 and MDCK cell lines. Since the mechanism of action of PKIs dictates how potent and specific they are towards a particular kinase, as mentioned previously, this may explain why PKIs from the GlaxoSmithKline library gave contradictory results compared to the commercially available compounds. For instance, GSK-3 and CDK inhibitors including azakenpaullone and roscovitine, respectively, can cross react with both GSK-3 and CDK protein kinases because of their structural similarity within their ATP biding domains (Zhang, Yang et al. 2009; Eldar-Finkelman and Martinez 2011). Therefore, these compounds may have not been as potent and specific as the P-gp activity hits identified in the GlaxoSmithKline library screen and may require higher concentrations or more selective compounds, to see an effect on P-gp activity.

Commercially available GSK-3 inhibitors that have different mechanisms of action in inhibiting GSK-3, ranging from non-specific inhibition to specific inhibition were also screened in the MDCK-MDR1 cell line and included tideglusib (ATP non-competitive GSK-3 inhibitor), indirubin-3-oxime (ATP competitive inhibitor, more selective towards GSK-3 compared to CDK2), kenpaullone (ATP competitive GSK-3 and CDK2 inhibitor), TDZD-8 (ATP non-competitive GSK-3 inhibitor) and L803-mts (substrate competitive GSK-3 inhibitor). Briefly, substrate competitive inhibitors are more selective towards GSK-3 protein kinase, compared to ATP competitive inhibitors, and ATP non-competitive inhibitors are the most selective (Eldar-Finkelman and Martinez 2011). After 24 h incubation of these compounds ranging from 1 nM to 10 µM treatments in the MDCK-MDR1 cell line, none were found to affect P-gp activity. Previously establishing that LiCl only had an effect on P-gp activity and expression when present in the 10 and 100 mM range, these compounds may significantly modify P-gp at higher concentrations in the MDCK cell line.

5.5. CDCAs Effect on Bi-Directional Permeability, Mediated through P-gp

CDCA, a primary bile acid, has been found to influence P-gp expression by acting as a ligand activator of the orphan nuclear receptor, FXR, and as a complex with RXRα can bind FXR response elements within the mouse *Abcb1a* (P-gp) promoter of the mouse *Abcb1a* gene (Jiang, Jin et al. 2013). In addition, it was found that exposure of 100 µM CDCA for 24 -96 h was sufficient enough to increase P-gp expression and activity in human P-gp transfected canine kidney epithelial cells (Kneuer, Honscha et al. 2007).

As mentioned previously, compounds from the GlaxoSmithKline PKI library were no longer available, and none of the commercially available compounds were showing any

substantial impact on P-gp activity. Therefore, as a proof of concept, we utilized known P-gp inducer CDCA as a positive control, to analyze its effects on P-gp mediated permeability. After confirming that our bi-directional equilibrium Permeability model was acceptable in the MDCK cell line under non-treated conditions over a 24 h period, we treated MDCK-MDR1 cells with CDCA at a 100 µM over a 24 h period, to analyze its effects on r123 permeability across a monolayer. Our results showed that 100 µM CDCA treatment increased r123 permeability across a monolayer (P-gp activity) by 41.6 % compared to non-treated matched control in the MDCK-MDR1 cells. Therefore, when focusing on the In-Cell Western analysis, where 100 µM CDCA 24 h treatment increased P-gp expression by only 10 %, demonstrates that a slight change in P-gp expression may result in a large change in permeability of a P-gp substrate across a monolayer. Conversely, when focusing on western blot analysis of 100 µM CDCA treatment in MDCK-MDR1 cells, P-gp expression increased by 75 % compared to control, indicating that a large change in P-gp expression may only result in a small change in permeability of P-gp substrate across a monolayer.

5.6. CDCAs Effect on P-gp Expression, Analyzed by In-Cell Western in the MDCK Cell Line

As a proof of concept, to establish that our In-Cell Western technique used to quantify the effects of PKIs on P-gp expression was appropriate, known P-gp expression inducer, CDCA was added at 100 µM (Kneuer, Honscha et al. 2007) to MDCK-MDR1 and -WT cells and incubated for 24 h. Our data supports previous data from Kneuer, Honscha et al. (2007) where the 100 µM CDCA treatment was shown to increase mRNA expression in MDCK cells over 24 h. Here we demonstrated that the 100 µM CDCA treatment in MDCK-MDR1 cells increased P-gp expression by 10 % compared to MDCK-MDR1 non-treated matched control, over a 24 h period. Even though this increase in P-gp expression by CDCA was not considered a P-gp expression

hit, it still provided evidence that the In-Cell Western technique is sensitive enough to detect PKI influenced changes in P-gp expression.

5.7. CDCA's Effect on P-gp Expression, Analyzed by Western Blot in the MDCK Cell Line

Western blot analysis was performed using the 100 µM CDCA treatment in the MDCK cell line. Our data supported our previous In-Cell Western data, where 100 µM CDCA treatment significantly increased P-gp expression in the MDCK-MDR1 cell line. Conversely, while both the In-Cell Western data and western blot analysis correlate, the western blot analysis showed a higher amount of P-gp expression at the 100 µM CDCA MDCK-MDR1 treatment (increase of 75% above control), compared to the In-Cell Western (increase of 10 % above control). This suggests that the western blot analysis might be a more sensitive technique in detecting P-gp expression, compared to the In-Cell Western. One possible explanation is that the In-Cell Western requires the cells to be fixed, and the western blot does not. Therefore, fixation may change the epitope slightly, causing the binding affinity of the primary antibody to decrease, leading to an underestimation of the actual P-gp protein expression content. Therefore, our previous assumptions of PKI modulation being more pertinent to P-gp activity compared to P-gp expression based on the In-Cell Western data, may not hold true. In addition, based on western blot analysis, a substantial increase in protein expression may only lead to a modest increase in P-gp activity.

5.8. P-gp Activity Hits Effect on P-gp Expression, Analyzed by In-Cell Western, in the MDCK, Caco-2 and hCMEC/D3 Cell Line

Subsequently, P-gp hits identified in either the MDCK, Caco-2 or hCMEC/D3 cell lines were looked at to see if they modulate P-gp expression, in addition to P-gp activity. Of the 30

PKI hits identified in the MDCK cell line, two were identified to increase P-gp expression above 25% and included the GSK-3 PKI (SB-675259-M) of 25.2 ± 1.0 % and IGF-1R PKI (GSK2220400A) of 29.3 ± 1.5 %. Both GSK-3 PKI (SB-675259-M) and IGF-1R PKI (GSK2220400A) were found to increase P-gp activity by 84.5 ± 0.2 % and 31.7 ± 6.2 %, respectively. As mentioned above, data that supports an increase in both P-gp expression and activity, when GSK-3β is inhibited, is most likely done through the subsequent activation of the wnt/β-catenin pathway (Lim, Kania et al. 2008).

Conversely to our studies, previous studies by Qosa, Abuznait et al. (2012) and Wang, Kathawala et al. (2014) have found that activation of IGF-1R leads to increased expression of *MDR1* gene, and inhibition of IGF-1R leads to increased sensitivity of tumors to cytotoxic drugs corresponding with decreased MDR, respectively. In addition, Tan, Killeen et al. (2014) demonstrated that certain cyclolignan PPP (IGF-1R inhibitor) treated tumour cell lines, resulted in increased IGF-1R gene expression without changes in *MDR1* expression compared to non-treated matched controls. Considering PKIs may be non-specific in nature and may inhibit additional kinases important in the regulation of P-gp activity and expression, this may explain why our studies showed compound IGF-1R PKI (GSK2220400A) increased P-gp activity and expression in the MDCK cell line. In addition, compound IGF-1R PKI (GSK2220400A) may also be a ligand for orphan nuclear receptors including PXR, which are known to increase P-gp activity and expression (Breier, Gibalova et al. 2013).

Of the six PKI P-gp activity hits identified in Caco-2 (Rho kinase (ROCK1) GSK317354A) and hCMEC/D3 (polo-like kinase (GSK1030062A and GW852849X), GSK-3 (GW811761X and GW827102X) and dual ErbB-1/ErbB-2 tyrosine kinase (GW615311X)) from the 30 PKI P-gp hits identified in MDCK, none were found to be P-gp expression hits. This

indicates that PKIs may modulate P-gp activity with minimal effects on P-gp expression, through mechanisms including post-translational modification of the transporter (phosphorylation and glycosylation), P-gp trafficking from the Golgi or nuclear envelope to the plasma membrane and/or P-gp localized lipid raft modification (Chambers, Pohl et al. 1993; Schinkel, Kemp et al. 1993; Gribar, Ramachandra et al. 2000; Yun, Lee et al. 2013)

P-gp activity hits identified in the Caco-2 complete PKI library screen were also analyzed for modulation of P-gp expression. GSK-3 (GW801372X) was the only PKI found to be a P-gp expression hit, increasing P-gp expression by 58.5 ± 3.77%. Previous data showing that GSK-3 (GW801372X) increased P-gp activity by 39.2 ± 7.74 %, in the Caco-2 cell line, possibly indicates that in the Caco-2 cell line, an increase in P-gp expression may not have as great as an effect on P-gp activity as witnessed in the MDCK cell line. Since certain GSK-3 PKIs tended to modulate P-gp expression significantly in both the MDCK and Caco-2 cell line, GSK-3 protein kinase may play an important role in P-gp expression independent of cell line. As mentioned previously, GSK-3β plays an important role in the regulation of the wnt/β-catenin pathways, which in turn influences P-gp expression and activity. This may explain why our results have found that certain GSK-3 PKIs modulate P-gp expression, in addition to P-gp activity (Yamada, Takaoka et al. 2000; Lim, Kania et al. 2008; Shen, Zhang et al. 2013)

5.9. LiCl Effect on P-gp Expression, Analyzed by In Cell Western, in the MDCK Cell Line

Potential effects of LiCl on P-gp expression was also looked at, considering its substantial effect on P-gp activity at both the 100 mM and 10 mM treatments in the MDCK cell line. Our data showed that over a 24 h period LiCl treatment at 100 mM in MDCK-MDR1 cell line was considered a P-gp expression hit, increasing P-gp expression of 81.8 ± 3.9 %, compared

to non-treated matched MDCK-MDR1 control. Augmenting our findings, were studies conducted by Lim, Kania et al. (2008), that showed exposure of hCMEC/D3 cells to LiCl over a 24 h period, substantially increased P-gp expression. Interestingly in the current study, LiCl at the 10 mM treatment increased P-gp activity substantially (82.7 ± 6.5%), but led to a non-significant decrease in P-gp expression. When looking at the wnt/β-catenin pathway, it has been suggested that there are two types of GSK-3β pools, one being membrane bound (Wu and Pan 2010), involved in the propagation of the wnt/β-catenin signal and increasing β-catenin driven P-gp expression and the other existing in the cytosol (Wu and Pan 2010), involved in decreasing the amount of β-catenin driving P-gp expression. Therefore, high doses of LiCl may inhibit both the membrane bound and cytosolic GSK-3β, allowing β-catenin to enter the nucleus and act as a P-gp inducer transcription factor. On the contrary, at lower concentrations, the membrane bound GSK-3β may only be inhibited, allowing the cytosolic GSK-3β to tag β-catenin for degradation, leading to a decrease in P-gp expression.

5.10. LiCl Effect on P-gp Expression, Analyzed by Western Blot, in the MDCK Cell Line

100 mM and 10 mM LiCl treatments with non-treated matched controls were also analyzed by western blot in the MDCK cell line. Our findings identified that the 100 mM and 10 mM LiCl treatments in the MDCK-MDR1 cells increased P-gp expression by 265% and decreased P-gp expression by 52%, respectively, compared to non-treated matched controls. In the In-Cell Western, the 100 mM and 10 mM LiCl treatment in the MDCK-MDR1 cell line increased P-gp expression by 81.8 ± 3.9 % and decreased P-gp expression by 8.52 ± 1.45 %, respectively. Therefore, these findings further support the notion that western blot analysis appears to have increased sensitivity in detecting changes in P-gp protein expression, compared to the In-Cell Western, as was mentioned above.

5.11. Conclusions

The main findings of our studies, are protein kinases tend to modulate P-gp activity and expression in a cell line dependent manner, where Craf1, VEGFR and Tie2 and GSK-3 PKIs appear to be important in modulating P-gp activity in the MDCK cell line, whereas Iκκ, Jnk2/3 and GSK-3 appear to be important in modulating P-gp activity in the Caco-2 cell line. In addition, considering GSK-3 PKIs were found to be important in regulating P-gp activity in both the MDCK and Caco-2 cell line, suggests that this PKI family regulates P-gp activity in a cell line independent manner, possibly through the ubiquitously expressed canonical wnt/β-catenin pathway.

Contrary to PKIs effect on P-gp activity, BCRP activity did not seem to be significantly modulated by PKIs. In addition, based on the In-Cell Western data, PKIs tend to modulate P-gp activity with minimal changes in P-gp expression. Of the compounds that did modulate P-gp expression, were inhibitors of the GSK-3 protein kinase family. Therefore, like P-gp activity, GSK-3 inhibitors appear to be important in modulating P-gp expression, independent of cell line.

When looking at the western blot analysis for CDCA and LiCl and comparing it to the In-Cell Western analysis for P-gp expression, the western blot analysis showed higher expression levels for both CDCA and LiCl treatments in the MDCK cell line. Therefore, western blot analysis appears to be a more sensitive technique in detecting P-gp expression in the above studied cell lines, and consequently the conclusion that PKIs tend to modulate P-gp activity more than expression may not hold true. Thus based on the western blot analysis in MDCK LiCl treated cells, a large change in P-gp expression may lead to a modest change in P-gp activity.

Permeability experiments performed on CDCA showed that a 10% increase in P-gp expression can lead to approximately 42 % increase in P-gp substrate permeability across a monolayer. Therefore, a minimal change in P-gp expression may lead to a large change in P-gp directed permeability across a membrane. Conversely, when focusing on the western blot analysis a large change in P-gp expression may only lead to a modest change in P-gp substrate permeability.

Lastly, most changes in P-gp expression and/or activity by PKIs resulted in maximally one fold change in r123 uptake. Therefore, these changes in P-gp expression and activity by PKIs may not be important from a drug distribution standpoint, but may be important in significantly altering the distribution of known endogenous P-gp substrates including hormones, steroids and lipids (Borst and Elferink 2002; Breier, Gibalova et al. 2013).

5.12. Future Directions

The future directions of these studies continue to be a multistep process. Compounds that led to a decrease in P-gp activity must be analyzed using ATPase studies to determine if the PKI is not interacting directly with the binding site of the transporter, allowing increased r123 levels to accumulate in the cell. In addition, compounds that significantly altered P-gp activity and were screened for alterations in P-gp expression using the In-Cell Western technique, should also be analyzed and validated using western blot, considering the substantial differences that were noted when looking at CDCA and LiCl, as mentioned previously. Furthermore, compounds that were found to significantly alter P-gp activity need to be analyzed using bi-directional studies to evaluate the impact these compounds have on permeability, providing insight into possible drug-drug interactions and multi-drug resistance directed by P-gp. These bi-directional studies can be

used to evaluate PKIs effect on permeability of both endogenous and xenobiotic P-gp substrates including Aβ (Qosa, Abuznait et al. 2012) and chemotherapeutics, respectively and eventually moved into in vivo studies. Ultimately, PKIs may be used to modulate P-gp activity in a direction and magnitude suitable in certain disease settings including Alzheimer's disease, where Aβ has been identified as being a P-gp substrate and increasing P-gp activity may increase Aβ clearance (Hartz, Miller et al. 2010; Qosa, Abuznait et al. 2012) or in cancer, where up regulation of P-gp plays a substantial role in tumor multidrug resistance (Wang, Kathawala et al. 2014; Zandvliet, Teske et al. 2014)

6.0. References

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