10th Annual North American ABC Genetic Workshop

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PROGRAM

10th North American ABC Workshop 2013 Program

September 19th 2013

9:00 –10:00	**Registration**	
10:00	**Welcome**	**Michael Dean**
10:10-10:40		**Lei Zhang, FDA** **Regulatory Science Perspectives on Transporter-Mediated Drug Interactions**
10:40-12:30	**Session 1**	**P-Glycoprotein in Disease and Drug Resistance** **Chair: Jason Chen**
10:40	**Talk 1**	**Yi-jun Wang** **Motesanib, a potent multikinase inhibitor, reverses ABCB1- and ABCG2-mediated drug resistance in cancer cells.**
11:00	**Talk 2**	**Jian-ye Zhang** **Apoptosis sensitization by Euphorbia factor L1 in ABCB1-mediated MDR K562/ADR cells**
11:20-11:50	**BREAK/Posters**	
11:50	**Talk 3**	**Suneet Shukla** **Elucidation of the structural basis of interaction of BCR-ABL kinase inhibitor, nilotinib (Tasigna®) with the human ABC drug transporter P-glycoprotein**
12:10	**Talk 4**	**Sascha C.T. Nicklisch** **Identification and characterization of the molecular interactions of persistent organic pollutants with the multidrug resistance transport protein ABCB1**
12:30-2:00		**Group photograph, Lunch Break and Posters**
2:00-3:20	**Session 2**	**ABC transporters in disease and drug resistance** **Chair: Bjoern Bauer**
2:00	**Talk 5**	**Bjoern Bauer** **Blood-Brain Barrier Dysfunction in Epilepsy: New Targets for Therapy.**

2:20	**Talk 6**	**Amit Tiwari** **Complimentary functions of efflux transporters in anticancer elimination**
2:40	**Talk 7**	**Yu Fukuda** **A porphyrin exporter is required for optimal survival of N-MYC myeloid leukemias**
3:00	**Talk 8**	**Jie Geng** **Conformational changes of the transporter associated with antigen processing (TAP)**
3:20-4:40	**Poster Session I**	
4:40-5:40	**Session 3.**	**Stem cells and ABCG2** **Chair: Olivier LeSaux**
4:40	**Talk 9**	**Laszlo Homolya** **ABC-transporter-based selective marker for isolation of hepatocyte-like cells from stem cell-derived cultures**
5:00	**Talk 10**	**Deshen Wang** **Icotinib (BPI-2009H) reverses multidrug resistance by antagonizing the activity of ATP-binding cassette subfamily G member 2**
5:20	**Talk 11**	**Matt Hall** **Direct bioluminescent imaging of ABCG2 function at the blood-brain barrier using the specific substrate D-luciferin**
5:40	**Adjourn for the day**	
6:00	**Dinner at Restaurant tbd**	

September 20th 2013

9:30-12:30	**Session 4**	**The ABCC family of transporters** **Chair- John Schuetz**
9:30	**Talk 13**	**Chris Brampton** **ABCC6 influences plaque formation in mouse model of atherosclerosis**
9:50	**Talk 14**	**Satish Cheepala** **A role for Abcc4/Mrp4 in platelet function**
10:10	**Talk 15**	**Yousef Hassan** **Role of the PDZ domains of human NHERF1 in regulating surface expression and cellular trafficking of the multidrug resistance protein-4, MRP4 (ABCC4)**
10:30	**Talk 16**	**Shangli Lian** **A role for Abcc4/Mrp4 in Pediatric Acute Myeloid Leukemia (AML)**
10:50-11:20	**BREAK/Posters**	
11:20-12:40	**Session 5**	**Mechanism and model systems to study drug transporters** **Chair- Suneet Shukla**
11:20	**Talk 17**	**Doug Swartz** **A fully functional cysteine-less P-glycoprotein built by directed evolution.**
11:40	**Talk 18**	**Khyati Kapoor** **Conserved residues in the intracellular loop 3 are critical for proper folding of human P-glycoprotein (ABCB1)**
12:00	**Talk 19**	**Irwin Arias** **Hepatocyte polarization: a unique response to stress and a target for liver injury**
12:20	**Talk 20**	**Lisa Murray** **Test System Selection: Enhanced Insights into Transporter Interactions**
12:40		**Concluding remarks- Mike Dean**
12:50		**Conference ends.**

ORAL PRESENTATIONS

Regulatory Science Perspectives on Transporter-Mediated Drug Interactions

Lei Zhang

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Pharmacokinetic drug interactions can lead to serious adverse events. Evaluation of a new molecular entity (NME)'s drug-drug interaction potential is an integral part of drug development and regulatory review prior to its market approval. Transporters are expressed in varying abundance in all tissues in the body where they govern the access of molecules to cells or their exit from cells, thereby controlling the overall distribution of drugs to their intracellular site of action. Clinically relevant interactions mediated by transporters are of increasing interest in drug development. Research in this emerging area has revealed that drug transporters, acting alone or in concert with drug metabolizing enzymes, can play an important role in modulating drug absorption, distribution, metabolism and excretion, thus affect the pharmacokinetics and/or pharmacodynamics of a drug. The newly released revised draft drug interaction guidance by the FDA in 2012 includes updated recommendations in addressing transporter-mediated drug interactions with various decision trees to help guide drug development. The presentation will discuss from regulatory science perspectives, role of transporters in drug development with a focus on transporter-mediated drug-drug interactions. Transporter-related recommendations in the recent FDA's revised draft drug interaction guidance will be discussed. Recent review examples and transporter-related labelings will also be presented and future directions including emerging ITC transporters will be discussed.

Motesanib, a potent multikinase inhibitor, reverses ABCB1- and ABCG2-mediated drug resistance in cancer cells.

Yi-Jun Wang, Rishil J. Kathawala, Yun-Kai Zhang, Kamlesh Sodani, Atish Patel, Priyank Kumar, Tanaji T. Talele, Zhe-Sheng Chen

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Chemotherapy has been the first-line approach for cancer treatment for several decades and involves systemic administration of combination of anticancer drugs from different chemical groups with different mechanisms of action. However, cancer cells often exhibit either intrinsic or acquired resistance to cheomotherapy through a phenomenon known as multidrug resistance (MDR). Several factors are responsible for the development of MDR, preeminent among them being the accelerated drug efflux mediated by overexpression of ATP binding cassette (ABC) transporters. Among them, ABCB1, ABCG2 and ABCCs are the primary contributors of MDR in cancer cells. Motesanib (AMG706), a novel nicotinamide, was identified as a potent, orally bioavailable inhibitor of the VEGFR1/Flt1, VEGFR2/kinase domain receptor/Flk-1, VEGFR3/Flt4, platelet-derived growth factor receptor, and Kit receptors in preclinical models. Therefore, the purpose of this *in vitro* study was to determine whether motesanib could reverse MDR in cancer cells that overexpress the ABCB1 and ABCG2 transporter proteins. The results showed that motesanib (1 μM and 3 μM) significantly reversed MDR in both ABCB1 overexpressing drug selected and transfected cells to paclitaxel, colchicine and vincristine. Similarly, motesanib also significantly reversed MDR in ABCG2 overexpressing drug selected cells to mitoxantrone. The [^{3}H]-paclitaxel accumulation and efflux assays indicated that motesanib increases the intracellular accumulation of ABCB1 substrates by blocking effluxing function of ABCB1 transporter. In contrast, Western blot analysis and immunofluorescence assay revealed that motesanib (3 μM) did not significantly alter the expression and localiztion of ABCB1 in KB-C2 cells. Additionally, a docking model predicted that the binding conformation of motesanib to be within the transmembrane region of homology modeled human ABCB1. We conclude that motesanib, at clinically achievable plasma concentrations, reverse ABCB1- and ABCG2-mediated MDR by blocking drug efflux function of these transporters. These findings may be useful in developing combination therapy for cancer treatment with TKIs.

Apoptosis sensitization by Euphorbia factor L1 in ABCB1-mediated MDR K562/ADR cells

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In this report, reversal activities of Euphorbia factor L1 (EFL1) against ABCB1-mediated multidrug resistance (MDR) and apoptosis sensitization in K562/ADR cells are reported. EFL1 decreased the IC_{50} values of anticancer agents in K562/ADR cells over-expressing ABCB1. However, EFL1 did not change the IC_{50} values of anticancer agents in sensitive K562 cells. Additionally, EFL1 increased the intracellular accumulation of rhodamine 123 and doxorubicin in K562/ADR cells without affecting their accumulation in K562 cells. Furthermore, EFL1 sensitized the apoptosis triggered by vincristine in K562/ADR cells, as confirmed by Annexin V-FITC/PI detection and Western blot. At the same time, EFL1 did not influence the apoptosis induced by vincristine in K562 cells. Western blot results showed that EFL1 did not affect the phosphorylation level of AKT and ERK in K562 and K562/ADR cells. Finally, EFL1 did not down-regulate protein expression of ABCB1.

Elucidation of the structural basis of interaction of BCR-ABL kinase inhibitor, nilotinib (Tasigna®) with the human ABC drug transporter P-glycoprotein

Suneet Shukla,[†] Eduardo E. Chufan,[†], Satyakam Singh,[‡] Amanda P. Skoumbourdis,[‖] Khyati Kapoor,[†] Matthew B. Boxer,[‖] Damien Y. Duveau,[‖] Craig J. Thomas,[‖] Tanaji T. Talele,[‡] Suresh V. Ambudkar.[†]

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Nilotinib (Tasigna®) is a tyrosine kinase inhibitor (TKI) that is used to treat imatinib resistant chronic myeloid leukemia patients. It is also a substrate of the ATP binding-cassette (ABC) drug efflux transporters P-glycoprotein (P–gp, ABCB1) and ABCG2 (BCRP). Identification of nilotinib's binding site in the drug-binding pocket of these transporters is important to gain an understanding of its interactions and can be exploited to design novel TKIs that would not interact with ABC drug transporters. Molecular docking of nilotinib in a homology model of human P-gp was performed to map its binding site in the substrate-binding pocket of P-gp. The docked model showed that nilotinib occupied site-1 (primary binding site) in the P-gp drug-binding pocket where aromatic residues, hydrogen bond acceptors and donor groups of nilotinib interact with amino acid groups lining the pocket. This docked model of nilotinib was evaluated by mutational mapping studies replacing representative amino acids Y307, M949 and A985 with cysteine, which resulted in the loss of nilotinib's activity to inhibit substrate binding to this pocket. The orientation of nilotinib in this binding pocket was further substantiated by assessing the interaction of nilotinib's structural derivatives with P-gp using ATP hydrolysis, photoaffinity labeling with a transport substrate and transport assays. These results demonstrate that the pyridine and pyrimidine ring in nilotinib play a key role in its interaction with the drug-binding pocket, while orientation of the imidazole ring determines the affinity of nilotinib to P-gp. The data presented in this study for the first time identifies the primary binding site of nilotinib in the drug-binding pocket of P-gp, a major ABC drug transporter.

Identification and characterization of the molecular interactions of persistent organic pollutants with the multidrug resistance transport protein ABCB1

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Multidrug resistance (MDR) transporters, from the ATP binding cassette (ABC) superfamily, are thought to act as determinants of chemical accumulation in cells. Despite a long history of research on the interactions of MDR proteins with pharmaceuticals, little is known about the molecular basis of their interactions with environmental compounds such as persistent organic pollutants (POPs). Here, we used purified recombinant P-glycoprotein (ABCB1) from mouse to measure pollutant-transporter interaction kinetics via ATPase stimulation. Our results show that many persistent pollutants are inhibitors of mouse ABCB1. For example, PBDE-209 and PCB-145 are potent inhibitors with IC_{50} values of 4.09±0.4μM and 4.4±0.4μM, respectively. In addition we found that isomeric forms of the same pollutant compound, such as Dieldrin (9.2±1.8μM) and Endrin (0.88±0.1μM), can differ dramatically in their IC_{50} values by up to an order of magnitude and thereby reaching inhibition coefficients in the range of the model inhibitor cyclosporine A. Finally we found that certain common pollutants, such as DEHP and BPA, do not appear to stimulate mouse ATPase activity indicating non-recognized substrates for this major MDR transporter. Our future directions will be to determine the kinetics and general regions of pollutant binding to transporters using fluorescence polarization and molecular docking. In addition, we plan to extend these findings to ABCB1 of the food fish yellowfin tuna (*Thunnus albacares*) to investigate whether evolutionary conserved structural features of ABC transporters and pollutants can govern pollutant transfer from oceans to man. Analytical chemistry on "real-world" pollutant levels in tuna will be used to guide the selection of pollutants for further study. These results will shed light on the question of how transporters can direct pollutant movement through the environment and will lead to new avenues for design of more potent pharmaceuticals and safer industrial chemicals.

Blood-Brain Barrier Dysfunction in Epilepsy: New Targets for Therapy

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The blood-brain barrier is dysfunctional in patients with epilepsy. Barrier dysfunction is due to seizure-induced decrease of influx transporter expression, increase of efflux transporter and metabolic enzyme expression, and development of barrier leakage. These changes likely reduce brain uptake of antiepileptic drugs (AEDs), thereby contributing to AED resistance. In addition, blood-brain barrier leakage is a consequence of seizures, and itself a trigger of seizures. Thus, blood-brain barrier dysfunction may contribute to both seizure genesis and AED resistance. However, the mechanism(s) responsible for these pathologies is/are unknown. We have addressed this clinical challenge and identified the LOX/COX pathway to be critical for seizure-induced barrier dysfunction.

We found that glutamate signaling through the COX arm (Glu→NMDAR→cPLA2→COX-2→Prostaglandins) decreased expression of AED influx transporters (MCT1, LAT, Oatp2) and increased expression of AED efflux transporters (P-gp, BCRP, Mrp2) and metabolic enzymes (Cyp2c8, 2c9, 3a4). These changes could result in AED resistance. We also found that glutamate activation of the LOX arm (Glu→NMDAR→cPLA2→5-LOX→Leukotrienes) increased permeability of brain microvessels, which could contribute to barrier leakage. Importantly, blocking the LOX/COX pathway in chronic epileptic rats abolished the seizure-induced changes in transporter and enzyme expression levels and prevented barrier leakage.

Our data show that seizure-induced release of glutamate activates the LOX/COX pathway, thereby changing expression of influx and efflux transporters, metabolic enzymes, and causing barrier leakage, which altogether leads to barrier dysfunction. Our findings suggest new therapeutic targets for designing a novel therapy with a two-fold benefit: 1) reduced AED resistance and 2) reduced seizure genesis for improved epilepsy treatment and better seizure control in patients.

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Complimentary functions of efflux transporters in anticancer elimination

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Chemotherapy involves concomitant administration of poly-drugs and with this, frequency of possible drug interactions increases, leading to serious adverse effects in patients. This is further complicated by the differential presence of ATP-binding cassette (ABC) multidrug transporters, which have different capability to handle variety of drugs. In this study we show that the ABC transporters ABCB1, ABCC2, ABCC4 and ABCG2 can markedly affect the pharmacokinetics of topotecan and topotecan lactone. These findings may be important for the treatment of cancer patients with topotecan, because for each of these drug transporters polymorphisms and mutations have been identified that affect their expression and/or activity. These polymorphisms and mutations may partly explain the high variability in the oral availability of topotecan observed among cancer patients. On the other hand, we also show that these ABC transporters have redundant functions in the elimination of topotecan. This overlap in functionality is favorable during patient treatment, as it reduces the risk of dramatically increased topotecan exposure and toxicity due to variable expression and/ or function of just one of these multidrug transporters. These insights may be used to further optimize pharmacotherapy with topotecan and possibly with related anticancer agents.

Optimal survival of N-MYC myeloid leukemias require a porphyrin exporter

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Identifying pathways facilitating the survival of certain leukemias may reveal unique vulnerabilities. Here we determined that pediatric AML with a unique N-MYC gene signature strongly upregulated genes in the porphyrin biosynthetic pathway. However, the significance and relative importance of porphyrin biosynthesis and homeostasis in myeloid leukemias is unknown. We show N-MYC strongly activates *de novo* porphyrin biosynthesis. Using a murine model of N-MYC myeloid leukemia we demonstrated that absence of the porphyrin exporter, *Abcg2*, impaired N-MYC leukemogenesis and blocked self-renewal of N-MYC expressing hematopoietic progenitors. Consistent with this, in N-MYC hematopoietic progenitors, *Abcg2* absence *in vivo* strongly reduced the number of leukemic stem and myeloid progenitors. Inhibition of porphyrin biosynthesis or re-introduction of ABCG2 in N-MYC expressing hematopoietic progenitors mostly restored self-renewal. In human myeloid leukemias expressing ABCG2, inhibition of ABCG2 produced porphyrin elevation that was accompanied by mitochondrial ROS as well as loss of both mitochondrial membrane potential and viability. In total, there results reveal a critical dependence of N-MYC myeloid leukemias upon the porphyrin exporter, Abcg2, in mice and suggest that modulating porphyrin levels in patients with these AMLs may be a promising approach to improve therapeutic efficacy.

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Conformational changes of the transporter associated with antigen processing (TAP)

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The transporter associated with antigen processing (TAP) plays a critical role in the major histocompatibility complex (MHC) class I antigen presentation pathway. TAP translocates cellular peptide across the endoplasmic reticulum (ER) membrane, in an ATP hydrolysis dependent manner. We used fluorescence resonance energy transfer (FRET) spectroscopy in permeabilized cells to delineate different conformational states of TAP in a native cellular membrane environment. For these studies, we tagged the TAP1 and TAP2 subunits with ECFP and EYFP respectively, C-terminal to their nucleotides binding domains (NBD), and measured FRET efficiencies under different conditions. Our data indicate increases in FRET efficiency following nucleotide binding, with the most significant effects induced by ATP. Peptide binding alone also induces a large and significant increase in the FRET efficiency, with a concentration dependence that is reflective of individual peptide affinities for TAP. Maximal FRET efficiency is induced by the combination of peptide and ATP/orthovanadate or AMP-PNP. Taken together, these findings show that at least three steps are involved in transport of peptide across the ER membrane for antigen presentation, corresponding to three dynamically and structurally distinct conformational states of TAP.

ABC-transporter-based selective marker for isolation of hepatocyte-like cells from stem cell-derived cultures

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The liver, the major organ of detoxification, is mainly composed of polarized hepatocytes, which secrete bile and selectively excrete various toxic compounds into the bile. This process involves ABC transport proteins and fundamentally determines the absorption, distribution, excretion, and toxicity (ADME-Tox) of endo- and xenobiotics. Pharmacological and toxicology studies often employ liver model cells. These hepatic model systems are mostly based on primary cell cultures or cancerous cell lines, therefore, their applicability and availability is greatly limited. Human pluripotent stem cells may offer an alternative cell source providing an unlimited number of progeny cells theoretically of any tissue type. However, even complex, sophisticated directed differentiation protocols results in heterogeneous cell cultures containing a limited number of target cell type. To identify hepatocyte-like cells in a mixed cell culture, we generated a transposon-based selective marker, which can readily be introduced in human embryonic stem cells. The construct harbors liver-specific promoter-driven fluorescent proteins, i.e., alpha fetoprotein promoter-driven GFP and ABCB11 (BSEP) promoter-driven mCherry. During differentiation of stem cells toward the hepatic lineage this marker makes the progeny cells green or red fluorescent depending on the stage of differentiation. In the present work we demonstrate the design, generation, and characterization of this selective marker in various cellular model systems.

This work has been supported by grants from the National Development Agency (KTIA_AIK_2012_0025, OTKA_A08_CK80283), and the Lendület Program of the Hungarian Academy of Sciences.

Icotinib (BPI-2009H) reverses multidrug resistance by antagonizing the activity of ATP-binding cassette subfamily G member 2

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Background: Icotinib (BPI-2009H) is a well tolerated, potent and specific small-molecule inhibitor of EGFR tyrosine kinase, which has showed clinical anticancer activity in patients with advanced non-small cell lung cancer (NSCLC). We conducted this study to investigate whether Icotinib could interact with ATP-binding cassette subfamily G member 2 (ABCG2)-mediated multidrug resistance (MDR).

Material and methods: Lung cancer cell line A549, NCI-H460 and its drug-selected derivative ABCG2-overexpressing NCI-H460/MX20, as well as the ABCG2-, ABCB1-, and ABCC10-overexpressing transfected cell lines were used for this study.

Results: Our results showed that Icotinib at 5.0 μmol/L significantly reversed ABCG2-mediated drug resistance by antagonizing the drug efflux function of ABCG2 and increasing the intracellular accumulation of [3H]-MX in ABCG2-overexpressing cells. However, Icotinib at reversal concentration did not reverse ABCB1- and ABCC10-mediated drug resistance in HEK/ABCB1 and HEK/ABCC10 cell lines. In addition, Icotinib at reversal concentration did not affect the expression level and stimulate translocation of ABCG2.

Conclusion: We concluded that Icotinib reversed MDR by antagonizing the activity of ABCG2.

Direct bioluminescent imaging of ABCG2 function at the blood-brain barrier using the specific substrate D-luciferin

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ABC transporters play a key role in protecting the brain parenchyma by exerting their action at the blood-brain barrier (BBB). However, these transporters also block the entry of therapeutic drugs, including cancer chemotherapeutics, limiting their efficacy against primary malignancies and metastases. One of the key transporters playing this role is ABCG2. While other ABC transporters can be studied through PET and SPECT, no probe exists for directly imaging ABCG2 function at the BBB. D-luciferin, the endogenous substrate of fLuc, has been shown to demonstrate decreased bioluminescence in ABCG2-expressing cells. Biodistribution and cell uptake kinetic studies have confirmed low brain distribution of D-luciferin. Given that the BBB probably restricts entry of D-luciferin, we hypothesized that we can image ABCG2 function at the blood-brain barrier using bioluminescent imaging in transgenic mice expressing fLuc in the brain. The selectivity of D-luciferin among other transporters was measured in vitro by the accumulation of fluorescence in human and mouse cells that overexpress each transporter. Accumulation of D-luciferin was lowest in cells overexpressing ABCG2, and the accumulation increased when co-administered Ko143, a potent and selective ABCG2 inhibitor. Among the three most prevalent efflux transporters at the blood-brain barrier, D-luciferin is a specific substrate for ABCG2. Using a mouse model expressing fLuc behind the GFAP promoter, mainly expressed in astrocytes of the brain, D-luciferin BLI signal was found to be low. However, it increased in a dose-dependent fashion with co-administration of the ABCG2 inhibitor Ko143, but not the P-gp inhibitor DCPQ. This method for directly imaging ABCG2 function at the BBB will be of use in understanding pharmacokinetic inhibition of the transporter. Furthermore, ABCG2's role in the efflux of D-luciferin at the blood-brain barrier should be considered for future bioluminescence neuroimaging protocols.

ABCC6 influences plaque formation in mouse model of atherosclerosis

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Cardiovascular disease is the leading cause of death and morbidity worldwide. The main pathological processes associated with this are arteriosclerosis and atherosclerosis resulting from an imbalance in lipid metabolism and early cardiovascular manifestations. Arteriosclerotic disease may be caused by acquired (aging, diabetes kidney disease) or genetic pathological conditions such as pseudoxanthoma elasticum(PXE) or generalized arterial calcification of infancy (GACI). PXE and GACI are characterized by variable levels of ectopic mineralization resulting from mutations in ABCC6, a protein primarily expressed in liver and kidneys. *Abcc6*-null mice develop calcification recapitulating the human PXE phenotype including mineralization of the skin and arteries. In addition, aging *Abcc6*-/- mice have low HDL-cholesterol levels when fed a normal diet and HDL levels inversely correlates with arteriosclerosis and vascular calcification in human PXE patients. How ABCC6 deficiency leads to arterial mineralization remains unclear. Here, we investigated whether ABCC6 was able to influence arterial calcification in a model of atherosclerosis using *Ldlr*-deficient mice. One month old *Ldlr-/-*, *Abcc6-/-* and *Ldlr-/- x Abcc6-/-* or +/- were fed either normal diet or high fat chow for 16 weeks. Aortic plaque formation and total calcium content were assessed and interestingly, we found no significant increase in calcium content between *Ldlr-/- x Abcc6-/-* mice and the control *Ldlr-/-* animals on high fat diet. However, both *Ldlr-/- x Abcc6-/-* and *Ldlr-/- x Abcc6*+/- fed high fat chow had significantly more plaque than *Ldlr-/-* mice while maintaining identical total cholesterol levels. These results suggested that ABCC6 has a more complex role in the onset of arterial disease than just calcium deposition for which it is commonly associated. This has important implications for both sufferers of PXE and carriers of *ABCC6* mutations.

The Role of Abcc4 in Platelets Physiologic Function and Megakaryopoiesis

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Platelet activation is a highly regulated process, and cyclic nucleotide mediated signaling pathways play an important role in the activation process. Exposed collagen at the vascular injury site binds to circulating platelets through the platelet's "collagen" receptor, GPVI, and results in the activation of guanyly/adenlyl cyclases. These interactions cause rapid alterations in the cyclic nucleotide concentration inside the platelets and leads to activation of protein kinase A and G signaling pathways which in turn modulate platelet function.

The role of cyclic nucleotides in platelet aggregation has been widely established. Although, ABCC4 functions as a plasma membrane transporter for cyclic nucleotides its role as a plasma membrane transporter in platelet biology is unknown because it was reported as intracellularly localized in the platelets dense granules. This original report (Jedlitschky, Tirschmann et al. 2004) evaluated ABCC4 localization by immune-fluorescence of platelets attached to collagen coated coverslips. However, this attachment via collagen results in activation of platelets producing mobilization and fusion of alpha and dense granules to the plasma membrane. Clearly under these conditions, distinguishing between plasma membrane and dense granule localization is not possible. We resolved this problem by labeling the platelets membrane proteins using cell impermeable biotinylating agent (EZ-Link Sulfo-NHS-LC-LC Biotin) and demonstrated that both the human and mouse Abcc4 localizes to the plasma membrane.

We extended these studies to the *Abcc4*- knockout (KO) mouse model. The *Abcc4*- KO mouse does not have any change in the number of platelet or dense granules compared to the wild type mouse. Platelet activation *in vivo* can be initiated by interaction with collagen through the GPVI receptor that is expressed at the plasma membrane of the platelets. At the molecular level, the initiation of platelet activation by collagen results in an increase in the cyclic nucleotide concentration leading to activation of signaling cascade through protein kinase A or G. Exposing Abcc4-KO platelets to collagen revealed impaired activation in response to collagen. However, *Abcc4*-KO platelets activated by either thrombin or ADP (which activate either G-coupled PAR receptors or P2Y12 receptor respectively) exhibits an aggregation profile almost identical to wildtype platelets, thus indicating the defect in *Abcc4* -KO platelet aggregation is specific to the collagen pathway. Furthermore, in Abcc4-KO platelet lysates Abcg2 protein expression was slightly elevated compared to the wildtype. These results coincide with the fact that Abcg2 has capacity to transport cGMP, an important physiological substrate involved in collagen mediated platelet activation process. Based on these results we speculated that lack of Abcc4 may be compensated by increasing the expression of Abcg2. However,

Abcg2 KO or Abcc4/ Abcg2 double knockout platelets did not show any defect in collagen mediated aggregation.

We further investigated the basis for the impaired collagen aggregation of *Abcc4*-KO platelets. Interestingly, in the *Abcc4*-KO platelets, after collagen activation, cyclic nucleotide dependent phosphorylation of VASP through protein kinase A or G at Ser-157 or Ser-239 respectively is reduced. Notably, Abcc4-null platelets have reduced GPVI surface expression that correlated with the reduced phosphorylation of VASP after collagen stimulation. The similar, protein levels of Syk and Plcg2, (downstream signaling molecules of GPVI signaling pathway), in the *Abcc4* wildtype and KO platelets implies that GPVI expression is the primary defect in Abcc4 deficiency. While these results suggest that Abcc4 plays a crucial role in regulating cyclic nucleotides in response to GPVI activation by collagen the defect was not as profound as expected. One possibility is decreased phosphodiesterase expression PDE2in the Abcc4 KO platelets compared to wildtype. Interestingly, when PDE2 activity was inhibited with EHNA (a PDE2 inhibitor and non –Abcc4 substrate) we found that Abcc4 KO platelets have a greater aggregation defect compared to the wildtype. Based on these results we propose that Abcc4 plays an important role in platelet aggregation process by regulating cyclic nucleotide levels.

These findings suggest that ABCC4/Mrp4 loss of function or inhibition (by drugs) may disrupt platelet aggregation. Many phosphodiesterase inhibitors used in antiplatelet therapy are also potent inhibitors of Abcc4 (e.g., Dipyridamole, Cilostozal, EHNA, Anagrelide, and Sildenafil) suggest that anti-platelet therapeutic efficacy of these drugs is also due to inhibition of Abcc4 function. These findings have strong implications not just the development of antiplatelet drugs, but also for further understand the role of Abcc4 in regulating intracellular nucleotide levels during platelet activation process.

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Role of the PDZ domains of human NHERF1 in regulating surface expression and cellular trafficking of the multidrug resistance protein-4, MRP4 (ABCC4)

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The 170 kDa multidrug resistance protein 4 (MRP4) or ABCC4 is a 4-domain membrane protein (encoded by *ABCC4*) with established roles in mediating cellular efflux of hydrophilic anti-cancer and antiviral agents and their metabolites, as well as signalling molecules that influence cell proliferation and pain (eg. cAMP and PGE_2). Elevated MRP4 levels are also associated with poor prognosis in neuroblastoma and an *ABCC4* polymorphism has been linked with hematopoietic toxicity associated with thiopurine-based chemotherapy regimens. MRP4 may traffic to either the basolateral or apical membrane in normal polarized epithelial cells, depending on the tissue in which it is expressed. We showed previously that Na^+/H^+ exchanger regulatory factor 1 (NHERF1), which contains two PDZ domains, plays a pivotal role in membrane trafficking, internalization, and consequently, the substrate efflux function of MRP4. Using pull-down and overlay assays, we have now determined that the C-terminal PDZ motif of MRP4 interacts with the PDZ1 domain of human NHERF1. In addition, *in vitro* NHERF1 interactions with MRP4 appear to be affected by the modification status of Ser^{77} in NHERF1-PDZ1. Thus a phosphomimetic mutation of Ser^{77} (Ser to Asp) decreases hNHERF1 interactions with MRP4 while a phosphodeficient mutation (Ser to Ala) does not. Moreover, a NHERF1-PDZ1 polymorphism (rs139622189) that results in substitution of Glu^{68} with Ala, and is associated with hypophosphatemia and defective trafficking of the phosphate transporter NPT2a, also abrogates binding of NHERF1 to MRP4. Ectopic expression of the PDZ1 phosphomimetic NHERF1-S77D mutant increases plasma membrane levels of endogenous MRP4 in non-polarized HEK293T cells as assessed by cell surface biotinylation assays. It also enhances levels of MRP4 at the apical membrane in polarized MDCKI cells as detected by confocal microscopy.
Together, our observations indicate that MRP4 interactions with NHERF1 are dependent on the first PDZ domain (PDZ1) of this adaptor protein and suggest that these interactions can be regulated by the phosphorylation of Ser^{77} or attenuated by NHERF1 polymorphisms near that region.

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A role for Abcc4/Mrp4 in Pediatric Acute Myeloid Leukemia (AML)

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Pediatric acute myeloid leukemia (AML) is an aggressive malignancy comprising 15-20% of childhood acute leukemias and remains a challenging disease with cure rates of only 60% using conventional therapy. However, unlike ALL de novo AML is characterized by a very low burden of genomic alterations suggesting other genetic and/or epigenetic alterations occur within hematopoietic stem cells (HSC) or progenitors to affect their normal self-renewal, proliferation and/or differentiation in this disease. We have determined that recurrent copy number alterations of ABCC4/MRP4 occur in some *de novo* AML, suggesting MRP4 has a role in myeloid leukemia. Moreover, gene expression profiling from 155 pediatric AML patients showed that MRP4 is differentially expressed among AML subtypes with M7 having the highest expression.

To characterize the function of MRP4 in normal hematopoiesis and in the etiology of AML, we use a murine Mrp4 knockout model. Mrp4KO mice had a similar number of BM progenitors as WT mice did. However, hematopoietic stem cells of Mrp4KO appeared to have an intrinsic defect because short- and long-term repopulation is impaired in a competitive repopulation (CR) mouse model.

To examine the role of MRP4 in AML etiology, we generated an NMYC-overexpressing AML model because NMYC overexpression is a frequent event in pediatric AML and is highly oncogenic in mouse myeloid cells (1). In addition, NMYC expression was frequently associated with elevated MRP4 expression, a recently reported target of NMYC (2, 3). We showed that NMYC-overexpressing WT BM cell had an increased progenitor population, which was absent in Mrp4KO cells. Because overexpression of MRP4 in lineage-depleted bone marrow cells increased the CD34+ population which correlated with increased expression of progenitor marker such as cKit, we tested whether exogenous MRP4 could rescue the Mrp4KO progenitor population. We demonstrated that putting back MRP4 in Mrp4KO cells indeed recued the progenitor populations. Furthermore, Mrp4-deficiency delayed in vivo NMYC-dependent leukemogenesis in AML mouse model, possibly by decreasing the proliferation/survival of the tumor cells. Cox-1, Cox-2, EP1, or PKA inhibitor all led to inhibition in colony formation of the WTNMYC tumor cells, indicating that the PGE2 signaling pathway is important for the survival of the cells. Current studies are further elucidating pathways whereby MRP4 provides a biological advantage.

In summary, Mrp4 is important for both normal and deregulated progenitor functions, especially during leukemogenesis when progenitor populations greatly increase. It is likely MRP4 alters survival and/or proliferation of progenitors. This work will further our understanding of ABC transporters in the normal biology and pathology of hematopoietic cells and has the potential to facilitate identification of new therapeutic targets for AML treatment.

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A fully functional cysteine-less P-glycoprotein built by directed evolution

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P-glycoprotein (Pgp) contributes to multidrug resistance in cancers and many other diseases through a poorly understood polyspecific drug transport mechanism. Understanding the steps required for Pgp drug binding and transport would allow for rational design of novel Pgp inhibitors, and provide a deeper knowledge of the general ABC transport mechanism. The objective of this project was to employ directed evolution to construct a highly expressed, fully active cysteine (Cys)-less Pgp that can be used as a template for site-specific incorporation of thiol reactive probes. Mouse Pgp contains 3 Cys in the transmembrane domains (TMDs), 3 in the first nucleotide binding domain (NBD) and adjacent flexible linker, and 3 in the second NBD. Site-saturation mutagenesis replaced the two sets of intracellular Cys in our codon-optimized mouse Pgp (Opti-Pgp) with degenerate codons that encode all 20 amino acids. Mutants were then subjected to a stringent selection in *S. cerevisiae*, followed by DNA sequencing of single surviving colonies to identify active mutants. Generally, Cys mutations were biased towards Ser, or Gly and other small hydrophobic amino acids except C1121, which had a strong preference for Arg. A subset of these mutants was successfully combined to form mutants with all six intracellular Cys removed that were highly active in quantitative yeast drug resistance assays. One of these mutants was chosen as the template to successively remove the three remaining TMD Cys, generating single and double Cys mutants, and a fully Cys-less mutants that all maintain high levels of yeast drug resistance. These mutants were purified in milligram quantities and found to be fully active in drug-stimulated ATPase assays. Overall, our novel directed evolutionary approach has provided a Cys-less protein that has overcome the limitations of previous Cys-less Pgp molecules.

Conserved residues in the intracellular loop 3 are critical for proper folding of human P-glycoprotein (ABCB1)

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P-glycoprotein (P-gp, ABCB1) is an ATP-Binding Cassette (ABC) transporter that effluxes a variety of structurally diverse amphipathic or hydrophobic compounds from cells, including anticancer drugs. The X-ray structures of *C. elegans* and mouse P-gp in the apo conformation show that the Nucleotide Binding Domain-Transmembrane domain (NBD-TMD) interface is important for transmission of conformational changes upon ATP hydrolysis, which results in substrate translocation. Intracellular loop 3 (ICL3) together with other ICLs in the homology model of human P-gp appear to form this NBD-TMD interface. We hypothesized that the conserved residues in this region might play a role in maintaining the integrity of the P-gp structure. To investigate this, we mutated the five conserved residues (R798, D800, D805, D806 and K808) in ICL3 to alanine in cysless WT P-gp and expressed them in HeLa cells using BacMam baculovirus transient expression system to study their cell surface expression and transport function. We found that expression levels of R798A and K808A were similar to those of cysless-WT and their transport function was not affected. On the other hand, three other mutants (D800A, D805A and D806A) had reduced cell surface expression and also showed decreased transport of calcein-AM, rhodamine 123 and daunorubicin. We further observed that D800A, D805A and D806A mutant P-gps could be rescued to the cell surface by treatment with 6.25 μM of the pharmacological chaperone cyclosporine A. The rescued mutant proteins exhibited normal transport of all the fluorescent substrates tested. The homology model data supports that some of the mutations (D800A, D805A and D806A) have a more drastic effect than others (R798A and K808A). In aggregate, our results demonstrate that ICL3, which forms part of the interface between NBD2 and the TMDs, is important for proper folding of P-gp.

Hepatocyte polarization: a unique response to stress and a target for liver injury

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Hepatocyte depolarization, which occurs in response to infection, drug induced liver injury, cholestasis and shock, is not frequently recognized because bile canaliculi are not stained by hematoxylin-eosin. Depolarization prompts internalization of canalicular ABC B11 and other transporters resulting in bile acid-mediated injury to organelles, particularly mitochondria. We studied how polarization as manifested by bile canalicular network formation is restored after the stress of rat hepatocyte isolation (PNAS 2013). Additional studies concern the polarization response to cell starvation on removal of glucose. We examined whether human hepatocytes containing HBV are polarized.

METHODS: Using collagen sandwich cultures of rat hepatocytes, sequential measurements were made of mitochondrial fission-fusion proteins, ATP production and its source, AMPK activation and its consequences, and apical polarization using biochemical, biophysical, immunocytochemical, EM and live cell imaging. In human liver biopsies, HBV containing hepatocytes were stained for HBsAg (orcein), ABCB11 and (in progress) other canalicular markers.

RESULTS; The stress of hepatocyte isolation resulted in depolarization, substantial ATP reduction and mitochondrial fission. Repolarization was associated with up-regulation of mitochondrial fusion proteins (Mfn1/2, opa1), inhibition of fission regulator (Drp1) and ATP synthesis, which was initially generated by oxidative phosphorylation facilitated by beta-oxidation of lipid droplets and autophagy. AMPK was sequentially activated over 5 days thereby augmenting ATP production by stimulating glycolysis. Over-expression of DN-AMPK prevented polarization but did not acutely affect mitochondrial fusion. Glucose deprivation also accelerated canalicular network formation. In human liver biopsies, many hepatocytes which contained HBV were not polarized as determined by altered distribution of ABCB11.

SUMMARY: Mitochondria and AMPK coordinately control restoration of hepatocellular polarization after stress. Under stress, hepatocytes preferentially use their sparse newly synthesized ATP to restore cell polarization. In human liver biopsies, many HBV infected hepatocytes were not polarized.

CONCLUSION: This unexpected pattern of initial stress response is likely evolutionarily conserved in other epithelial cells and in regeneration and development. This study supports the hypothesis that stress, as occurs with infection (here illustrated by HBV), drugs, shock, etc, may overwhelm this system resulting in depolarization, mitochondrial dysfunction, cholestasis and liver failure.

Test System Selection: Enhanced Insights into Transporter Interactions

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Increasing pressures to achieve clinically-relevant, site-specific concentrations under in vitro conditions as well as test systems most relevant to the site of interaction have added to the complexity of transporter study designs. Membrane transporters play a clinically important role in drug absorption and disposition;Caco-2 and MDCK cells are the most widely used *in vitro* models for studying the functions of these transporters and associated drug interactions. Transport studies using these cell models are mostly focused on apical transporters, while basolateral drug transport processes are largely ignored. However, for some hydrophilic drugs, a basolateral uptake transporter may be required for drugs to enter cells before they can interact with apical efflux transporters. This presentation evaluates potential differences in drug transport across Caco-2 and MDCK basolateral membrane that could cause discrepancies in the identification of efflux transporter substrates, and elucidates the underlying factors that may cause such differences, using rosuvastatin as a model substrate. Bidirectional transport results in Caco-2 and BCRP-MDCK cells demonstrate the necessity of an uptake transporter at the basolateral membrane for rosuvastatin. Kinetic study reveals saturable and nonsaturable processes for rosuvastatin uptake across the Caco-2 basolateral membrane, with the saturable process encompassing >75% of overall rosuvastatin basolateral uptake at concentrations below the *Km* (4.2 μM). Furthermore, rosuvastatin basolateral transport exhibits *cis*-inhibition and *trans*-stimulation phenomena, indicating a facilitated diffusion mechanism. This basolateral transporter appears to be a prerequisite for rosuvastatin and perhaps for other hydrophilic substrates to interact with apical efflux transporters. Deficit of such a basolateral transporter in certain cell models may lead to false negative results when screening drug interactions with apical efflux transporters. Test system selection is critical in achieving clinically relevant transporter results.

POSTER PRESENTATIONS

Studies of the C-terminal extension of NBD1

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The sulfonylurea receptor (SUR) is an ATP-binding cassette transporter that performs as the regulatory subunit of the ATP-sensitive potassium (K_{ATP}) channel. Binding and hydrolysis of MgATP at the SUR nucleotide binding domains (NBDs) results in channel opening. Several aspects of the regulation of the K_{ATP} channel by the NBDs are yet to be elucidated at the molecular level. Regulation of K_{ATP} channel gating by the NBDs is critical as mutations of the NBDs result in diseases. For example, mutations of the NBDs of the cardiac-specific SUR protein (SUR2A) cause cardiovascular disorders. Thus, studies of the NBDs are critical to understanding SUR2A-mediated regulation of K_{ATP} channels.

The region of SUR2A between the canonical end of NBD1 and beginning of the second membrane spanning domain contains a region of fifteen consecutive negatively charged amino acids, ^{947}EDEDEEEEEEEDEDD^{961,} known as the ED region. This set of residues is conserved among species and has been shown to affect NBD1/NBD2 dimerization, and to influence drug action on K_{ATP} channel gating. In an effort to understand the molecular basis underlying the effect of the ED region on NBD1, we performed NMR and fluorescence studies on SUR2A NBD1 proteins with and without the ED region. NMR data comparing the two proteins, as well as nucleotide and drug binding data will be presented. Our results will further highlight the role of the ED region in controlling the activity of NBD1, which leads to a greater understanding of the regulation and behaviour of the K_{ATP} channel.

Enhancing Chemosensitivity in ABCB1- and ABCG2-Overexpressing Cells and Cancer Stem-Like Cells by An Aurora Kinase Inhibitor CCT129202

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Imidazopyridine CCT129202 is an inhibitor of Aurora kinase activity and displays favorable antineoplastic effect in preclinical studies. Here, we elucidate the enhanced effect of CCT129202 on the cytotoxicity of chemotherapeutic drugs in multidrug resistant (MDR) cells and cancer stem-like cells via interacting with ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2). In MDR cells overexpressing ABCB1 or ABCG2, nontoxic dosage of CCT129202 significantly enhanced the cytotoxicity of ABCB1 or ABCG2 substrate drugs and increased the intracellular accumulations of doxorubicin and rhodamine 123, while no effect was found on parental cells and cells overexpressing ABCC1. Interestingly, CCT129202 also potentiated the sensitivity of cancer stem-like cells to doxorubicin. Importantly, CCT129202 increased the inhibitory effect of vincristine in ABCB1 overexpressing KBv200 cell xenografts in nude mice and that of paclitaxel in human esophageal cancer tissue overexpressing ABCB1 *ex-vivo*. In addition, the ATPase activity of ABCB1 was inhibited by CCT129202. Homology modeling predicted the binding conformation of CCT129202 within the large hydrophobic cavity of ABCB1. However, CCT129202 did not apparently alter the expressions of ABCB1 or ABCG2, as well as the phosphorylated Aurora A, the phosphorylated histone H3, p21 and c-Myc in MDR cells at a resistance reversal concentration. In conclusion, CCT129202 significantly reversed ABCB1- and ABCG2-mediated MDR *in vitro*, *in vivo* and *ex-vivo* by directly inhibiting the function of their transporters and enhanced the eradication of cancer stem-like cells by chemotherapeutic agents. CCT129202 may be a candidate as MDR reversal agent for antineoplastic combination therapy and merits further clinical investigation.

Heme disruption of pyruvate dehydrogenase complex as a mechanism of hypoxic cell survival.

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Cellular heme levels are tightly regulated and may contribute to cell survival under various stresses. In this study, we show that the mitochondrial porphyrin importer, ABCB6, a member of the ATP-binding cassette family, is upregulated under hypoxic conditions in multiple cell types and *in vivo* in a HIF-1α dependent fashion. ABCB6 induction protects against hypoxic cytotoxicity, as does ABCB6 overexpression, whereas ABCB6 deficiency in mouse embryonic fibroblasts or siRNA knockdown sensitizes cells to hypoxia. ABCB6 expression is required for maximal porphyrin biosynthesis under hypoxic conditions. The hypoxic survival advantage is blocked by inhibition of heme synthesis. As reduced pyruvate dehydrogenase (PDH) activity provides a survival advantage under hypoxic conditions, we evaluated PDH activity in cells overexpressing ABCB6. PDH activity was reduced in ABCB6 overexpressing cells. PDH activity was potently reduced by heme. Heme-agarose affinity chromatography demonstrated that PDH specifically bound heme, but the binding site was distinct of the pyruvate binding site. Studies employing glycerol gradient separation of complexed and free PDH subunits show that heme promotes a dissociation of the PDHA1 subunit, leading to the observed inhibition. This inhibition is reversible, as dilution of the heme concentration restores activity and promotes assembly of the PDH complex. These findings suggest that an ABCB6 elevation in *de novo* heme synthesis leads to a reduction in PDH activity. We propose that elevated mitochondrial heme concentrations restrict further oxidative metabolism by directly interacting with PDH leading to inhibition of PDH by subunit dissociation and consequently an increased ability to survive hypoxic stresses.
This work was supported by NIH and by ALSAC.

Systematic replacement of Tryptophan residues for structure-function study in the human P-glycoprotein (ABCB1)

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The drug transport function of human P-glycoprotein can be inhibited through modulator interaction at a site distinct from the site of substrate recognition. Binding of monoclonal antibody UIC2 specific to a conformation-sensitive external epitope of Pgp as well as altered susceptibility to limited protease digestion, indicated that conformational changes induced by Pgp transport substrates (verapamil) and allosteric modulators (such as *cis*-flupentixol) are distinct from one another. To describe the exact nature of those conformational rearrangements, we intend to use "Change in intrinsic tryptophan (Trp) fluorescence of Pgp" as a tool. Since there are Trp residues in Pgp at 11 different positions (W45, W136, W162, W212, W232, W315, W698, W708, W803, W855 and W1108) assigning relative contribution of any individual Trp is virtually impossible. In this study, employing systematic replacement with phenylalanine (Phe), we identify the maximal allowable substitutions from the 11 Trp residues in Pgp without abrogating the modulatory potential of the verapamil and cis-flupentixol. The recombinant Pgps generated, with differential Trp substitutions, were expressed in insect cells and subjected to biochemical characterization. Results indicate that out of the 11 Trp residues present, those that are from the TM domains (TMD1 and 2) W45, W136, W162, W212, W232, W315, W803 and W855 can be collectively substituted with Phe without significantly affecting ATP-site modulation by the transport substrate verapamil. However, for modulation by the allosteric modulator *cis*-flupentixol, two additional Trp residues, both from the C-terminal TM regions (W803 and W855), also seem to be essential. Interestingly, substitution of the two Trp residues from the linker region (W698 and W708), just by themselves, abolish ATP-site modulation by both modulators, suggesting an important role for those two residues in interdomain crosstalk. Although W1108 from NBD2 also appear to be important in ATP-site modulation, substitution of this residue, all by itself, only has a moderate effect on ATP-site stimulation. In summary, we successfully established the Pgp construct with only three Trp residue, two in the linker region (W698 and W708) and one in the NBD2 as the best candidate for studying conformational changes associated with verapamil interaction with Pgp. In addition, the two Trp residues from the C-terminal TM region (W803 and W855) need to be intact for preserving the conformational change associated with the allosteric modulator *cis*-flupentixol.

Cell-surface MRP4 (ABCC4) Levels are Down-regulated by the NH_2-truncated CortBP1 Isoform of SHANK2

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Multidrug resistance protein 4 (MRP4/ABCC4) is a "short" member of subfamily C of the ABC superfamily of membrane transport proteins (ABCC). Previously, we have shown that down-regulation of PDZ-based adapter protein NHERF1 (*Na^+/H^+ exchanger regulatory factor 1*) increases plasma membrane expression and function of MRP4 in HEK and HeLa cells [Hoque & Cole, 2008]. In the present study, the role of a second adaptor protein, the 165 kDa CortBP1, an NH_2-tuncated isoform of the PDZ-based adapter protein SHANK2 (*SH3 and multiple ANKyrin repeat domain protein* 2) which lacks both the SH3 domain and ankyrin repeats of the full-length protein has been investigated. Pull-down experiments indicate that, like the MRP4/NHERF1 interaction, the interaction between CortBP1 and MRP4 occurs via the last four amino acids ^{1322}ETAL of the transporter. Because CortBP1 is known to recruit proteins (eg. dynamin, cortactin, β-pix) involved in endocytosis, we hypothesized that CortBP1 plays a role in MRP4 internalization from the plasma membrane and its insertion into the endocytic pathway for subsequent degradation. In support of this hypothesis, biotinylation experiments showed that CortBP1 significantly decreases cell-surface MRP4 in HEK293 cells and pull-down experiments have indicated CortBP1 exists in a complex with MRP4, dynamin, cortactin and β-pix. Current ongoing studies are aimed at determining how internalized MRP4 is routed between various subcellular compartments.

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Next Generation Sequencing Identifies Novel Mutations in ABCC6 and Modifier Genes

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Pseudoxanthoma elasticum (PXE) is an autosomal recessive disorder that is characterized by the mineralization of connective tissues in the body. Primary manifestation of PXE occurs in the tissues of the skin, eyes, cardiovascular system, and gastrointestinal system, and is estimated to affect anywhere between 1 in 100,000 - 1 in 25,000 people. PXE is primarily caused by mutations in the *ABCC6* gene. The *ABCC6* gene encodes the trans-membrane protein ABCC6, which is highly expressed in the kidneys and liver. PXE has high phenotypic variability, which may be affected by a number of modifier genes (in addition to the *ABCC6* gene). Previously it has been been proposed that mutations in the *VEGFA* gene are linked to ocular manifestations of PXE. We have used NextGen Sequencing on the 454 platform to identify variants in *ABCC6*, *VEGFA*, and 11 other modifier genes (including *AHSG, XYLT1, XYLT2, ENPP1, CAT, GGCX, GPX1, MGP, MMP2, SOD2, SPP1* and *VKORC*1) in patients with PXE. We have validated each recorded variant and determined which variants are novel. We have also found several SNPs (Single Nucleotide Polymorphisms) in the modifier genes that may be important in PXE patients. By using this new information about the genetics of PXE, we may identify how amino acid variability affects ABCC6 and modifier gene proteins.

Nucleotide-Binding Domain Association and Dissociation During the Hydrolysis Cycle of P-glycoprotein

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Often found over-expressed in cancer cells, P-glycoprotein (Pgp) is an ATP-binding cassette (ATP) transmembrane protein that plays a key role in multidrug resistance by transporting a wide variety of chemically diverse compounds across the plasma membrane. Pgp does this in an energy-dependent manner requiring the binding and hydrolysis of ATP at the two nucleotide-binding domains (NBDs). Despite the recent crystallization of murine Pgp, the relationship between Pgp structure and function has yet to be fully understood during the hydrolysis cycle. Using luminescence resonance energy transfer (LRET), optical probes placed at the NBDs in a double-cysteine mutant suggest that the NBDs of Pgp appear to remain closely associated even in the absence of ATP, that the proportion of tightly dimerized NBDs increases in the presence of ATP and that the NBDs may not fully dissociate following each hydrolysis cycle. In the case of Pgp, these results support a constant-contact model for ATP hydrolysis.

The ABC transporter Mrp4/Abcc4 is required for Leydig cell protection from chemotherapeutic drugs

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The ABC transporter Mrp4/Abcc4 is expressed in both human and mouse testes, specifically in Leydig cells, the primary testosterone synthesizing cells in males. In an Mrp4 knockout (KO) model, we demonstrated that Mrp4 is required for optimal testosterone production*. Because cancer chemotherapeutic drugs used to treat childhood acute lymphoblastic leukemia can produce detrimental reproductive effects related to impaired testosterone production, we hypothesized that in Leydig cells, Mrp4 may play a protective role. Many cancer chemotherapeutics such as methotrexate, dasatinib, sunitinib, and imatinib are Mrp4 substrates including the nucleotide metabolites of 6-mercaptopurine (6MP). We show that *in vivo* administration of a therapeutically relevant concentration of 6MP to Mrp4 WT and KO mice produces a substantial reduction in Leydig cells, testicular testosterone concentration, and increased Leydig cell death only in KO mice. However, as Leydig cells are post-mitotic, the mechanism of death is unrelated to 6MP nucleotide incorporation into DNA. We show that cultured Mrp4 KO Leydig cells are highly susceptible to 6MP, exhibiting cytotoxicity at doses 6-fold lower than WT Leydig cells. Exposing Leydig cells to a pan-caspase inhibitor (ZVAD) blocks death by 6MP in cultured Mrp4 KO Leydig cells, indicating the activation of a cell death pathway. The executioner caspase, caspase 3, is downstream of two apoptotic pathways: the caspase 8-dependent extrinsic pathway and the caspase 9-dependent intrinsic pathway. We are investigating if either or both of these pathways contribute to Mrp4 KO Leydig cell toxicity induced by 6MP. Our data shows that 6MP is toxic to Leydig cells by activating a caspase-dependent pathway, and Mrp4 protects Leydig cells by export of 6MP nucleotides. These data show that, in mice, the absence of Mrp4 produces substantial toxicity to Leydig cells. It suggests that, in humans, individuals harboring non-functional Mrp4 alleles may be at risk for Leydig cell toxicity and consequently, reduced testosterone concentrations.

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Suppression of ABCG2 mediated MDR *in vitro* and *in vivo* by a novel inhibitor of ABCG2 transport.

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Cancer is a disease whose treatment is often limited due to the development of a phenomenon known as multidrug resistance (MDR) that is its Achilles' heel. Hence there is an immense demand for development of novel agents that can overcome the emergence of multidrug resistance (MDR) in cancer. A group of transmembrane proteins called ABC (ATP- binding cassette) transporters, present ubiquitously in the human body possesses a modular architecture, contributing immensely towards the development of MDR in various tumor types. An analysis of structural congeners among a group of compounds led to the discovery of 15.23 (2,2-dichloro-N-(3',4'-dimethoxy-[1,1'-biphenyl]-3-yl) acetamide) that could selectively reverse ABCG2-mediated MDR in cancer cells both *in vitro* and *in vivo.* 15.23 at concentrations upto 5 mM sensitized the cancer cells as well as transfected cells overexpressing ABCG2 to the substrate chemotherapeutic drugs. The reversal ability of 15.23 was primarily due to the inhibition of the active efflux function of the transporter; also there was no change in the protein expression of the ABCG2 transporter in the presence of 15.23 at reversing concentrations. Importantly the reversal effect produced by 15.23 was reversible. Co-administration of 15.23 restored the *in vivo* antitumor activity of doxorubicin in ABCG2 overexpressing tumor xenografts without added toxicity. Taken together, our findings indicate that 15.23 is a potent and reversible, modulator of ABCG2 export function that may offer therapeutic promise for multidrug- resistant malignancies.

The Role of Mrp4 in Erythropoiesis

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The production of erythrocytes is a tightly regulated process. It has been postulated that cAMP plays an important role in the commitment to differentiation of erythroid cells (1). While mature erythrocytes have been shown to export cAMP (2), it is unknown if cAMP export affects erythroid differentiation processes. Mrp4 is expressed in erythrocytes, but it is unknown if its expression is regulated during erythroid differentiation or if it has a functional role.

To investigate the role of Mrp4 in erythroid differentiation, Mouse Erythroleukemia (MEL) cells were initially used because they represent a pro-erythroblast cell that can recapitulate erythroid differentiation. Western blot analysis revealed that Mrp4 is induced by differentiation, with low expression of Mrp4 in the precursor BFU-E like cells and increased expression in the more differentiated CFU-E cells. This pattern of low to high Mrp4 expression during differentiation correlates with the internal cAMP levels, having the highest levels in the undifferentiated MEL cells and the lowest levels in the differentiated cells. Furthermore, inhibition of Mrp4, using the Mrp4 inhibitor MK571, suppressed erythroid differentiation, keeping the treated cells in less differentiated state.

To test Mrp4's role in an *in vivo* system, we created a conditional knockout mouse, in which exons two and three of Mrp4 are flanked by loxP sites. By using an inducible Cre system, we can delete the Mrp4 gene at any time during the animal's life, thus minimizing the potential compensation seen in a constitutive knockout mouse. Hematopoietic progenitor cells isolated from adult $Mrp4^{flox/flox}$ and $Mrp4^{\Delta/\Delta}$ mice and grown in culture show that cAMP levels are increased following Mrp4 deletion. Proliferation of hematopoietic progenitor cells from the $Mrp4^{\Delta/\Delta}$ mice was increased, indicative of a more undifferentiated phenotype. Methocult cultures of these progenitor cells revealed that the mice lacking Mrp4 showed a sharp decrease in the number of early erythroid progenitor (BFU-E) colonies, but did not show any change in late erythroid progenitors (CFU-E) colonies. These cells, however, were capable of differentiating into other myeloid lineages (granulocyte/monocyte), indicating this was not a general defect in MEP differentiation. Together, our data suggests that Mrp4 plays a role in erythropoiesis by facilitating the transition from BFU-E to CFU-E as well as a crucial, but yet to be defined, role in the commitment to the erythroid lineage from the MEP to the BFU-E population. We hypothesize that in the undifferentiated BFU-E precursor cells, the low expression of Mrp4 results in increased internal cAMP levels, resulting in maintenance of the precursor phenotype. However, as differentiation occurs and Mrp4 expression increases, internal cAMP levels decrease, and commitment to an erythrocyte occurs.

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An attempt to verify the model of MRP1 structure by chemical protease FeBABE

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Multidrug Resistance Protein MRP1 (ABCC1) is a broad-specificity membrane transporter. MRP1 can efflux a vast array of amphiphilic organic anions, including many GSH, glucuronate and sulfate conjugates, as well as free GSH. As yet no crystal structure has been determined for MRP1. The model of MRP1 which is currently in use is based on the bacterial transporter Sav 1866. Although the best available, the "template" is less then ideal since it is only 22% identical with MRP1 and much of this identity is contributed by the generally conserved NBDs. Thus the model requires experimental validation and refinement.

We proposed to test the model using internal cleavage of MRP1 by chemical protease FeBABE. Previously in our lab almost Cys less MRP1 has been created. It was shown that three cysteines (Cys 388, 1439, 1479) are vital for preserving functionality of MRP1. We have chemically attached FeBABE reagent to sulfhydryl group of cysteine residues in 3Cys MRP1. It is known that FeBABE moiety is able to cleave the peptide backbone of the protein at locations which are expected to be within approximately 10Å from it. Preliminary experiments using crude membranes, have allowed us to tentatively map the approximate location of 3 such cleavage sites. Based on cleavage pattern of MRP1 we assume that Cys 388 in TM7 is in close proximity with TM8, 14 and 15.

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EXPERIMENTAL STRATEGIES FOR *IN VIVO* RESCUE OF DISEASE-CAUSING MUTATIONS OF ABCC6

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Mutations in *ABCC6* can cause chronic or acute forms of dystrophic mineralization described in disease models such as pseudoxanthoma elasticum (PXE, OMIM 26480) and generalized arterial calcification of infancy (GACI, OMIM 614473) in human. The major aim of our study was to identify mutants with preserved transport activity but failure in intracellular targeting, as these mutants are candidates for functional rescue. Ten missense mutations were investigated; we found that seven variants retained significant transport activity. All mutants were transiently *in vivo* expressed in mouse liver via hydrodynamic tail vein injections. We tested whether 4-phenylbutyrate (4-PBA), a drug approved for clinical use, could restore their intracellular trafficking to the plasma membrane in mouse liver. The cellular localizations of four mutants were significantly improved by 4-PBA treatments, thus potentially rescuing its physiological function.
Silencing of the Abcc6a gene in zebrafish morpholinos triggered a developmental phenotype which can be rescued by injecting wt human ABCC6 mRNA. Consequently, zebrafish provides an in vivo model to study disease-associated ABCC6 variants functionally. Indeed, all ten of disease-causing mutants fail to rescue the phenotype, irrespective whether they are transport- or localization mutants. Interestingly, V1298F, which is correctly situated in the plasma membrane, but shows minimal residual transport activity gave a partial (30%) rescue. These results indicate that – although the (patho)physiological consequence of lack of ABCC6 function in human and zebrafish is different, zebrafish provides a promising animal model to study ABCC6 mutations.
Our work demonstrates the feasibility of the *in vivo* rescue of cellular maturation of ABCC6 mutants in physiological conditions very similar to the biology of the fully differentiated human liver and could have future allele-specific therapeutic application. Furthermore, our studies can provide a model for systematic investigation of disease-causing mutations of membrane proteins and for pharmacologically assisted maturation of this class of proteins.

ABCC10 and its Role in Taxane Resistance in Non-Small Cell Lung Carcinoma

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ATP binding cassette transporter member 10, ABCC10, a multidrug resistance protein has been shown to confer resistance to various anti-neoplastic agents including docetaxel and paclitaxel in vitro. More importantly, Abcc10 null mice have been shown to exhibit increased tissue sensitivity to paclitaxel in vivo. In this study, we investigate the contributions of ABCC10 to taxane resistance and tumor biology in NSCLC. ABCC10 expression analysis was performed and detected in 6 non-drug selected human NSCLC cell lines by western immunoblotting. ABCC10 expression in A549 and H1299 cell lines was suppressed by short-hairpin RNAs and the cells analyzed for changes in accumulation and efflux of [3H] docetaxel; in vitro and in vivo drug sensitivity and changes in molecular expression profiles. Comparison of control scrambled shRNA to ABCC10 knockdown cell lines showed that ABCC10 loss resulted in increased intracellular accumulation of [3H] docetaxel and a reduction in the efflux of th e drug out of the cells. ABCC10 loss also increased the sensitivity of the cells to docetaxel in in vitro cytotoxicity assays and in vivo xenografts. Increased apoptosis was demonstrated by an increase in PARP and Caspase 3 cleavage. Reverse phase protein array and western blot analysis interestingly demonstrated decreased expression of epidermal growth factor receptor (EGFR) and diminished activation of the PI3K/AKT pathway as a result of ABCC10 loss. These results suggest that ABCC10 may be involved in chemoresistance in NSCLC, but that it may also have a role in tumorigenic processes modulated by EGFR and PI3K/AKT signaling. Further studies are needed to determine the precise mechanisms through which ABCC10 modulates signaling processes in NSCLC.

β-Elemene, a compound derived from *Rihizoma zedoariae*, reverses multidrug resistance mediated by ABCB1 transporter

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In this *in vitro* study, we examined the effect of the compound β-elemene on the response of KB-C2 cells overexpressing the ABCB1 transporter to specific antineoplastic compounds. The MTT assay was used to determine the effect of β-elemene in combination with other anticancer drugs on ABCB1 overexpressing cancer cell lines. Furthermore, we used [^{3}H]-paclitaxel accumulation, efflux assay, immunofluorescence experiments, Western blotting assays and docking analysis to ascertain the mechanism of action of β-elemene. The incubation of KB-C2 cells overexpressing ABCB1 transporters with β-elemene (100 μM) significantly augmented the antineoplastic efficacy of colchicine, vinblastine and paclitaxel compared to KB-C2 cells incubated with these drugs alone. In HEK293 cells overexpressing the ABCB1 transporter, β-elemene significantly increased the cytotoxicity of paclitaxel. In addition, 100 μM of β-elemene significantly increased the accumulation of [^{3}H]-paclitaxel and this was due to a decrease in [^{3}H]-paclitaxel efflux compared to controls. The incubation of KB-C2 cells with β-elemene (100 μM) for 72 h did not significantly alter the expression of ABCB1 protein levels. Immunofluorescence experiments indicated that β-elemene did not significantly alter the subcellular localization of the ABCB1 transporter. Docking analysis indicated that β-elemene binds to the drug binding site of ABCB1transporter. Finally, β-elemene at 100 μM partially increased the sensitivity of the BCRP overexpressing cell line, NCI-H460/MX20, to mitoxantrone but β-elemene did not significantly alter the resistance of MRP1 transfected HEK293/MRP1 cells to vincristine. Overall, our findings indicated that β-elemene potentiates the cytotoxic effects of various antineoplastic drugs in cell lines overexpressing the ABCB1 transporter and that this is due to the inhibition of the efflux component of the ABCB1 transporter.

Ibrutinib (PCI-32765) Reverses Multidrug Resistance by Inhibiting the Efflux Function of Multiple ATP-Binding Cassette Transporters

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The multidrug resistance (MDR) phenotype presents an insurmountable obstacle towards achieving a favorable chemotherapeutic response. A few members of the ATP-binding cassette (ABC) transporter family are often associated with the MDR phenotype, wherein these membrane transporters actively efflux various substrate anticancer drugs. Blocking these efflux transporters results in an increased intracellular accumulation of the anticancer drugs leading to an effective chemotherapeutic response. Recent advent of tyrosine kinase inhibitors provided a competent approach towards blocking the efflux function of these membrane transporters. Ibrutinib, is a first in class of a selective and irreversible inhibitor of Burton's tyrosine kinase (BTK), currently in phase III clinical trials for patients with relapsed or refractory chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL). In this study, we investigated the effect of ibrutinib to reverse MDR due to over expression of ABC transporters such as ABCB1, ABCG2 and ABCC10. Our results showed that non-toxic doses of ibrutinib significantly increased the effective intracellular concentration of ABCB1 or ABCC10 substrate drugs in cells expressing these transporters. In contrast, ibrutinib did not alter the cytotoxicity of specific substrates in the parental cells and cells overexpressing ABCG2. Furthermore, studies on [^{3}H]-paclitaxel accumulation and efflux have shown that ibrutinib increases the intracellular accumulation of [^{3}H]-paclitaxel in KB-C2 and HEK293/ABCC10 cells by directly inhibiting the ABCB1- and ABCC10-mediated drug efflux respectively. Western blot analyses indicated no significant alterations of ABCB1 or ABCC10 protein levels after treatment with ibrutinib up to 72 h. Docking experiments revealed the binding conformation of ibrutinib within the large cavity of the transmembrane region of ABCB1. Our finding suggests that combination of ibrutinib with chemotherapeutic agents may be able to overcome ABCB1- or ABCC10-medicated drug resistance to attain a successful chemotherapeutic response in cancer.

An Animal Model of Intrahepatic Cholestasis of Pregnancy

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The maternal symptoms of intrahepatic cholestasis of pregnancy (ICP) often resolve, but fetal complications frequently do not, and include perinatal mortality. The severity of fetal complications is positively related to maternal serum total bile acids, but the genetic basis of this disease remains unsolved. Respiratory distress syndrome (RDS) occurs in 28.6% of newborns of ICP patients. Neonatal RDS occurrence is positively related to maternal serum bile acids (1). Bsep KO females show elevated serum bile acids (2). Therefore, we hypothesized that Bsep KO females might be a model to test the effect of maternal bile acids on neonates.

Offspring from KO dams showed 100% mortality and appeared pale and did not survive over 24 hours, regardless of paternal genotypes. Necropsy only revealed atelectasis in neonates from KO dams. Gene and protein expression of pulmonary cell type markers (Spc, Aqp5, and Abca3) did not change. Histopathological analysis did not reveal difference in lungs at E17.5 and E18.5 between embryos of WT and KO dams. These data suggest that pulmonary development is not defected in the neonates of KO dams.

However, electron microscopy revealed thickened septa and super-coiled surfactant in the lungs of neonates from KO dams. The lipid components of pulmonary surfactant, dipalmitoylphosphatidylcholine or unsaturated lysophosphatidylcholine, did not differ between neonates of WT and KO dams. The expression of surfactant proteins was no different. Serum bile acid levels were significantly higher in KO dams during pregnancy. Accordingly, they were higher in neonates of KO dams than of WT dams. However, only taurine conjugated muricholic acid (T-MCA) was elevated in the lungs of neonates from KO dams. Our data suggests that elevated T-MCA affects pulmonary surfactant function, leading to poor oxygenation. This is consistent with our findings that Hif target genes are activated in the postnatal but not embryonic lungs of KO dams

To rescue of the mice, we backcrossed Bsep KO into either CAR or PXR-null background. Bsep/CAR double KO mice rendered 100% neonatal mortality rates. In contrast, maternal absence of PXR with Bsep produces strong neonatal rescue with about 60% of neonates surviving longer than 24 hours.

The studies have identified Bsep as a major gene that accounts for neonatal RDS of cholestatic mothers. The Bsep KO female could be an animal model to develop therapeutic approach.

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Human ABCB1 (P-glycoprotein) and ABCG2 Mediate Resistance to BI 2536, a Potent and Selective Inhibitor of Polo-like Kinase 1

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The overexpression of the serine/threonine specific polo-like kinase 1 (Plk1) has been detected in various types of cancer, and thus has fast become an attractive therapeutic target for cancer therapy. BI 2536 is the first selective inhibitor of Plk1 that inhibits cancer cell proliferation by promoting G2/M cell cycle arrest at nanomolar concentrations. Unfortunately, alike most chemotherapeutic agents, the development of acquired resistance to BI 2536 is prone to present a significant therapeutic challenge. One of the most common mechanisms for acquired resistance in cancer chemotherapy is associated with the overexpression of ATP-binding cassette (ABC) transporters ABCB1, ABCC1 and ABCG2. In this study, we discovered a novel mechanism of acquired resistance to BI 2536 in human cancer cells. We found that cancer cells overexpressing either ABCB1 or ABCG2 are highly resistant to BI 2536. Furthermore, we show that BI 2536 stimulates the ATPase activity of both ABCB1 and ABCG2 in a concentration-dependent manner, as well as inhibits the drug substrate transport mediated by these transporters. More significantly, we found that the reduced chemosensitivity and BI 2536-mediated G2/M cell cycle arrest in cancer cells overexpressing either ABCB1 or ABCG2 can be significantly restored in the presence of selective inhibitor or other chemotherapeutic agents that also interact with ABCB1 and ABCG2, such as tyrosine kinase inhibitors nilotinib and lapatinib. Taken together, our findings indicate that in order to circumvent ABCB1 or ABCG2-mediated acquired resistance to BI 2536, a combined regimen of BI 2536 and inhibitors or clinically active drugs that potently inhibit the function of ABC drug transporters, should be considered as a potential treatment strategy in the clinic.

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