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Identification of FDA-Approved Drugs as Modulators of Multidrug Resistance Protein 2 (MRP2/ABCC2) Expression Levels in MRP2-Overexpressing Cells: Preliminary Data

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Abstract

Multidrug Resistance Protein 2 (MRP2) is an ATP-dependent transmembrane protein that plays a pivotal role in the efflux of a wide variety of physiological substrates across the plasma membrane. Several studies have shown that MRP2 can significantly affect the absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles of many therapeutic drugs and chemicals found in the environment and diet. This transporter can also efflux newly developed anticancer agents that target specific signaling pathways and are major clinical markers associated with multidrug resistance (MDR) of several types of cancers. MDR remains a major limitation to the advancement of the combinatorial chemotherapy regimen in cancer treatment. In addition to anticancer agents, MRP2 reduces the efficacy of various drug classes such as antivirals, antimalarials, and antibiotics. The unique role of MRP2 and its contribution to MDR makes it essential to profile drug-transporter interactions for all new and promising drugs. Thus, this current research seeks to identify modulators of MRP2 protein expression levels using cell-based assays. A unique recently approved FDA library (372 drugs) was screened using a high-throughput In-Cell ELISA assay to determine the effect of these therapeutic agents on protein expression levels of MRP2. A total of 49 FDA drugs altered MRP2 protein expression levels by more than 50% representing 13.17% of the compounds screened. Among the identified hits, thirty-nine (39) drugs increased protein expression levels whereas 10 drugs lowered protein expression levels of MRP2 after drug treatment. Our findings from this initial drug screening

showed that modulators of MRP2 peregrinate multiple drug families and signify the importance of profiling drug interactions with this transporter. Data from this study provides essential information to improve combinatorial drug therapy and precision medicine as well as reduce the drug toxicity of various cancer chemotherapies.

Keywords

ABC Transporters, Multidrug Resistance, MRP2/ABCC2, MRP2 Modulators, ELISA

1. Introduction

MRP2 (ABCC2) is a member of the ATP-binding cassette superfamily of transporters, MRP1 and MRP2 are homologous members of this superfamily [1]. In humans, it is encoded by the gene ABCC2 [2]. Structurally, MRP2 is a 190-kDa membrane protein consisting of 1545 amino acids. The predicted membrane topology consists of 17 transmembrane spanning domains (MSD0, MSD1, MSD2) which are linked together by conserved linker regions, and two highly conserved nucleotide-binding domains (NBD1 and NBD2) that serve as substrate–binding sites [3]. Whilst MRP1 localizes at the basolateral membrane of endothelial cells, MRP2 localizes at the apical membrane of polarized cells of hepatocytes, renal proximal tubular cells, enterocytes, and syncytiotrophoblasts of the placenta [4]. It is known to play critical roles in the export of conjugated bile salts in the liver as well as the transport of physiologically important substrates such as glutathione-S-conjugates, 17-beta-glucuronosyl estradiol, leukotriene C4 [5]. Overexpression of MRP2 is associated with multidrug resistance of tumor cells such as hepatocellular, ovarian, colorectal, lung, breast, and gastric carcinomas [6], where it pumps drug conjugates and drug complexes across the plasma membrane into the extracellular space [5]. Thus, affecting the bioavailability and efficacy of anticancer drugs like cisplatin and methotrexate. Aside from cancer drugs, MRP2 also affects the efficacy of a broad spectrum of drug classes including HIV drugs (lopinavir), antibiotics (ampicillin, azithromycin), and antihypertensives (Olmesartan, Temocaprilate) [7]. With MDR being a major impediment to the chemotherapy regime and the overexpression of the MRP2 transporter being a major factor in this phenomenon, it is of great clinical interest to find ways of addressing this canker. Researchers have proposed two main approaches. One of which, is to completely block the efflux or pump activity of the transporter in these cells [8]. However, this approach would be destructive to the cells since it may also impede some important physiological activities of the transporter, thereby jeopardizing the overall well-being and physiological homeostasis of the cell or tissue [9]-[11]. Another possible approach that was proposed, was to modulate the activity of this transporter using biochemical modulation [9] [12] [13]. Using biochemical modulation, exogenously supplied metabolites can be used to selectively manipulate

the activity of MRP2 in tumor cells to ensure the more selective response of cancer cells to the action of administered anticancer agents [14]. This would go a long way to improve the bioavailability and efficacy of anticancer drugs in tumor cells. Hence the identification of possible modulators of the MRP2 transporter is of great clinical importance [8] [13]. Moreover, the broad impact of the efflux activity of this transporter on the efficacy of a broad class of drugs makes it essential to investigate the possible interactions between various therapeutic drugs (both approved and those in clinical trials) and this transporter. Thus, in this study, a unique set of drugs from the FDA-approved drug library was screened using In-Cell ELISA to identify modulators of MRP2 protein expression.

2. Materials and Methods

2.1. Chemicals

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

2.2. Cell Lines and Cell Culture

Madin-Darby Canine Kidney cells (MDCKII) and Madin-Darby Canine Kidney cells overexpressing MRP2 (MDCKII/MRP2) cells were kindly provided by Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCKII cell lines were grown in Dulbecco's modified Eagle medium (DMEM) (GE Healthcare, Marlborough, MA, USA) enriched with 10% fetal bovine serum (FBS). Cell lines were cultured in a humidified incubator at 5% CO_2 and 37˚C. This incubation condition was retained in all subsequent cell culture procedures.

2.3. Screening of FDA-Approved Drug Library Using In-Cell ELISA Assay in MDCKII/MRP2 Cells

In-cell ELISA assay development and optimization were performed with MDCKII and MDCKII/MPR2 cells and used to screen the FDA-approved drug library for modulators of MRP2 protein expression. MDCKII/MRP2 cells were seeded at 7×10^4 cells per well in 96-well plates with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and incubated for 24 hours. Cells were then treated with drugs (10 uM), and 0.1% DMSO (control and Parental) and incubated for 48 hours at 37˚C. At the end of the incubation period, treatment was removed, and cells were rinsed twice with 150 µL of PBS. Cells were fixed with 3.7% paraformaldehyde and permeabilized with Triton-X 100. The cells were blocked using fish gel (MB-066-0100, Rockland) and incubated overnight at 4˚C with monoclonal anti-MRP2 antibody (MABN1545, EMD Millipore) or anti-α tubulin antibody (T5168, Sigma-Aldrich) in a 1:1000 dilution. Secondary antibody

incubation was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific, Waltham, MA) for an hour at room temperature. Target proteins were detected using Super Signal West Dura® Extended Duration Substrate chemiluminescence substrate (21EAPI34076, Thermo Fisher Scientific) and read using Hidex Sense Beta Plus plate reader (Turku, Finland). Treatments were performed in triplicates and expressed as means. Data analysis was performed using Microsoft Excel Office 19 and GraphPad Prism TM software (GraphPad Software version 8.4.3, San Diego, CA, USA). Drugs that showed modulation of MRP2 protein levels above 50% were selected as hit compounds.

3. Results

Screening of FDA-Approved Drug Library for Modulators of MRP2

The modulatory effect of the FDA-approved drug library containing 372 drugs on MRP2-overexpressing MDCKII cells was successfully screened using an In-Cell ELISA assay. Treatments were performed in triplicates, and experiments were done using the 96-well format. Data obtained was statistically analyzed and expressed as means. Drugs showing more than 50% modulation on MRP2 protein expression were selected as "Hit compounds". The results revealed 49 hit compounds that changed the MRP2 protein expression by more than 50%, representing 13.17% of the total compounds screened. Among the identified hits for MRP2, 39 drugs increased expression levels whereas 10 drugs lowered expression levels of MRP2 after drug treatment as shown in Figure 1. Details on the hit test compounds identified from screening are listed in Tables 1-4.

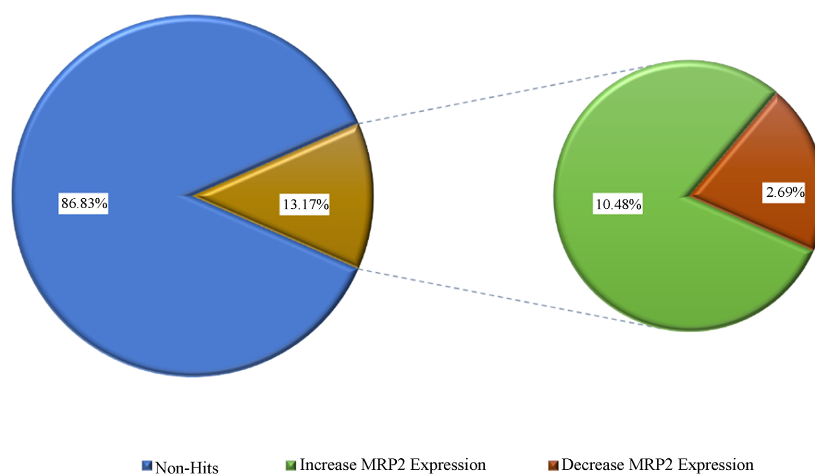

Figure 1. Screening of 372 FDA-approved drug library using In-Cell ELISA assay.

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

Table 1. List of hit compounds identified from drug screening on MRP2 protein expression.

Drug	Target	% Modulation on MRP2 protein levels[a]
Pralatrexate (Folotyn)	DHFR	−57.39 ± 3.85
Cetirizine Dihydrochloride	Histamine Receptor	106.29 ± 22.62
Mercaptopurine	DNA/RNA Synthesis	112.77 ± 15.36
Streptozotocin (Zanosar)	Nicotinamide adenine dinucleotide	107.57 ± 13.87
Dexamethasone	IL Receptor	257.86 ± 10.58
Megestrol Acetate	Androgen Receptor	124.35 ± 26.22
Trilostane	Dehydrogenase	66.04 ± 4.96
Ranolazine dihydrochloride	Calcium Channel	93.67 ± 39.42
Repaglinide	Potassium Channel	94.45 ± 12.23
Sildenafil Citrate	PDE	70.30 ± 17.82
Gestodene	Estrogen/progestogen Receptor	54.07 ± 4.05
Isotretinoin	Hydroxylase	67.87 ± 5.05
Nafamostat Mesylate	Proteasome	84.25 ± 14.38
Ondansetron hydrochloride (Zofran)	5-HT Receptor	68.23 ± 7.83
Oxcarbazepine	Sodium Channel	75.54 ± 22.66

[a]Mean ± SD.

Table 2. List of hit compounds identified from drug screening on MRP2 protein expression.

Drug	Target	% Modulation on MRP2 protein levels[a]
Afatinib (BIBW2992)	EGFR, HER2	−62.24 ± 20.67
Gefitinib (Iressa)	EGFR	85.68 ± 14.81
Crizotinib (PF-02341066)	C-Met	56.41 ± 9.37
Sunitinib Malate	VEGFR, PDGFR, c-Kit, Flt	71.59 ± 11.40
Cladribine	DNA/RNA Synthesis	−74.96 ± 2.30
Evista (Raloxifene Hydrochloride)	Estrogen/progestogen Receptor	69.87 ± 7.06
2-Methoxyestradiol	HIF	148.42 ± 46.91
Asenapine	Adrenergic receptor, 5-HT receptor	−73.49 ± 5.67
Adrucil (Fluorouracil)	DNA/RNA Synthesis	−62.54 ± 1.78
Vincristine	Microtubule Associated	216.85 ± 46.37
Oxaliplatin (Eloxatin)	DNA/RNA Synthesis	100.88 ± 10.98

[a]Mean ± SD.

Table 3. List of hit compounds identified from drug screening on MRP2 protein expression.

Drug	Target	% Modulation on MRP2 protein levels[a]
Glyburide (Diabeta)	Potassium channel	−77.59 ± 3.61
Adefovir Dipivoxil (Preveon, Hepsera)	Reverse transcriptase	−87.87 ± 5.68
Sulfadiazine	Anti-infection	69.784 ± 19.01
Suprofen (Profenal)	COX-1/COX-2	108.14 ± 22.69
Cefditoren pivoxil	5-alpha Reductase	68.25 ± 9.12
Rifabutin (Mycobutin)	Antineoplastic and Immunosuppressive Antibiotics-Anti-infection	96.66 ± 30.90
Esomeprazole Magnesium (Nexium)	proton pump	68.11 ± 20.06
Ethionamide	Anti-infection	52.59 ± 27.15
Vidarabine (Vira-A)	5-alpha Reductase	88.61 ± 18.51
Deferasirox (Exjade)	Ferroptosis P450 (e.g. CYP17)	78.46 ± 19.75
Methylprednisolone	Immunology and Inflammation related, Glucocorticoid Receptor, Interleukins, ACE, Apoptosis related, Autophagy	93.67 ± 25.54
Metolazone (Zaroxolyn)	Treatment of congestive heart failure and high blood pressure	155.75 ± 34.37
Darunavir Ethanolate (Prezista)	HIV Protease	56.94 ± 18.04
Prednisone (Adasone)	Glucocorticoid receptor	76.69 ± 23.89

[a]Mean ± SD.

Table 4. List of hit compounds identified from drug screening on MRP2 protein expression.

Drug	Target	% Modulation on MRP2 protein levels[a]
Rasagiline Mesylate	MAO	−58.99 ± 4.72
Dronedarone HCl	Anti-infection	−56.17 ± 5.53
Conivaptan HCl (Vaprisol)	Vasopressin receptor	−57.59 ± 0.83
Eltrombopag (SB497115-GR)	C-mpl (TpoR) receptor	79.40 ± 18.03
Paeoniflorin	COX, HIF	53.80 ± 20.35
Benserazide	Dopamine Receptor	54.48 ± 22.74
Lovastatin	HMG-CoA Reductase	77.64 ± 17.81
Lafutidine	Histamine Receptor	55.36 ± 19.22
Erythromycin	Anti-infection, Antibiotics	104 ± 18.32

[a]Mean ± SD.

4. Discussion

Test compounds that modulated the protein expression levels of MRP2 in MDCKII/MRP2 cells above 50% (Hit compounds) in this study cut across a broad spectrum of drug classes and exhibit great diversity in their structure, molecular targets, and mode of action. This included anticancer drugs, antibiotics, antivirals, and anti-inflammatory drugs. This finding reaffirms the promiscuous nature of the MRP2 transporter, and how important it is to investigate the interaction between both old and newly developed drugs with MRP2. Although several studies have aimed at investigating the impact of various therapeutic agents on MRP2 efflux activity, it is necessary that researchers also pay critical attention to how these drugs may affect the protein expression levels of this transporter. From our screening, about 10.48% of the hit compounds increased the expression of MRP2 protein levels, this included drugs like vincristine (anticancer drug), oxaliplatin (antineoplastic medication), and irinotecan (anticancer drug). MRP2 has been reported to be one of the major ABC transporters that affect the bioavailability and therapeutic potency of anticancer drugs in both polarized and unpolarized cells [9] [15]. The anticancer drugs: Vincristine, Oxaliplatin, and Irinotecan have earlier been reported in other studies as substrates of MRP2 [7] [16] [17]. This indicates they are actively transported by MRP2/ABCC2 transporter thus the increase in MRP2 protein expression as observed in this study provides the possible explanation that more MRP2 is expressed in these cells to catalyze and ensure successful transport or efflux of these drugs across the plasma membrane.

Glucocorticoids like dexamethasone and prednisone also upregulated MRP2 protein expression in our screening. The ability of dexamethasone to increase the expression levels of MRP2 protein as observed from the screening also reaffirms the observation reported by Narang and his colleagues [18] who reported that dexamethasone increased the expression and activity of multidrug resistance transporters at the rat blood-brain barrier. Prednisone, on the other hand, has also been reported to induce the activity of the MRP2 promoter [19], thus providing a possible reason for the increase in MRP2 protein levels observed in this study. Methylprednisolone, another glucocorticoid with anti-inflammatory and immunomodulating properties, also upregulated MRP2 protein levels in this screening. However, to the best of our knowledge, the interaction between MRP2 and methylprednisolone is yet to be reported, and it would be enlightening for further studies to be carried out to investigate how these drugs may affect MRP2 activity. Erythromycin (an antibiotic) and Lovastatin (a hypolipidemic agent and an HMG-CoA reductase inhibitor) which has been reported by other studies to be substrates of MRP2 [20] [21] also elevated the protein levels of MRP2 in this present study. Hence, this finding provides useful information on the modulatory effect of these drugs that can be further explored.

Furthermore, findings from this study also suggest that anticancer drugs like Pralatrexate, Afatinib, and Cladribine (an immunosuppressant) may decrease MRP2 protein expression levels in MDCKII MRP2-overexpressing cells. Pralatrexate has

been reported in earlier studies to act as both a substrate and an inhibitor of MRP2 [22], interestingly, results from our present study demonstrate that pralatrexate may downregulate the expression levels of MRP2. Further investigation can be conducted to provide more insight into the effect of pralatrexate on gene expression and other effectors that regulate MRP2 protein expression. Moreover, the effect of pralatrexate on MRP2 protein expression levels in other MRP2-overexpressing cell lines can be explored to confirm this initial finding. Afatinib is a known moderate inhibitor of Permeability glycoprotein (P-gp) [23] [24], and a substrate/inhibitor of Breast Cancer Resistance Protein (BCRP) [24]. Results of this current study show that Afatinib may reduce MRP2 protein levels. A thorough search of current literature revealed that little is known about the impact of Afatinib on MRP2 activity and protein expression. Thus, it would be enlightening to conduct further investigation to confirm and ascertain how Afatinib affects the efflux activity of this transporter in other MRP2 overexpressing cells. Cladribine is an FDA-approved drug used in the treatment of multiple sclerosis and hairy cell leukemia. It is a known substrate of BCRP [25] [26] but proved otherwise on MRP2 when its impact on MRP2 membrane vesicles was explored [27]. Nonetheless, cladribine downregulated the protein expression levels of MRP2 in MDCKII MRP2 overexpressing cells in our present study. Thus, it would be illuminating to investigate the impact of cladribine on other MRP2-overexpressing cells since the specific interactions between Cladribine and MRP2 remain uncertain. Further probing using cell lines overexpressing this transporter would be a step in the right direction. Adrucil (Fluorouracil), a DNA and RNA synthesis inhibitor that irreversibly inhibits thymidylate synthase, and Asenapine, an antipsychotic medication belonging to the dibenzooxepino pyrrole class [28] downregulated the expression levels of MRP2 in our present study. To the best of our knowledge, the impact of Adrucil and Asenapine on MRP2 activity and expression has not been reported in literature.

Although HIV Protease Inhibitors (HPIs) have been reported to be substrates of MRP1 and MRP2, the majority of tested HPIs are transported by MRP2. As such the overexpression of MRP2 has great pharmacological implications on administered HPIs [29] [30]. Darunavir Ethanolate is the ethanolate form of darunavir and an antiretroviral drug that inhibits the human immunodeficiency virus type-1 (HIV-1) protease [31]-[33]. In this present study, Darunavir Ethanolate increased MRP2 protein expression in MDCKII overexpressing MRP2 cells. Although to the best of our knowledge, Darunavir Ethanolate has not been reported as a substrate of MRP2, its ability to increase MRP2 protein levels suggests that this antiretroviral drug may also be a prey of MRP2 efflux activity. Interestingly, Darunavir the parent compound of Darunavir Ethanolate has been reported in other studies to induce P-gp mRNA activity and expression *in vitro* as well as induce MRP1 protein expression in CD4 (+) T cells from healthy human volunteers [34] [35]. On the Contrary, Adefovir dipivoxil, a diester prodrug of adefovir and an antiviral medication used in the treatment of chronic Hepatitis B infection in

adults, also lessened the protein levels of MRP2 in this screening. This is not surprising since adefovir is reported to be a known inhibitor of MRP2 [36]. However, no information has been reported on the interaction between this diester derivative of adefovir and MRP2. Investigating the modulatory effect of Darunavir Ethanolate and Adefovir dipivoxil on MRP2 activity and expression in other MRP2 overexpressing cells would be enlightening.

Our screening also identified other novel drugs whose effect on MRP2 activity or expression levels is yet to be reported or explored to the best of our knowledge. This included glyburide (medication for diabetes), Rasagiline mesylate (medication for Parkinson's disease), dronedarone HCl (antiarrhythmic drug), and conivaptan HCl (vasopressin antagonist, endocrine-metabolic agent). These novel drugs downregulated the protein expression levels of MRP2 in MDCKII MRP2-overexpressing cells in this study. Other non-reported drugs that upregulated the expression levels of MRP2 in our present study included Streptozotocin, Megestrol acetate, Gestodene, Trilostane, and Ranolazine dihydrochloride among others. This initial data on these novel drugs would provide foreknowledge that can further be explored. Like most proteins, MRP2 can be regulated at the transcriptional and post-transcriptional levels. Studies have revealed that alterations in the intracellular concentrations of bile acids and of some lipophilic compounds that are ligands for nuclear hormone receptors can regulate MRP2/ABCC2 transcription levels [6]. Nuclear hormone receptors for hydrophobic molecules such as steroid hormones (estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, vitamin D3, ecdysone, oxysterols, and bile acids), retinoic acids (all-trans and 9-cis isoforms), thyroid hormones, fatty acids, leukotrienes and prostaglandins [37] [38]. Research has shown that the hormone response element in rat MRP2/Abcc2 promoter (ER-8) is bound by heterodimers of the retinoid receptor [39] with the ligand-activated transcription factors, pregnane X receptor (PXR), farnesoid X receptor (FXR) or constitutive androstane receptor (CAR). Thus, various xenobiotics that regulate bile acid concentration can activate these receptors which in turn upregulates the promoter region of the ABCC2 transporter [40]-[44]. This finding provides a possible explanation for the increase in MRP2 protein expression observed in this study after MDCKII/MRP2 cells were treated with Megestrol acetate (androgen receptor), Gestodene (estrogen/progestogen receptor), Methylprednisolone and Prednisone (Glucocorticoid receptor), and Evista (estrogen/progestogen receptor). Data from this study provides important information on otherwise potent drugs that can regulate the protein expression of MRP2. The early detection of drugs that upregulate MRP2 expression levels provides vital information on the performance of these drugs which is essential for clinical trials. Drugs that showed the ability to decrease protein expression could be used together with drugs that are known to be efficacious in treating cancer and other diseases but are unfortunately substrates of the MRP2 transporter. As such, in the presence of a drug that decreases protein expression of the MRP2 transporter, the more potent drug would have higher bioavailability to exert its effect thereby enhancing combinatorial drug

therapy for the treatment of diseases.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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