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REVIEW ARTICLE

MRP4/ABCC4 As a New Therapeutic Target: Meta-Analysis to Determine cAMP Binding Sites as a Tool for Drug Design

Agustín Yaneff[a,#,*], Ana Sahores[a,#], Natalia Gómez[a], Alejandro Carozzo[a], Carina Shayo[b] and Carlos Davio[a]

[a]*Instituto de Investigaciones Farmacológicas (ININFA-UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina;* [b]*Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires, Argentina*

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Abstract: MRP4 transports multiple endogenous and exogenous substances and is critical not only for detoxification but also in the homeostasis of several signaling molecules. Its dysregulation has been reported in numerous pathological disorders, thus MRP4 appears as an attractive therapeutic target. However, the efficacy of MRP4 inhibitors is still controversial. The design of specific pharmacological agents with the ability to selectively modulate the activity of this transporter or modify its affinity to certain substrates represents a challenge in current medicine and chemical biology. The first step in the long process of drug rational design is to identify the therapeutic target and characterize the mechanism by which it affects the given pathology. In order to develop a pharmacological agent with high specific activity, the second step is to systematically study the structure of the target and identify all the possible binding sites. Using available homology models and mutagenesis assays, in this review we recapitulate the up-to-date knowledge about MRP structure and aligned amino acid sequences to identify the candidate MRP4 residues where cyclic nucleotides bind. We have also listed the most relevant MRP inhibitors studied to date, considering drug safety and specificity for MRP4 in particular. This meta-analysis platform may serve as a basis for the future development of inhibitors of MRP4 cAMP specific transport.

Keywords: MRP4/ABCC4, cystic fibrosis transmembrane conductance regulator (CFTR), SUR1, SUR2.

1. INTRODUCTION

The multidrug-resistance protein 4 (MRP4 or ABCC4) belongs to the large family of ATP binding cassette (ABC) transporters, which has been grouped into seven subfamilies named A to G based on sequence homology and phylogeny in humans [1]. MRP4 belongs to the human type C subfamily (ABCC) that clusters twelve members: nine MRP, cystic fibrosis transmembrane conductance regulator (CFTR), and two sulfonylurea receptors, SUR1 and SUR2.

MRP4 is expressed in several human tissues; high levels have been reported in prostate glandular and renal tubular epithelium [2, 3]. Low levels have been documented in liver, testes, ovaries, lung, adrenal gland, neurons, smooth muscle, platelet, and various blood cells. MRP4 is unique among ABC transporters due to its dual localization in polarized cells; having a basolateral membranous localization the prostate gland and liver [4, 5], while it is restricted to the apical membrane of brain capillary endothelium [6] and renal proximal tubule [7, 8].

MRP4 expression is finely regulated by a wide variety of factors. For example, several sex steroids and sex-specific growth hormones, including oestradiol-17-beta-D-glucuronide ($E_2$17βG), androgens and anti-androgens have been reported to regulate the transporters expression in prostatic and ovarian tissue [9-11].

*Address correspondence to this author at the Laboratorio de Oncología y Farmacología Molecular, Instituto de Investigaciones Farmacológicas, ININFA-UBA-CONICET, Facultad de Farmacia y Bioquímica, Junín 956, (C1113AAD), Ciudad Autónoma de Buenos Aires, Argentina. TE: +54-11-5287-4526.
E-mail: agustinyaneff@hotmail.com
[#]*These authors contributed equally*

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Although MRP4 expression is low in hepatocytes, bile acid in cholestatic situations enhances its expression [4, 12]. Also, oxidative stress induces higher MRP4 levels due to a NRF2 binding site in the promoter region [13].

Given that MRP4 promoter has several response elements for cAMP, this cyclic nucleotide also modulates MRP4 mRNA and protein levels. Enhanced cAMP levels up-regulate MRP4 expression through the Exchange Proteins Activated by cAMP (EPAC) pathway, causing an increase in cAMP efflux in a variety of cell types [14]. Cyclic AMP appears to have a dual role in the regulation of MRP4 expression in pancreatic adenocarcinoma cell lines. While intracellular cAMP up-regulates MRP4 through an EPAC2- and Rap1-mediated mechanism, extracellular cAMP reduces MRP4 promoter activity by a MEK/ERK-mediated pathway [15]. Lastly, in lung cancer models, COX-2 can also induce overexpression of MRP4, but not of other MRP transporters, through a PGE_2-dependent pathway [16]. A detailed description of the regulation of MRP4 expression in hepatocytes is revised in *Gu and Manautou 2010* [17].

MRP4 has been linked to several disease states and has been proposed as a promising new therapeutic target for pathophysiological processes in which the transporter is involved [18-22]. Given that most of the available MRP4 inhibitors are not very effective, the design of more potent and specific inhibitors and the improvement of the existing ones is indispensable. In order to reduce the adverse effects of the candidate drugs it is necessary to revise the structure of similar proteins and to consider all the physiological functions of the target. In this review, both aspects are thoroughly studied and using available MRP homology models together with mutagenesis assays, we carefully scanned MRP4 structural characteristics and compared them with other MRP family members. This analysis allowed us to postulate several candidate residues as highly probable cAMP binding sites.

2. MRP4 PHYSIOLOGICAL FUNCTIONS

MRP4 has multiple physiological and pharmacological roles given that it transports a wide variety of substrates out of the cell [2, 23]. These include endogenous molecules (Table **1**) and exogenous drugs, from antiviral to chemotherapeutic agents.

Some of the endogenous molecules transported by MRP4 include cyclic nucleotides (cAMP and cGMP), ADP, eicosanoids, prostaglandins, leukotrienes, urate, and conjugate steroid hormones [3, 18]. All of these physiological substrates participate in intra- and extracellular communication pathways, thus MRP4 is powerfully linked to signaling and cellular communication in healthy and pathological conditions (Fig. **1**). Of all the MRP, MRP4 particularly ensures cyclic nucleotides homeostasis, given that it regulates the intra- and extracellular levels of cAMP and cGMP [7, 24-27].

An example of MRP4 physiological role includes sperm capacitation. Early events in sperm capacitation include, among several processes, an increase in intracellular cAMP followed by efflux of this cyclic nucleotide [28]. Extracellular cAMP induces sperm hyperactivation and capacitation through a broad range of signaling cascades, including the activation of PLC, PKC and ERK1-2, which finally increases Ca^{2+} levels [29]. MRP4 pharmacological inhibition impairs sperm capacitation, and supplementation of cAMP in the culture media reverses the effect, increasing *in vitro* fertilization rate in a bovine model [29, 30]. Although studies in other species are needed to extrapolate these findings, the evidence suggests that the exclusion of the nucleotide might be critical to guarantee the cAMP tone required for the capacitation-associated events to take place. Modulating MRP4 activity might be beneficial in the generation of clinical advances in the treatment of infertility and the development of novel contraceptive strategies.

MRP4 physiological participation through cyclic nucleotides homeostasis has also been suggested for cell migration in fibroblasts associated with wound healing [21], and endothelial cells associated with angiogenesis [31]. Silencing MRP4 in human pulmonary artery smooth muscle cells, as well as in dendritic cells, resulted in a significant decrease in cell motility [32]. These findings indicate that cAMP tone determines migration capacity in various cell types and that targeting MRP4 could represent a novel therapeutic strategy to modulate cell migration.

Given that MRP4 avoids the accumulation of biological metabolites and participates in the removal of a variety of xenobiotics in several tissues, it is considered a vital organ protector. MRP4 is crucial for renal excretion of organic anions and drugs [2], and transports bile acids in the presence of glutathione in hepatocytes [4, 33]. In fact, the up-regulation of MRP4 in the liver of humans and rats with obstructive cholestasis provides an adaptative mechanism to eliminate excess bile salts [12, 17, 34]. MRP4 also prevents harmful substances from entering the brain through active drug efflux [6, 35]. Interestingly, MRP4 deficiency allows topotecan to cross the blood brain barrier and to penetrate the central nervous system and brain [6].

Table 1. Overview of MRP endogenous substrates transport.

	MRP1	MRP2	MRP3	MRP6	MRP7	MRP4	MRP5	MRP8
Eiconsanoids								
LTC_4	[84]	[84]	[85]	[86, 87]	[88]	[89]	[90]	[91, 92]
LTB_4	[89]					[89]		
LTD_4	[93]					[89]		
LTE_4	[93]							
PGE_1	[79]	[79]	[79]			[79, 94]	[79]	[79]
PGE_2	[79]	[79]	[79]			[79, 94]	[79]	[79]
$PGF_{2\alpha}$						[79, 94]		
TXB_2						[79, 94]		
PGA_1	[95]	[95]						
15d-PGJ_2	[96]		[96]					
Bile salts								
Bilirubin Glucuronide	[97]	[98]	[99]					
Taurocholate			[85, 100]		[92]	[4, 101]		[91]
Cholate						[4, 101]		[91]
Glycocholate			[85, 102]		[92]	[101]		[91]
Taurolithocholate-3-sulfate	[93]	[103]	[100]					
Folates								
Folate	[104]	[105]	[104]		[92]	[106]	[107]	[91]
Cyclic Nucleotides								
cAMP	[95]	[108]			[92]	[36, 109]	[90]	[91, 110]
cGMP	[111]				[92]	[36, 109]	[90]	[91, 110]
cCMP	[112]	[112]	[112]			[112]	[112]	[112]
cUMP	[112]	[112]	[112]			[112]	[112]	[112]
Steroids and conjugates								
$E_2$17βG	[84]	[84]	[85]	[87]	[88]	[36]	[90]	[91]
$E_1$3SO_4	[113]	[114]						[91, 115]
DHEAS	[33]	[33]	[33]			[33]		[91, 115]
Purine analogs								
Urate		[109]				[109]		
Other								
GSSG	[116]	[117]					[118]	
GSH	[119]	[117]	[120]	[86]		[4]		

Table 1. Records of positive (light or dark grey), co-transported with glutathione (dark grey) or negative (black) substrate transport were obtained through a rapid vesicle filtering technique, using cells that overexpress the corresponding MRP transporter. Most studies use HEK-293 [33, 79, 84, 85, 87-89, 91, 98, 101, 104, 107, 109, 112, 118, 121] or Sf9 insect cells [86, 102, 106, 108, 120]. In some cases, V79 [4, 89, 90, 94, 101], HeLa [89, 93, 97, 101, 113, 116, 119], MDCKII [95, 99, 103, 114, 115, 117], U937 [101], H69AR [113, 119], MCF7 [96], LLC-PK1 [110], NIH3T3 [36], CHO [87], ovarian carcinoma cell line 2008 [105], human erythrocytes [111] or DTY168 yeast cells [95] were used to produce the corresponding vesicles. Data about MRP transport capacity was also obtained from a review [92]. In some particular cases (blank spaces), transport was not determined or has not been reported in bibliography yet.

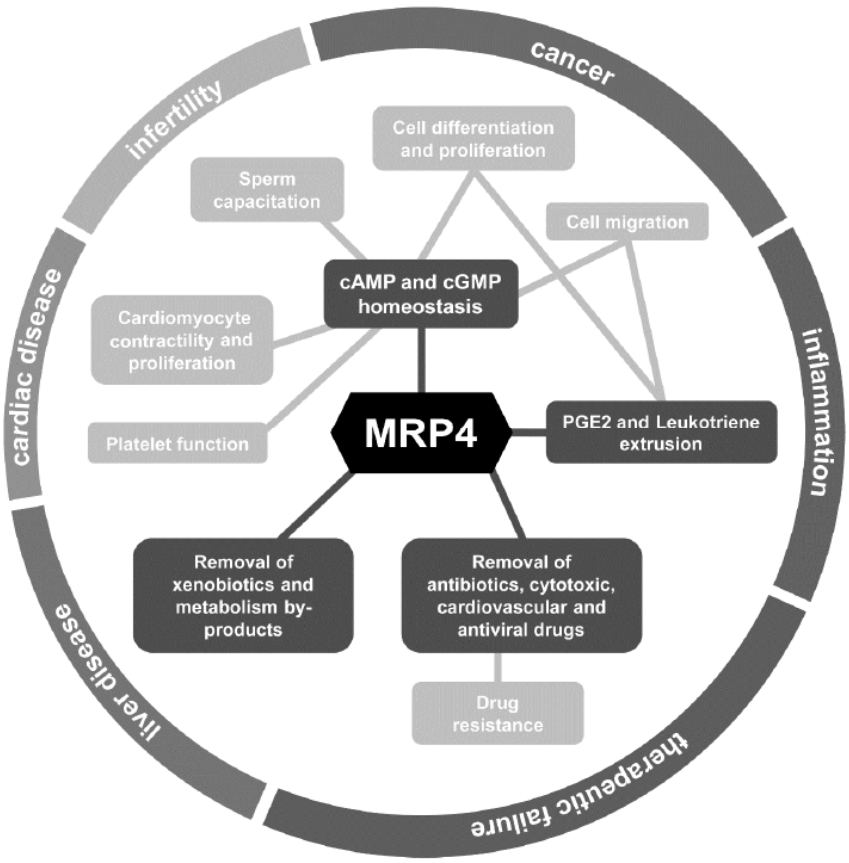


Fig. (1). MRP4 has pleiotropic functions and is associated with multiple physiological and pathological conditions. Inserted in the plasma membrane, MRP4 avoids the accumulation of metabolic by-products and participates in the removal of a variety of xenobiotics, including antibiotics, antiviral, and chemotherapeutic drugs, which explains the role of MRP4 in therapeutic failure. MRP4 also extrudes cyclic nucleotides (cAMP and cGMP) and prostaglandins (such as PGE_2), and regulates their intracellular levels. In turn, these signaling molecules may module fundamental processes in platelet function, cardiomyocyte proliferation and contractility, sperm capacitation, cell differentiation, proliferation, and migration, among other processes. Due to this, dysregulation in MRP4 expression or activity is associated with several pathological conditions, including liver disease and multiple types of cancers.

As a member of the ABC transporter family, the study of MRP4 was naturally focused on its role in cytotoxic drug resistance and consequential failure of multiple chemotherapeutic agents [23]. This is essentially due to its ability to increase the efflux of cytotoxic agents [18, 36], which results in the decline of intracellular drug levels and consequent drug insensitivity [37]. Since MRP4 also transports antibiotics, cardiovascular and antiviral drugs [38, 39], the protein has an effect not only in anticancer therapies, but in multiple therapies as well [37].

3. MRP4 IN PATHOLOGICAL CONDITIONS

Taking into account the diverse localization of MRP4 and the wide variety of substrates it transports, it is only natural that changes in its expression or activity may disturb cellular homeostasis by altering normal transport and disposition of endogenous and xenobiotic molecules with critical cell functions. Dysregulation of MRP4 expression has been linked to several pathological conditions.

3.1. MRP4 Associated with Cardiovascular Disease

MRP4 is expressed in smooth muscle cells, including cardiac myocytes [40, 41]. As mentioned before, this transporter is vital in cAMP and cGMP homeostasis which in part controls cardiac hypertrophy and myocardial fibrosis [40-42]. The reduction in the intracellular levels of these cyclic nucleotides activates signals related with proliferation and migration in mice cardiomyocytes [43] and reduces cell contractility [40, 41, 44]. Moreover, MRP4 is highly expressed in human platelets where intracellular ADP and cAMP accumulation is key for controlling the activation of platelets

[45]. Recent studies using MRP4-null mice platelets [46, 47] or pharmacological inhibition with MK571 on normal platelets [48] indicate that the transporter plays a key role in platelet activation, aggregation and thrombus formation. Altogether, these findings show that MRP4 plays an important role in the vascular biology and that inhibiting its activity might have therapeutic implications in many vasculo-proliferative disorders.

3.2. MRP4 Associated with Hepatic Disease

MRP4 is expressed at very low levels in the human, mouse, and rat liver. However, as previously cited, these levels significantly increase in patients with cholestasis [12, 49] or primary biliary cirrhosis [50], presumably serving to protect the liver from accumulation of hydrophobic bile acids, bilirubin, and other potentially toxic endogenous and exogenous chemicals. In fact, MRP4-null mice are more susceptible to obstructive cholestasis, which supports a role for this transporter in mitigating bile acid toxicity and oxidative-type cell necrosis [51]. Moreover, numerous publications inform that xenobiotics may increase the expression of hepatic MRP4 as well [17]. Toxic ingestion of acetaminophen [49] and patients with gallstones scheduled for cholecystectomy treated with ursodeoxycholic acid [52] showed an increase in hepatic MRP4 mRNA and protein levels. The same pattern was observed in mice fed with acetaminophen, ursodeoxycholic acid or carbon tetrachloride [53, 54]. Treatment with PPARα agonists, perfluorodecanoic and perfluorooctanoic acids also induce hepatic MRP4 mRNA and protein expression in mice [55]. Altogether, these findings highlight MPR4 role in detoxification and liver damage, and suggest its modulation could influence hepatocyte repair and recovery during hepatocellular injury.

3.3. MRP4 in Cancer

MRP4 is largely known to play a role in non-neoplasic conditions, however, there is an increasing body of evidence that indicates that the endogenous signaling molecules transported by MRP4 might have important roles in tumorigenesis as well [20, 56-58]. ABC transporter expression is linked to tumor aggressiveness in different tumor types, as shown by several observations from clinical studies [59]. MRP4 is dysregulated in several cancer types, such as acute myeloid leukemia (AML) [20, 60], multiple types of gastrointestinal cancers [56, 57], lung cancer [61], hepatocellular carcinomas [62], colorectal cancer [63], ovarian carcinoma [64, 65], prostate cancer [9, 66] and neuroblastoma [19], among others. In fact, given that high levels of MRP4 have been significantly associated with poor clinical outcomes of neuroblastoma, it has been proposed as a prognostic marker for this type of cancer [19, 67].

As mentioned above, the MRP family is well known for its contribution to the resistance to cytotoxic drugs and consequent failure of anticancer therapies. This is mainly due to their capacity to increase the efflux of chemotherapeutic drugs against the gradient, hydrolyzing ATP [68]. In particular, MRP4 is capable of exporting nucleotide base, nucleoside and nucleotide analogues [36], as well as structurally dissimilar chemotherapeutic compounds [69]. Therefore, the levels of MRP4 and its activity have been explored in the context of drug resistance in a number of cancer cell types, such as osteosarcoma [70] and ovarian cancer [64]. Indeed, the high expression of ABC transporters in cancer stem cells has been found to protect these cells from chemotherapeutic agents [71]. Moreover, several publications inform the overexpression and polymorphisms of members of the MRP family, including MRP4, in refractory/resistant tumors [64, 65, 67, 72, 73]. Despite these findings, and the relationship between MRP4 expression and drug resistance in several types of cancer cell lines, few studies have satisfactorily explored the association between MRP4 expression and cancer clinical outcome, and with the exception of neuroblastoma, no association has been reported.

The study of MRP4 in cancer has been classically focused on its role in chemotherapy resistance. However, the available evidence also indicates that MRP4 influences cancer cell biology independently of anticancer agent exposure [20, 63, 74, 75]. For example, human gastric cancer cell lines resistant to cisplatin overexpress MRP4, and silencing the transporter with siRNA restores sensitivity to this chemotherapeutic agent [72]. Interestingly, the reversion of this phenotype is produced even if cisplatin is not a substrate of MRP4. Moreover, in neuroblastoma cell lines, the silencing and pharmacological inhibition of MRP4 inhibits cell proliferation and clonogenicity, and induces morphological changes related to cell differentiation [19]. In this kind of neoplasia, MRP4 expression correlates with a worse prognosis, even if none of the routinely used chemotherapeutics is a substrate of the transporter [67, 74]. Studies using pancreatic Capan-1 clones, which are resistant to 5-FU, reveal that they overexpress MRP4 although the transporter is not able to transport the nucleoside out of the cell [76]. Altogether, these findings suggest that MRP4 levels are

important for tumor biology independently of its role as a xenobiotic transporter and suggest that an endogenous signaling molecule transported by MRP4 could be responsible for tumor progression. In this regard, MRP4 could promote cell survival through an increase in the efflux of autocrine signaling molecules, such as leukotrienes, prostaglandins, and cyclic nucleotides.

Signaling via leukotrienes promotes cell survival and proliferation through the activation of both autocrine and paracrine pathways in several cell types [32, 77]. It has also been reported that these bioactive lipid mediators can influence angiogenesis, inflammation, as well as migration and invasion of neuroblastoma cells [78]. As pro-inflammatory mediators, leukotrienes and thus, MRP4, appear to have a vital role in T-cell migration; where inhibition of the transporter reduces the ability of interstitial human dendritic cells to migrate towards lymph node chemotaxis [32]. Altogether, these findings raise the possibility that MRP4 could influence the migratory properties of cancer cells as well as their neighbors.

Regarding prostaglandins, MRP4 regulates PGE_1 and PGE_2 levels in the extracellular microenvironment by transporting them out of the cell [79]. The pleiotropic effects of higher levels of PGE_2 contribute to key steps on cancer development, including stimulation of cell proliferation, motility, and invasiveness, promotes tumor-associated neovascularization, inhibition of programmed cell death and immunosurveillance [80-82].

Lastly, intracellular cAMP levels play a key role in leukemic cell maturation and the extrusion of this cyclic nucleotide is ATP-dependent and probenecid-sensitive, supporting that the transport is MRP-mediated [20]. Knockdown strategy by shRNA revealed that this process is mediated by MRP4. What is more, genetic silencing and pharmacological inhibition of the transporter reduced tumor growth in a xenograft model, inducing cell arrest and an increase in the apoptotic index [60]. Interestingly, when leukemic stem cells population was isolated, increased cAMP levels were observed and MRP4 blockade resulted in differentiation of these cells. In a recent discovery, targeting protein interaction between MRP4 and another protein with a PDZ domain prevents MRP4 to arrive to plasma membrane and impacts in differentiation and drug sensitivity in AML cell lines and primary patient AML cells [83]. Also, *Carozzo et al.* describe that the balance between cAMP intra- and extracellular levels is critical in the regulation of MRP4 expression in pancreatic adenocarcinoma cell lines [15]. Moreover, the activity of the transporter is critical for PANC-1 cell proliferation, in culture and *in vivo*, and its inhibition causes a clear decrease in malignancy and invasive capacity of this cell line (*unpublished data*). Collectively, these findings postulate that MRP4 could represent a new potential therapeutic target to modulate cAMP levels in several kinds of neoplasias.

4. GROUP-ANALYSIS REGARDING MRP SUBSTRATE SPECIFICITY

As summarized above, several pathological processes show MRP4 overexpression, which leads to an enhanced activity of the transporter and an elevated efflux of certain substrates. Understanding the link between each substrate and pathology is critical for the design of specific pharmacological therapy. Efficient inhibitors are those that modulate the transport of certain molecules without affecting others that could be important for physiological processes. Given that MRP family members share many of the transported substrates, an overview of each of the substrate profiles is key in the delineation of the structural characteristics that give each transporter its particular activity and specificity.

We first performed a group-analysis of the substrate specificity for some of the reported MRP endogenous substrates (Table **1**). MRP9 was not included in this analysis because there is no information about its substrate specificity. MRP6 and MRP7 were included though, up to this date, only a few endogenous substrates have been tested.

ABCC proteins have two Nucleotide Binding Domains (NBD) and two or three Membrane Spanning Domains (MSD), depending on the isoform. Thus MRP1, MRP2, MRP3, MRP6, and MRP7 present three MSD (MSD0, MSD 1 and MSD2), whereas MRP4, MRP5, MRP8, and MRP9 present only two (MSD1 and MSD2). Although it has been proposed that MSD0 does not determine the selectivity of the transporter [122], the MRP substrate profile seems to agree with a sub-classification between those that have the MSD0 and those that do not. MRP containing MSD0 mainly transport endogenous phase II metabolites, such as glutathione (GSH), sulfates, and glucuronide conjugates. Remarkably, unlike members lacking the MSD0, none of these transporters can extrude cyclic nucleotides. The substrate profile of the transporters is clearly associated with the homology between MRP. Phylogenetic dendogram analysis groups MRP1-3 and MRP6 in the same cluster [123]. These channels share a similar substrate profile, except for the capacity of exporting GSH

and conjugated products, which is impaired in MRP3 [124]. MRP6 is located only 9Kb apart from the *ABCC1* gene (coding for MRP1 protein) in human chromosome 16, which suggests that it is probably the product of a genetic duplication [125]. Experimental evidences show that MRP6 has a limited variety of endogenous substrates compared to MRP1 [86, 87]. However, the affinity constants for leukotriene C4 (LTC_4) and dehydroepiandrosterone sulfate (DHEAS) are similar to those of MRP1, suggesting that both transporters have similar binding pockets. Although MRP7 belongs to the sub-group of transporters containing the MSD0, this domain is evolutionarily remote from the one in MRP1-3 and MRP6 [126]. This evolutionary divergence is evidenced by its sequence homology and by the fact that its N-terminal domain is encoded by only three exons, in contrast to the eleven to twelve present in other MRP. To date, only LTC_4 and estradiol 17-β-D-glucuronide ($E_217\beta G$) transport has been confirmed for this transporter [88, 127].

In this review we will refer to the "MRP1 group" when mentioning MRP1, MRP2, and MRP3. Despite sharing a similar sequence homology, MRP6 and MRP7 have not been included in this group-analysis because there is little experimental data about their substrate specificity. MRP4, MRP5, and MRP8 belong to the sub-group of MRP transporters lacking the MSD0. These MRP have similar DNA identities and also share many of their endogenous substrates. In this review we will refer to the "MRP4 group" when mentioning MRP4, MRP5, and MRP8 because they share cyclic nucleotides transport as a main characteristic [90, 110, 128].

A plethora of evidence highlights the participation of MRP4 in several pathological processes, mainly involving cAMP and PGE_2 deregulated transport. Cyclic AMP extrusion was first reported in 1963 as an active process sensitive to probenecid [129]. Over the next few years, several studies have described that the synthesis of cAMP and cGMP is followed by their exclusion, in an energy dependent process [130-132]. In this sense, clinical studies have shown that patients with hyper-proliferative disorders, such as leukemias and certain solid tumors, display higher levels of cAMP in plasma and urine compared to healthy individuals, postulating this second messenger as a prognosis biomarker in these pathologies [133]. Interestingly, these studies were forgotten for many years probably due to the lack of knowledge about the molecular mechanisms underlying the exclusion process. It was not until the end of the 20th century that the proteins responsible for the transport of this cyclic nucleotide could be identified. As mentioned before, to date only three members of the MRP family (MRP4, MRP5 and MRP8) with the capacity of transporting cyclic nucleotides out of the cell have been described (Table **1**). Using both intact HEK293 cells or membrane vesicles generated from hamster V79 cells transfected with MRP5 or from insect Sf9 cells transfected with MRP4, it was proven that these channels are responsible for the extrusion of cAMP and cGMP [7, 36, 90, 128]. MRP4 and MRP5 have been extensively studied and present significant differences in their kinetic parameters. For instance, MRP4 has a lower affinity for cGMP (Km = 180μM or Km = 9.7μM) than MRP5 (Km = 2.1μM) [36, 90, 109]. In contrast, the affinity of MRP4 for cAMP (Km = 44.5μM) [36] is nine times greater than that of MRP5 (Km = 379μM) [90]. This evidence, together with the characterization of the cyclic nucleotide transport in erythrocytes by elucidation of the affinity constants and the characterization of inhibitors that interfere in each process outlines MRP4 as the main transporter of cAMP and MRP5 as the main transporter of cGMP [134].

In line with this, MRP4-dependant cAMP extrusion has been proposed to play a significant role in the regulation of intracellular cAMP levels [135]. Nonetheless, the relevance of this process in cAMP homeostasis has been questioned, mainly due to the high energetic cost of cAMP extrusion, the possible associated depletion of purine reserves [136, 137] and the powerful and rapid control already given by Phosphodiesterase E (PDE) mediated degradation [127]. In fact, this controversy is further supported by the fact that in some systems no substantial changes were observed in the intracellular concentration of cAMP after inhibiting or over-expressing MRP4 [128]. Therefore, several reports indicate that the transport of cAMP mediated by MRP4 acts as an accessory regulatory mechanism only in cases where cyclic nucleotide levels are significantly high [128, 138]. Although inhibition of MRP4 does not affect global cytosolic cAMP levels, mathematical simulations propose it may control cAMP levels in restricted sub-membrane compartments with high MRP activity [139]. This assumption is supported by the fact that MRP4 protein is mainly confined to caveolae, which are specialized membrane micro-domains where multi-molecular complexes of signaling molecules are compartmentalized [35]. In addition, the MRP4 C-terminal portion presents a PDZ domain that mediates physical association between MRP4 and other molecules, such as CFTR chloride-channel through PDZ scaffolding proteins [24, 83, 140].

Finally, multiple hypotheses have been proposed regarding the role of cAMP in the extracellular compartment. Some researchers suggest that cAMP could act as a "third messenger" by providing an intercellular signal through an unknown receptor, or through its metabolism which leads to the production of adenosine in the extracellular space [137, 141].

As previously mentioned, PGE_2 belongs to another particular group of MRP4 endogenous substrates, which includes the prostanoids PGE_1, PGE_2, $PGF_{2\alpha}$ and TXB_2. Arachidonic acid derivatives are lipid hormones that contribute to various physiological processes such as renal function, inflammation, platelet aggregation, vasoconstriction, vasodilation, modulation of the immune system, and neurotransmission [142]. These molecules exert their effects through extracellular protein G-coupled receptors [143, 144] or nuclear receptors [145]. In order to act on extracellular receptors in the parental or neighbor cells, prostanoids exit the cell mainly through passive diffusion that happens at a very slow rate. Consequently, some physiological conditions may require active transport [79]. A few studies have tested PGE_1, PGE_2, $PGF_{2\alpha}$ and TXB_2 transport in MRP4 using isolated vesicles [79, 94] and others have used whole cell systems [146]. Alternative prostaglandins, such as PGF_1 and PGA_1, inhibit PGE_1 and PGE_2 transport; therefore it is possible that they might also be MRP4 substrates [79]. Prostanoids derived from eicosapentaenoic acid (an omega-3 polyunsaturated fatty acid) are also transported by MRP4 [147].

5. STRUCTURAL CHARACTERISTICS AND TRANSPORT MECHANISM

MRP4, as many ABC proteins, has more than one substrate binding site [4, 94, 101, 109, 148-150]. The presence of multiple binding sites determines a variability of responses when substrate and inhibitors (that could be substrates or not) are combined. To comprehend how these binding sites interact it is important to understand current models of the transport mechanism.

As mentioned above, MRP4 is an ABC transporter, and as such, it couples ATP hydrolysis in the NBD with the movement of two MSD. Structure-function relationship has been thoroughly studied in some ABC proteins, such as MDR1 or MRP1. These biophysical studies have focused in two main aspects: the functioning of the NBD domains and their role as molecular motors [151, 152], and the binding sites for each of the transported substrates [153, 154].

Currently, there are three models that try to explain the temporal and spatial coupling of ATP hydrolysis with conformational changes that drive the MSD from an inward to an outward facing conformation. The "Switch model" was the first one proposed [155] and postulates that both NBD alter between monomer and dimer states. Briefly, MSD1 and MSD2 are in an inwardly oriented conformation only accessible from the cytoplasm in the dissociated state of the NBD. Substrate binding and subsequent binding of two ATP molecules to the NBD monomers induce dimer formation, directing the MSD to the outward conformation, only accessible from the extracellular space. The sequential hydrolysis of the two ATP molecules induces the separation of the dimer which favors the return to the inward conformation. The main objection to this model is that, after dissociation, the NBD have few probabilities of coming together to form a dimer as a consequence of ATP binding [156].

Unlike the previous model, the "Constant Contact model" proposes that the NBD open sequentially when ATP hydrolysis occurs. Pi and ADP are released immediately without needing the monomers to separate completely. A new ATP molecule binds and the NBD closes again when the opposite NBD is prepared for ATP hydrolysis and release. This cycle is repeated and as a consequence of each ATP molecule hydrolysis, produces the shift between inward and outward conformations necessary for the substrate to be transported [155].

Conversely, the "Reciprocating model" is more intricate but agrees with experimental data [157], especially regarding ABC proteins that present more than one substrate binding site and shows allosteric transcooperativity [109]. This model postulates that the hydrolysis of ATP in the NBD occurs alternately, and that each molecule couples with a functional translocation pathway in the MSD. In this way, ABC transporters would have two translocation pathways functioning alternately, extending the idea of reciprocal hydrolysis of ATP from the "Constant Contact model" to the entire transport cycle [157].

It is worth noting that all three models differ in the mechanical interpretation of two fundamental characteristics of MRP transporters: the functional asymmetry of NBD and the presence of allosteric binding pockets [155, 157, 158].

Regarding allosterism, the "Switch" and "Constant Contact" models imply that the binding of a substrate in an inward site is capable of altering a second binding site, allowing or impairing the binding of another substrate. Also, the binding of a substrate could modify the maximum velocity of transport (Vmax) of another sub-

strate without changing its affinity constant (Km). This happens when the binding of a given substrate favors or delays the catalytic process of ATP hydrolysis, which in turn accelerates or slows down the restitution of the transporter to its basal state [158]. When the "Reciprocating model" is applied, the explanation for substrate cooperativity is much more interesting, as it suggests that substrates bound to one translocation pathway can affect the binding and transport of substrates in the other translocation pathway. Thus, a substrate that binds to an inward or outward site of one translocation pathway could prevent or facilitate substrate binding to the inward site of the other translocation pathway, leading to positive or negative allosterism. Therefore, this model helps to understand instances in which a substrate that is not transported stimulates or inhibits substrate traffic or ATP hydrolysis [102, 159, 160].

Most crystallographic structures support either the "Constant Contact" or the "Switch" models [156, 161]. Both models present a transporter with both MSD1 and MSD2 oriented either towards the intracellular side (inward conformation) or facing the extracellular side (outward conformation). On the contrary, while the "Reciprocating model" fails to reconcile with available crystal structures, it is better supported by the biochemical and biophysical data reported [157]. Beyond these differences, all three models not only settle with the idea of the existence of more than one substrate binding site, but also agree that in order to ensure an efficient transport, the sites exposed in the inward conformation should have a high affinity for the substrate, while the sites exposed in the outward conformation should have low affinity and thus facilitate the substrate release.

The presence of more than one substrate binding site has been evidenced for MRP based on vesicular transport studies, kinetic parameters calculations [109] or transport inhibition experiments, together with radioactive photo-labeling [150] and homology modeling [148, 149, 162]. Accumulated evidence proves both the existence of more than one binding site [4, 94, 101, 109, 127, 163] and the presence of non-transporting allosteric sites [102, 150, 164].

Regarding MRP4, substrate interaction was assessed with many combinations of endogenous and exogenous substrates. Within endogenous substrates, prostaglandins, DHEAS (dehydroepiandrosterone-3-sulfate), cGMP, bile acids sulfates, and cAMP inhibit $E_2$17βG transport [33, 36, 109, 165]. Prostaglandins inhibit cAMP and cGMP transport [128] and cAMP also inhibits cGMP transport [109]. Most authors affirm that substrates compete for the same binding sites, thus indicating competitive inhibition between these substrates. However, some cases suggest the existence of a more complex mechanism than a simple competitive inhibition between substrates. One of these examples arises from the half-maximal inhibitory concentration (IC50) of several steroids on the transport of DHEAS and $E_2$17βG. Since MRP4 has more affinity for DHEAS than for $E_2$17βG, it is expected that any inhibitor with these characteristics would affect $E_2$17βG transport more than DHEAS. The fact that the IC50 of oestradiol 3,17-disulfate for inhibiting DHEAS transport is lower than that of $E_2$17βG, gives rise to the idea that inhibition is not exclusively competitive [33]. Another interesting case occurs in MRP8, where $E_2$17βG, DHEAS, and taurocholate apparently share a common binding site since they have inhibitory effects on each other's transport, with the exception of DHEAS which somehow stimulates $E_2$17βG transport [92].

Another proof of the existence of more than one binding site in a variety of members of the MRP family is the co-transport of several substrates together with GSH. The most studied case is the effect of GSH on the MRP1-mediated transport of a wide range of substrates [151]. Experiments show that GSH stimulates transport of many conjugated and unconjugated substrates, independently of their ability of being co-transported or not. In addition, stimulation of GSH transport mediated by other substrates has also been described. Co-transport of bile salts, LTB_4, and LTD_4 with GSH is another proof supporting that MRP4 has more than one binding site [4, 89, 101]. GSH is not the only substrate that acts as a cooperative partner with other substrate producing mutual stimulation of transport. This heterotropic allosteric effect has also been observed for $E_2$17βG and bile salts transported by MRP2 [163]. Moreover, some MRP2 ligands like taurocholate, penicillin G, and pantoprazole are also able to stimulate $E_2$17βG transport without being transported themselves [166]. Apparently, this transport enhancing effect is dependent not only on the stimuli but also on the substrate that is being transported, as probenecid stimulates the MRP2-mediated transport of $E_2$17βG but inhibits the transport of methotrexate (MTX) and S-(2,4-dinitrophenyl)-glutathione (GS-DNP) instead [166]. Similarly, LTC_4 and MTX were found to display positive cooperative allosteric effects in MRP3-mediated transport [167]. Interestingly, MRP4 also presents homotropic allostery, given that urate stimulates cGMP transport, inhibits MTX transport, and has no effect regarding cAMP transport [109]. The urate-mediated

stimulation of cGMP transport is due to an increment in the Vmax and rarely, to a decrease in the Km. Conversely, cGMP does not stimulate urate efflux. By analyzing Hill coefficients, the authors conclude that the binding of urate changes cGMP transport from an allosteric binding stimulated transport to a single binding transport, implicating urate displaces cGMP from one of its binding sites. Finally, stimulation of cGMP transport by MRP4 was achieved when urate was placed on either sides of the membrane (*cis* and *trans*). *Cis* incubation with substrates is the most common approach for substrate interaction studies. In these experiments, both substrates are placed outside the inside-out vesicles. So, if both substances are transported, they can accede to inward and outward transporter conformations. But when *trans* incubation is used, one substrate is placed outside and the other inside the inside-out vesicles. In this way, the substrate inside the vesicles only contacts the outward conformation of the transporter. The fact that urate *trans*- stimulates the transport of cGMP supports the idea of the presence of an allosteric site in the outward conformation that could affect cGMP Vmax [109]. In agreement with the existence of allosteric regulatory sites, stimulatory or inhibitory effects on the transport of certain substrates have been found to be concentration-dependent. For example, at low concentrations, phenylbutazone and celecoxib stimulate MTX transport by MRP2 and MRP4, respectively, probably through binding to a high affinity allosteric site. However, at higher concentrations, both molecules act as inhibitors competing for MTX transport sites [164].

6. PUTATIVE CYCLIC NUCLEOTIDE BINDING SITE ANALYSIS

Identifying the location of binding sites on proteins is of fundamental importance for a wide range of applications including molecular docking, *de-novo* drug design, structure identification and comparison of functional sites. Given that cAMP transport by MRP4 has been linked to several pathological processes, especially in cancer and cardiovascular diseases, we performed an analytical approach to try to identify cAMP binding sites in the transporter.

Our group-analysis regarding MRP substrate specificity led us to divide MRP transporters in two groups according to their capability to transport cyclic nucleotides. Thus, we performed a meta-analysis using sequence alignment, homology models, docking experiments and mutagenesis studies of MRP4 and other members of the MRP family. A systematical comparison of the primary amino acid sequence between members of the two groups allowed us to identify several candidate residues that could confer MRP4 the capacity of recognizing and transporting cyclic nucleotides. Finally, we built two homology models in order to corroborate the spatial localization of the suggested residues.

Firstly, we performed a sequence alignment using the amino acid sequence from some of the human MRP. MRP6, MRP7 and MRP9 were not included in this alignment analysis since the data available regarding their substrate specificity and potential binding sites was considered insufficient. In order to identify relevant sites within the entire MRP4 sequence we used available information from MRP4, MRP5 and MRP8 homology models [148, 149, 162]. We considered a MRP1 model generated using two cryo-electron microscopy (CEM) structures of apo and substrate-bound MRP1 [168]. Finally, mutagenesis studies of MRP1, MRP3 and MRP4 were key to identifying the possible cyclic nucleotide binding sites [121, 150, 169-193].

The amino acid sequence alignment of MRP4, MRP5, MRP8, MRP1, MRP2 and MRP3 are shown in Fig. (**2**). Aligned amino acids were colored according to their chemical and physical properties. The residues which have been suggested to be part of MRP4, MRP5 and MRP8 binding pockets by previous authors are also marked [148, 149, 162]. In this analysis, we included homology models of MRP4, MRP5 and MRP8 constructed using the outward facing *S. aureus* Sav1866 crystal structure (homologous to human ABCB1) as a template. In addition, an inward model of MRP8 obtained from mouse MDR3 (homologous to human ABCB4) structure was also included [149]. In these studies, the authors used ICMPocketFinder to identify the sites that participate in the substrate binding during transport by MRP4 and MRP5 [148, 162]. As to MRP5 and MRP8, the authors performed docking studies using cGMP [148, 149] and 5-Fd-UMP [149] as ligands. For practical purposes, the sites suggested for 5-Fd-UMP binding to MRP8 are not presented in Fig. (**2**). Thanks to these experimental approaches, two binding pockets have been predicted for MRP5 and MRP8: extracellular binding pockets 1 and 2 for MRP5 and MRP8, respectively (shown in light grey) and intracellular binding pockets 2 and 1 for MRP5 and MRP8, respectively (shown in dark grey) [148, 149]. Regarding the MRP5 model, the amino acids which belong to both binding sites are shown in a combination of both greys. Despite that MRP4 has multiple binding sites, this model makes no differentia-

tion between them (shown in blue) [162]. Regarding MRP1, we included data from *Jhonson and Chen's* recent publication [168], where two CEM structures for bovine MRP1 (bMRP1, 91% identity with hMRP1) were generated in the inward conformation: the first CEM structure consists of the transporters' structure alone and the second one depicts the structure of the compound in the presence of LTC_4. As a result of these CEM structures, the authors were able to describe a binding site consisting of two pockets: a positively charged section which binds to the GSH moiety of the conjugated ligand (p-pocket, shown in light grey with the respective amino acid and position in bMRP1) and a hydrophobic section which binds to the lipidic tail (h-pocket, shown in dark grey with the respective amino acid and position in bMRP1). We also took into consideration the information about MRP1 substrate binding sites that have been thoroughly analyzed by mutagenesis studies and naturally occurring polymorphisms that disrupt transport activity of one or multiple endogenous substrates. Several publications have mainly analyzed the transport capacity of five of its primary substrates: LTC_4, $E_217\beta G$, estrone sulfate (E_13SO_4), GSH, and MTX. Transport of E_13SO_4 was always analyzed with incubation in presence of GSH [184], while GSH transport was studied in combination with apigenin [174, 176, 177] or verapamil [189, 190]. In addition, available mutagenesis studies of specific MRP3 and MRP4 transmembrane sections (TM6 and TM12) were also included in the analysis. These studies were performed using either two different mutation strategies: i) replacing the residue for an equivalent one in a homologous MRP, in a polymorphic variant or in another species orthologous MRP [169-173]; or ii) replacing the residue by performing conservative or non-conservative mutations [121, 150, 174-191]. All the mutagenesis data included in this study were analyzed according to variations in substrate transport parameters. Cells painted in black correspond to point mutations that affect the transport of the studied substrate and light grey was used when no changes were observed. Blank cells correspond to residues which, to our knowledge, have not been mutated in transport studies.

In order to simplify the process of substrate translocation and identify the role of each of the binding sites, the whole process can be divided in three main stages. During the **first stage**, the substrate binds with high affinity to the sites which are exposed in the inward conformation of the transporter. During the translocation of the substrate, the **second stage** or **transition state**, the binding sites may or may not be the same as those of the other stages. Lastly, in the **third stage,** the substrate is released to the extracellular compartment when interaction ceases with a low affinity binding site exposed in the transporters' outward conformation. In addition, substrate binding can also occur in allosteric sites, modulating the traffic of other substances without being transported *per-se*. The inward and outward models can predict key residues that participate in the first and third stages, respectively, but fail to predict the sites that are involved in the second stage. On the other hand, mutagenesis studies can be applied to predict residues involved in the three stages. However, it is worth noting that mutagenesis *per-se* can potentially affect the transporters' functionality as a whole, without particularly changing the first, second or third stages, or the allosteric binding sites. This can be explained as a result of: i) MRP expression, trafficking or activity alteration, which includes disrupted communication between the TMD and the NBD; or ii) changes in the electrostatic, hydrophobic or hydrophilic environment in the binding pockets adjacent area.

Luckily, over the last few years, several tools and strategies have been developed that allow us to identify whether a mutated residue affects MRP activity or not. In these studies, levels of expression and trafficking of the transporters must be carefully controlled in order to draw valid conclusions about the effect of each mutation on the activity of the transporters [186-188]. As mentioned above, when analyzing mutagenesis studies it is imperative to consider if a mutated residue affects the transport of all tested substrates. If this happens, it could be speculated that the mutation has disrupted the entire transporters' functionality or that it has degenerated the binding sites in one or various stages involving all tested substrates. ATPase activity, 8-Azido-[α^{32}P]ATP labeling and orthovanadate-dependent trapping of 8-Azido-[α^{32}P]ADP are the most common strategies used to draw conclusions about the general functionality of mutated MRP [172, 177, 178, 189, 190]. Photo-labeling of LTC_4 or GSH has also been used to confirm whether a point mutation affects the binding capacity, especially regarding first stage high affinity binding [176, 177, 189, 190]. Finally, calculating the kinetic constants for each of the mutants helps to discern which of the three binding stages is affected by the mutation [186-188]. As a general interpretation, a specific mutation increases the affinity constant (Km) because the mutation of that residue leads to less binding during the first stage of the translocation pathway. On the other hand, when a mutation affects the maximum speed of transport (Vmax), it is generally concluded that the mutation is affecting any three stages of

the process. Unfortunately, mutations which affect allosteric binding sites are hard to discern and, thus have been poorly investigated. Furthermore, a particular residue may not only intervene in the binding of certain substances during one specific stage but may also affect the binding of other substances in a different stage [191].

Fig. (**2**) proposes the potential binding sites for each of the substrates in each of the MRP. However, our study was focused on the key residues within those sites that have been previously suggested by three-dimensional models and are conserved in the first group ("MRP4 group") compared to the second group ("MRP1 group"). We therefore believe these residues represent MRP4 specific cyclic nucleotide binding sites and are colored in blue in the last row of each of the TM blocks. The rest of the information shown in (Fig. **2**) constitutes a powerful tool for future studies given that it locates the binding sites of different solutes for the different MRP presented.

For instance, in **TM1** and **ECL1** (Fig. **2**, Block 1) the sites predicted by ICMPocketFinder or by docking studies of MRP4, MRP5, and MRP8 outward models overlap almost entirely. Remarkably, the amino acid sequence of these sites is significantly different when comparing the three transporters. These residues also overlap with two of the candidate sites for LTC_4 binding suggested in the bMRP1 inward CEM structure (K332 and H335). Mutagenesis and photo-labeling studies have shown that K332 appears to be crucial for LTC_4 binding to MRP1 during the first stage because mutations in this site completely abrogated LTC_4 photo-labeling and increased the Km [150, 181]. Additionally, non-conservative mutations of H335 showed a reduction in the Vmax without changing the Km, which suggests that this residue could participate in the second and third stages of LTC_4 transport. Also, photo-labeling of MRP1 H335 mutants showed a 45-50% photo-labeling reduction compared to the wild type variant. Therefore, MRP1 mutagenesis studies confirm the involvement of these two residues (K332 and H335) in substrate selectivity [150, 181, 184]. Interestingly, T99 in MRP4 (aligned with K332 from MRP1) has been suggested to interact with its substrates in the MRP4 outward model [162]. In a similar fashion, L186, a non-charged residue from MRP5, is also aligned with K332 from MRP1, and has been suggested to interact with cGMP in MRP5 outward docking experiments. Another interesting fact is that both ICMPocketFinder and MRP5 cGMP-docking studies identified two residues present in the intracellular and extracellular binding sites for this cyclic nucleotide (Q190 and G193). Docking experiments have shown that the purine base of cGMP interacts with Q190 in certain spatial poses of the cyclic nucleotide. This residue is not highly conserved among the MRP that bind cGMP, but in fact, corresponds to a charged glutamic acid residue in MRP4, similar to the aspartic acid residue found in MRP1, MRP2 and MRP3. In MRP8, a natural occurring polymorphism (G180R) takes place in this position, disrupting cGMP transport and highlighting its importance as a cyclic nucleotide binding site in MRP8 [173].

Candidate binding sites proposed by the MRP8 inward model appear to be located in the final section of the TM1 and in the first ECL, while binding sites in the outward models show localization only in TM1. The loop shows low amino acid conservation when comparing MRP4 sequence with the other transporters. In this loop, E194 from MRP8 was suggested to interact with cGMP in the inward-facing conformation [149]. In the proximity of this amino acid, MRP4 presents two negatively charged residues (E121 and D124), while MRP5 presents only one (E212). In addition, mutations in MRP1 neighboring residues show differential substrate transport properties suggesting that they determine substrate differential recognition. As an example, W361A mutant in MRP1 retains the ability to transport LTC_4 and E_13SO_4, but exhibits a 25-50% decrease in $E_217\beta G$, GSH, and MTX transport [174]. Members of the MRP1 group have a very conserved tryptophan residue in this site while the MRP4 group depicts an asparagine. After analyzing the complete TM1 and ECL1 alignment, we propose that T99, E102, E103, A105, K106 and K132 from this section of MRP4 may participate in cAMP binding (Fig. **2, 3**) and should be studied further.

When analyzing **TM2** (Fig. **2**, Block 2), only binding sites of the outward conformation have been proposed in MRP4, MRP5, and MRP8 homology models. Regarding MRP5, it is interesting to note that sites suggested by ICMPocketFinder and by cGMP docking-studies are different, with the exception of the R232 site. In fact, Ravna *et al.* inform that a salt bridge usually forms between cGMP and this positive charged residue in most of the tested docking conformations [148]. This amino acid is conserved in MRP8 but has not been identified as a binding pocket residue in neither of the MRP8 models [149]. Despite that the positive charge is not conserved in MRP4 in this position, L148 could be involved in cAMP binding. The H152 and H153 residues are the closest positively charged

amino acids in MRP4 and the former has been identified as part of the binding pocket in the outward model of this protein. MRP4 H152 corresponds to F220 in MRP5, an aromatic residue also identified as a potential binding site in the latter [148]. Conversely, the analog residue for MRP5 R232 in the "MRP1 group" was found to be an extremely conserved glutamine and its adjacent residues are also similar to that of MRP5, since it is preceded by a hydrophobic amino acid (Ile, Leu or Met) and followed by a hydrogen-bond donor (Thr or Ser). Based on this analysis, we suggest L148, H152, and H153 may be involved in cAMP binding (Fig. **2, 3**).

For **TM3** (Fig. **2**, Block 3) cGMP-docking analysis of MRP5 lead to the identification of three amino acids (L297, P301, and A304) that are extremely conserved in MRP4. One of them (L297) appears to participate in both, MRP5 intra- and extracellular cGMP-binding pockets. Interestingly, MRP4 L215 (corresponding to L297 from MRP5) and MRP4 P219 (corresponding to P301 from MRP5) were also identified as cGMP-binding sites for MRP4. Also, other amino acids in TM3 (W216, G218, Q221, and V225) have been suggested as MRP4 binding sites. All of these residues are extremely conserved in MRP transporters, and mutations on MRP1 corresponding residues impair or prevent transport for all of the tested substrates. W445A in MRP1 decreases 70-75% transport of LTC_4, $E_217\beta G$, E_13SO_4 and almost eliminates GSH and MTX transport compared to the wild type variant [174]. P448A in MRP1 completely abolishes GSH, MTX, and $E_217\beta G$ transport and decreases 40-60% transport of LTC_4 and E_13SO_4 [175]. The drop in LTC_4 transport is mostly due to a reduction in the Vmax, indicating that this site is involved in the second and third stages of LTC_4 transport. On the other hand, in the MRP1 inward CEM structure, the Y440 amino acid was suggested to form a hydrogen-bond with GSH through its hydroxyl-group, thereby forming part of the p-pocket. This MRP1 residue and adjacent sites have been implicated in substrate selectivity through mutational analysis. Mutations of Y440 with the corresponding MRP3 amino acid (Y440F) have shown to change LTC_4 and E_13SO_4 transport, while no variation was observed in MTX and $E_217\beta G$ transport [169]. Mutation of this residue for a bigger one (Y440W) not only decreased LTC_4 and E_13SO_4 transport but also impaired $E_217\beta G$ traffic. I441L also showed to reduce E_13SO_4 and $E_217\beta G$ transport activity, while LTC_4 and MTX traffic was similar to that of the wild type variant. M443L reduced LTC_4, $E_217\beta G$ and E_13SO_4 transport but did not change MTX traffic. GSH transport was not tested in neither of these mutants. Y440F and I441L produced significant changes in the Km for E_13SO_4, but did not affect the Vmax, while M443L diminished photo-labeling with azidophenacyl-[^{35}S]-GSH [169].

Despite its localization between two aminoacids that have been suggested in the MRP4 outward model analyzed by ICMPocketFinder, A217 is an interesting residue to study since it is a well conserved small aliphatic residue in the "MRP4 group", while the "MPR1 group" depicts a serine in this location (Fig. **2, 3**).

In the case of **TM5** (Fig. **2**, Block 4), candidate binding sites which have been suggested in the outward models of MRP4, MRP5, and MRP8 overlap almost perfectly. Interestingly, the amino acids corresponding to the proposed sites are not very conserved between these MRP. T550 and W553 were predicted to form part of the h-pocket in MRP1 and the former amino acid is in the exact same position of the suggested candidate residues for the outward MRP4, MRP5, and MRP8 models. Despite this, when this residue was replaced by an alanine in MRP1, no effect was observed on the transport of LTC_4, $E_217\beta G$, and GSH. Furthermore, W553A almost abolished $E_217\beta G$, E_13SO_4, GSH and MTX transport, while LTC_4 transport was reduced 50%, which confirms that the residue could participate in the h-pocket [174]. Importantly, an aromatic residue is conserved in all members of the "MRP1 group" as well as in MRP4, while MRP8 has an aromatic residue in the F391 adjacent site. No aromatic residues are present in the corresponding MRP5 section. This could be related to the fact that MRP5 is the only member of the "MRP4 group" that does not transport LTC_4 [90].

After analyzing the complete TM5 and ICL2 alignment, we observed that MRP4, MRP5, and MRP8 contain a highly conserved small aliphatic residue in the corresponding A322 site, while the "MRP1 group" depicts a phenylalanine. Also, aligned with MRP1 W553, MRP4 has a phenylalanine residue in position 324 and another in position 325 that has been suggested as a binding site for MRP4, MRP5 and MRP8 outward models. Additionally, S328 and K329 residues have been suggested as binding sites in the MRP4 outward model and also, K329 position (V411 in MRP5 and T395 in MRP8) has been suggested in both MRP5 and MRP8 models as well. It would be interesting to further analyze these two residues since they are a hydrogen bond donor and a positively charge amino acid, respectively and could participate in cAMP binding site (Fig. **2, 3**).

Concerning **TM6** and **ICL3** (Fig. **2**, Block 5), residues R362, l363 and T364 from MRP4 and the corre-

A.
BLOCK 1
ECL1
TM1
TM2
TM3
BLOCK 2
BLOCK 3
BLOCK 4
KCL2
TM5
MRP4 SITES
REFERENCE
D,E
S,T
P,G,C
Y, F, W
N,Q
A,L,I,M,V
K,R,H

(Fig. 2) contd….

B.

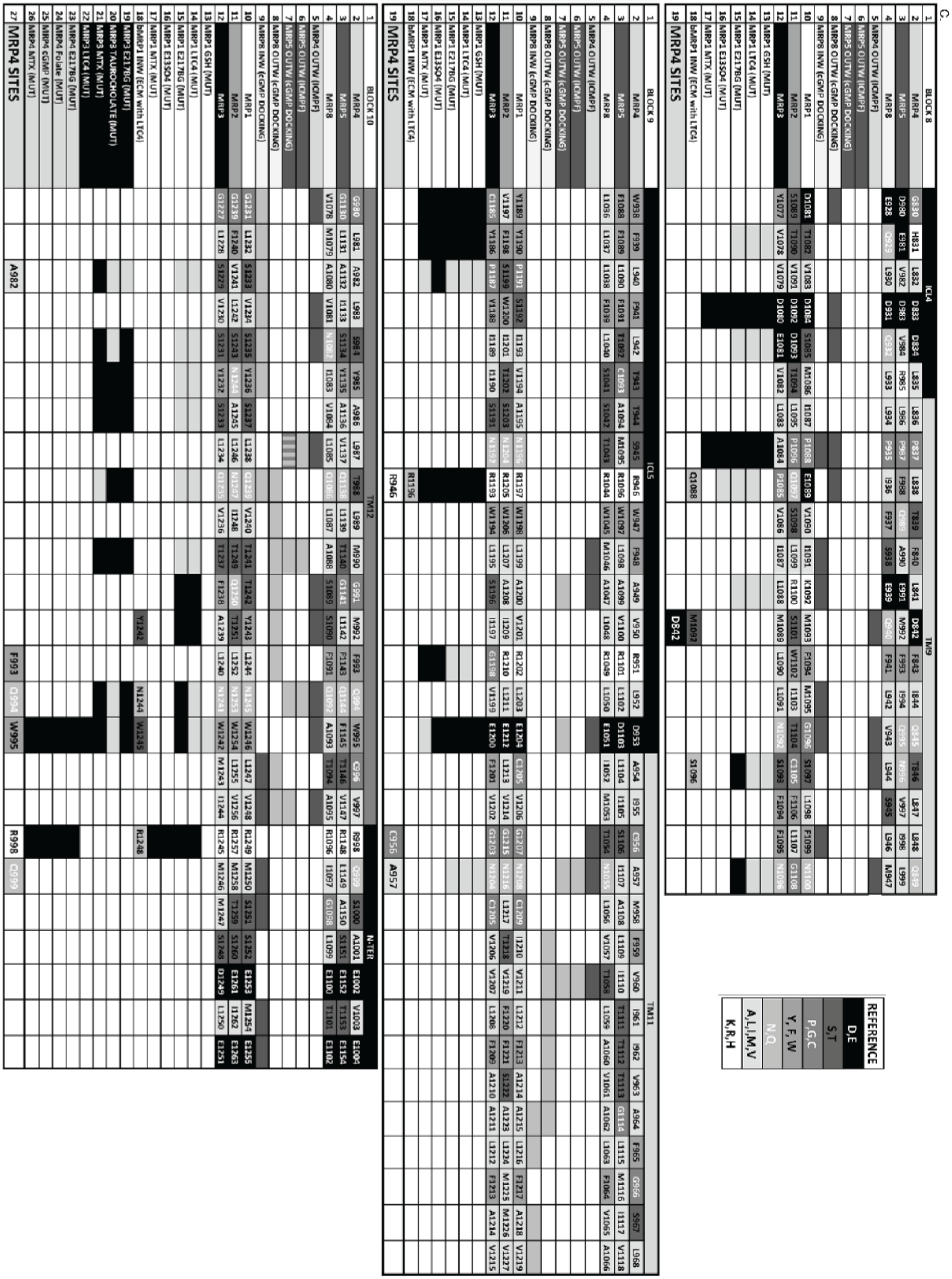
1	BLOCK 5	TM6																				ICL3						
2	MRP4	F352	V353	A354	V355	T356	L357	Y358	G359	A360	V361	R362	L363	T364	V365	T366	L367	F368	F369	P370	S371	A372	I373	E374	R375	V376	S377	E378
3	MRP5	F434	T435	V436	V437	T438	V439	F440	N441	S442	M443	T444	F445	A446	L447	K448	V449	-	T450	P451	F452	S453	V454	K455	S456	L457	S458	E459
4	MRP8	F418	S419	M420	L421	A422	S423	L424	N425	L426	L427	R428	L429	S430	V431	F432	F433	-	V434	P435	I436	A437	V438	K439	G440	L441	T442	N443
5	MRP4 OUTW (ICMPF)																											
6	MRP5 OUTW (ICMPF)																											
7	MRP5 OUTW (cGMP DOCKING)																											
8	MRP8 OUTW (cGMP DOCKING)																											
9	MRP8 INW (cGMP DOCKING)																											
10	MRP1	F583	V584	S585	L586	A587	L588	F589	N590	I591	L592	R593	F594	P595	L596	N597	I598	-	L599	P600	M601	V602	I603	S604	S605	I606	V607	Q608
11	MRP2	F580	T581	S582	I583	T584	L585	F586	N587	I588	L589	R590	F591	P592	L593	S594	M595	-	L596	P597	M598	M599	I600	S601	S602	M603	L604	Q605
12	MRP3	F570	V571	S572	V573	S574	L575	F576	N577	I578	L579	R580	L581	P582	L583	N584	M584	-	L586	P587	Q588	L589	I590	S591	N592	L593	T594	Q595
13	MRP1 GSH (MUT)																											
14	MRP1 LTC4 (MUT)																											
15	MRP1 E217BG (MUT)																											
16	MRP1 E13SO4 (MUT)																											
17	MRP1 MTX (MUT)																											
18	bMRP1 INW (ECM with LTC4)												F594								M601							
19	MRP4 E217BG (MUT)																											
20	MRP4 Folate (MUT)																											
21	MRP4 cGMP (MUT)																											
22	MRP4 MTX (MUT)																											
23	MRP4 SITES											R362	L363	T364				F368	F369					E374	R375			E378

1	BLOCK 6	TM7																ECL4										
2	MRP4	F716	L717	I718	L719	L720	N721	T722	A723	A724	Q725	V726	A727	Y728	V729	L730	Q731	D732	W733	W734	L735	S736	Y737	W738	A739	N740	K741	Q742
3	MRP5	V861	I862	M863	A864	L865	F866	M867	L868	N869	V870	G871	S872	T873	A874	F875	S876	T877	W878	W879	L880	S881	Y882	W883	I884	K885	Q886	G887
4	MRP8	I808	I809	F810	F811	F812	V813	V814	L815	I816	V817	F818	L819	T820	I821	F822	S823	F824	W825	W826	L827	S828	Y829	W830	L831	E832	Q833	G834
5	MRP4 OUTW (ICMPF)																											
6	MRP5 OUTW (ICMPF)																											
7	MRP5 OUTW (cGMP DOCKING)																											
8	MRP8 OUTW (cGMP DOCKING)																											
9	MRP8 INW (cGMP DOCKING)																											
10	MRP1	S978	I979	F980	L981	-	F982	M983	C984	N985	H986	V987	S988	A989	L990	A991	S992	N993	Y994	W995	L996	S997	L998	W999	T1000	D1001	D1002	P1003
11	MRP2	I982	I983	L984	A985	-	F986	V987	M988	N989	S990	V991	A992	F993	I994	G995	S996	N997	L998	W999	L1000	S1001	A1002	W1003	T1004	S1005	D1006	S1007
12	MRP3	I974	C975	L976	L977	-	Y978	V979	G980	Q981	S982	A983	A984	A985	I986	G987	A988	N989	V990	W991	L992	S993	A994	W995	T996	N997	D998	A999
13	MRP1 GSH (MUT)																											
14	MRP1 LTC4 (MUT)																											
15	MRP1 E217BG (MUT)																											
16	MRP1 E13SO4 (MUT)																											
17	MRP1 MTX (MUT)																											
18	bMRP1 INW (ECM with LTC4)																											
19	MRP4 SITES													Y728											A739		K741	

1	BLOCK 7	ECL4		TM8																					ICL4				
2	MRP4	S769	G770	L771	T772	V773	A774	T775	V776	L777	F778	G779	I780	A781	R782	S783	L784	L785	V786	F787	Y788	V789	L790	V791	N792	S793	S794	Q795	T796
3	MRP5	A919	L920	S921	M922	A923	V924	M925	L926	I927	L928	K929	A930	I931	R932	G933	V934	V935	F936	V937	K938	G939	T940	L941	R942	A943	S944	S945	R946
4	MRP8	G867	L868	N869	A870	L871	L872	L873	I874	C875	V876	G877	V878	C879	S880	S881	G882	I883	F884	T885	K886	V887	T888	R889	K890	A891	S892	T893	A894
5	MRP4 OUTW (ICMPF)																												
6	MRP5 OUTW (ICMPF)																												
7	MRP5 OUTW (cGMP DOCKING)																												
8	MRP8 OUTW (cGMP DOCKING)																												
9	MRP8 INW (cGMP DOCKING)																												
10	MRP1	G1020	A1021	L1022	G1023	I1024	S1025	Q1026	G1027	I1028	A1029	V1030	F1031	G1032	Y1033	S1034	M1035	A1036	V1037	S1038	I1039	G1040	G1041	I1042	L1043	A1044	S1045	R1046	C1047
11	MRP2	G1028	A1029	L1030	G1031	L1032	A1033	Q1034	G1035	I1036	F1037	V1038	F1039	I1040	A1041	H1042	F1043	W1044	S1045	A1046	F1047	G1048	F1049	V1050	H1051	A1052	S1053	N1054	I1055
12	MRP3	A1016	A1017	L1018	G1019	I1020	L1021	Q1022	G1023	F1024	L1025	V1026	M1027	L1028	A1029	A1030	M1031	A1032	M1033	A1034	A1035	G1036	G1037	I1038	Q1039	A1040	A1041	R1042	V1043
13	MRP1 GSH (MUT)																												
14	MRP1 LTC4 (MUT)																												
15	MRP1 E217BG (MUT)																												
16	MRP1 E13SO4 (MUT)																												
17	MRP1 MTX (MUT)																												
18	bMRP1 INW (ECM with LTC4)																												
19	MRP4 SITES										G779	I780	A781	R782											S793	S794	Q795	T796

REFERENCE
D,E
S,T
P,G,C
Y, F, W
N,Q
A,L,I,M,V
K,R,H

(Fig. 2) contd….


C.
REFERENCE
D,E
S,T
P,G,C
Y,F,W
N,Q
A,L,I,M,V
K,R,H
MRP4 SITES
BLOCK 8
BLOCK 9
BLOCK 10

(Fig. 2) contd….

Fig. (2). MRP4 sequence alignment and cAMP binding site identification. Amino acid sequence alignment for MRP4 (NP_005836.2), MRP5 (BAA76608.1), MRP8 (NP_115972.2), MRP1 (NP_004987.2), MRP2 (CAB45309.1), and MRP3 (CAA76658.2) is shown (rows 2, 3, 4, 10, 11, and 12, respectively) for each block. The alignments were performed using the T-Coffee software [194] and further analysis was achieved with MS Excel 2011 for Mac (v14.5.5). For rows 1-3 and 10-12, aligned amino acids were colored regarding chemical and physical properties as referenced in the figure. Each block corresponds to a different section of the MRP, and includes one transmembrane domain (TM) and adjacent intracellular and/or extracellular loops (ICL and ECL, respectively) where binding sites have been suggested by the homology models used as reference. TM4 and TM10 were not included in this analysis since no binding sites have been previously proposed in these sections. In row 1 areas corresponding to TM, ICL, and ECL of MRP4 are defined for each block (Uniprot accession number: O15439). Rows 5 to 9 highlight the amino acids which have been suggested to be part of the binding pocket of MRP4 (dark grey), MRP5 (dark and light grey), and MRP8 (dark and light grey) [148, 149, 162]. The second column specifies the MRP model studied (MRP4, MRP5 or MRP8), whether it corresponds to the outward model (OUTW) or the inward model (INW) and how it was generated (ICMPocketFinder or cGMP-docking). Regarding the MRP5 and MRP8 models, since two binding pockets have been suggested, different grey scales were used to identify each of them: the extracellular binding pocket 1 and 2 for MRP5 and MRP8 (light grey) and the intracellular binding pocket 2 and 1 for MRP5 and MRP8 (dark grey). Concerning the MRP5 model, the amino acids that belong to both binding sites are identified with both colors (dark and light grey) [149, 162]. Information was also gathered from a bovine inward-MRP1 model constructed with two CEM structures of apo and substrate-bound MRP1 [168]. The residues suggested by this model are indicated in row 18 for each block with the corresponding amino acid name and bMRP1 position. The positive charged pocket (p-pocket) is identified in light grey and the hydrophobic pocket (h-pocket) is identified in dark grey. Rows 13-17 assemble the information about MRP1 transport provided by mutagenesis studies and some natural occurring polymorphisms (MUT) where transport for the following substrates was tested: LTC_4, $E_217\beta G$, E_13SO_4, GSH, and MTX. Information about MRP3 and/or MRP4 transport regarding some assayed substrates obtained by mutagenesis studies were included in some of the blocks (rows 19-22 in Block 5 and rows 19-26 in Block 10). A grey scale code was used to define each situation: when the mutation of the residue affected the substrate transport (black), when the mutation of the residue did not affect the studied substrate transport (light grey), and when no transport studies were performed concerning mutation of a particular residue (white) [121, 150, 169-193]. Finally, the last row indicates which amino acids could be important for cAMP binding in MRP4 according to our analysis. These residues were colored regarding chemical and physical properties as referenced in the figure.

sponding aligned residues in the "MRP4 group" and "MRP1 group" are worth analyzing since both homology models and mutagenesis studies have suggested their participation in substrate transport.

The first position (corresponding to R362 in MRP4) is occupied by a positively charged amino acid with the exception of MRP5 which presents a tyrosine residue. None the less, MRP5 has a positively charged lysine four residues downstream (K448). In the MRP5 outward model, K448 is able of to interact with the phosphate group present in cGMP. Mutagenesis studies in R593 from MRP1 determined this residue is important in transport activity [181]. Regarding the second position (corresponding to L363 in MRP4), F594 of MRP1 was suggested to contact GSH by van der Waals interaction as part of the p-pocket. Mutation analysis of this residue and adjacent residues showed that all three amino acids are important in binding the five most tested MRP1 endogenous substrates. In MRP1, the F594 amino acid, together with some TM11 aromatic residues (W1198, W1246, and Y1243), are thought to form an aromatic region which shows to be important for substrate recognition. In fact, conservative and non-conservative mutations in this residue produce distinct effects regarding the transport of the five classical MRP1 tested substrates. Distinct mutations in F594 disturb substrate transport in different ways, and F594A completely abrogates LTC_4 photo-labeling. These observations indicate that F594 might participate in substrate selectivity during the first stage binding for MRP1 [176] while the corresponding residues in MRP4 (L363) and MRP5 (F445) have been suggested as binding sites of the outward conformation. In the third position (corresponding to T364 in MRP4), the "MRP1 group" has a proline, while the members of the "MRP4 group" show residues with a hydroxyl-group. Particularly, MRP5 has an alanine in this position and a threonine three positions upstream (T444). A proline residue in this position has shown to be important in MRP1 activity, since P595A mutation inhibits LTC_4 transport in 70% and almost eliminates GSH, $E_217\beta G$, MTX, and E_13SO_4 traffic [175]. The striking participation in substrate transport activity of these residues and sequence comparison leads us to suggest that R362, L363, and T364 are interesting residues which ought to

Table 2. MRP4 inhibitors.

Compound	MRP4	Other MRP
Bile acids and conjugates		
Cholic acid	250 (E217βG 30μM) [33] ND (TC 5μM) [4]	
Glycocholic acid	400 (E217βG 30μM) [33] ND (TC 5μM) [4]	ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
Glycolithocholic acid sulfate	10 (E217βG 30μM) [33]	
Lithocholic acid sulfate	10 (E217βG 30μM) [33]	
Taurochenodeoxycholic acid	55 (E217βG 30μM) [33]	
Taurocholic acid	350 (E217βG 30μM) [33] ND (LTB4 0.1μM &) [89] ND (LTC4 0.1μM &) [89]	2200 (cGMP) [206] **MRP5** ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
Taurodeoxycholic acid	60 (E217βG 30μM) [33]	
Taurolithocholic acid	20 (E217βG 30μM) [33]	
Taurolithocholic acid sulfate	10 (E217βG 30μM) [33]	
Ursodeoxycholic acid	ND (TC 5μM) [4]	
Tauroursodeoxycholic acid	ND (TC 5μM) [4]	
Endogenous substances		
cAMP	ND (cGMP 1μM) [7] ND (E217βG 1μM, 30μM) [36, 165] ND (MTX 20μM) [106]	ND (cGMP 1μM) [90] **MRP5** 296 (cGMP 3.3μM) [111] **MRP5** ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
cGMP	ND (E217βG 1μM, 30μM) [36, 165] ND (MTX 20μM) [106]	ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
DHEA-3-glucuronide	80 (DHEAS 0.025μM) [33] 80 (E217βG 1μM) [33]	
DHEAS	3 (E217βG 1μM) [33]	1.5 (cGMP 3.3μM) [207] **MRP5** STIM 10μM (E217βG 1μM) [91] **MRP8**
E217βG	30 (DHEAS 0.025μM) [33] ND (MTX 0.1 μM, 20μM) [7, 106]	0.47 (cGMP) [206] **MRP5** ND (DHEAS 100nM) [91] **MRP8**
Estradiol-3,17-disulfate	0.2 (DHEAS 0.025μM) [33] 2 (E217βG 1μM) [33]	
Estradiol-3-glucuronide	80 (DHEAS 0.025μM) [33] 120 (E217βG 1μM) [33]	
Estradiol-3-sulfate	70 (DHEAS 0.025μM) [33] 50 (E217βG 1μM) [33]	
Estrone-3-sulfate	95 (DHEAS 0.025μM) [33] 45 (E217βG 1μM) [33]	
Hydrocortisone	0.0007 (H)/53 (L) (MTX 0.5μM) [208]	0.0039(H)/788(L) (MTX 0.5μM) [208] **MRP2**
Progesterone		11.7 (cGMP) [206] **MRP5**
Indole-3-acetic acid	2000 (MTX) [209]	
Indoxyl sulfate	1000 (MTX) [209]	
Kynurenic acid	25 (MTX) [209]	

(Table 2) contd….

Compound	MRP4	Other MRP
LTB4	ND; STIM 5μM (LTC4 0.1μM &) [89]	
LTC4	ND (LTB4 0.1μM &) [89]	ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
LTD4	ND (LTB4 0.1μM &) [89] ND (LTC4 0.1μM &) [89]	
PGA1	ND (E217βG 1μM) [79]	ND (E217βG 1μM) [79] **MRP1** STIM 20μM (E217βG 1μM) [79] **MRP2** ND (E217βG 1μM) [79] **MRP3** 1.2 (cGMP 3.3μM) [207] **MRP5**
PGE1	4.1 (cAMP 2μM) [134] ND (E217βG 1μM) [79]	STIM 20μM (E217βG 1μM) [79] **MRP2** 4.2 (cGMP 2μM) [134] **MRP5** 1.8 (cGMP 3.3μM) [207] **MRP5**
PGE2	2.7 (cAMP 2μM) [134] ND (E217βG 1μM) [79] ND (LTB4 0.1μM &) [89]	STIM 20μM (E217βG 1μM) [165] **MRP2** 4.4 (cGMP 2μM) [134] **MRP5**
PGF1α	ND (E217βG 1μM) [79]	STIM 20μM (E217βG 1μM) [79] **MRP2**
PGF2α	ND (E217βG 1μM) [79]	STIM 20μM (E217βG 1μM) [79] **MRP2**
TXB2	ND (E217βG 1μM) [79]	ND (E217βG 1μM) [79] **MRP1** STIM 20μM (E217βG 1μM) [79] **MRP2** ND (E217βG 1μM) [79] **MRP3**
Hippuric acid	2500 (MTX) [209]	
Uric acid	NO INH (cAMP 1μM) [109] STIM (cGMP 10μM) [109] 235 (MTX 1μM) [109] ND (PAH 100μM) [8]	
NSAID and metabolites		
Celecoxib	ND (E217βG 1μM) [79] 35 (MTX 0.5μM) [164] ND (PGE2 0.02μM) [195]	100 (MTX 0.5μM) [164] **MRP2**
Diclofenac	ND (E217βG 1μM) [79] 0.006 (H)/326 (L) (MTX 1μM) [164] 332 (MTX 1μM) [210]	ND (E217βG 1μM) [79] **MRP1** 97 (MTX 0.5μM) [164] **MRP2** 139 (MTX 1μM) [210] **MRP2**
Diclofenac glucuronide	140 (MTX 1μM) [210]	18.6 (MTX 1μM) [210] **MRP2**
Etodolac	120 (MTX 0.5μM) [164]	480 (MTX 0.5μM) [164] **MRP2**
Flurbiprofen	ND (E217βG 1μM) [79]	
R-Flurbiprofen	10.6 (MTX 1μM) [210]	133 (MTX 1μM) [210] **MRP2**
S-Flurbiprofen	37.2 (MTX 1μM) [210]	58.4 (MTX 1μM) [210] **MRP2**
R-Flurbiprofen glucuronide	3.24 (MTX 1μM) [210]	29.5 (MTX 1μM) [210] **MRP2**
S-Flurbiprofen glucuronide	93 (MTX 1μM) [210]	21.5 (MTX 1μM) [210] **MRP2**
Ibuprofen	ND (E217βG 1μM) [79] 26.3 (MTX 0.5μM) [164] ND (PGE2 0.02μM) [195]	ND (E217βG 1μM) [79] **MRP1** 930 (MTX 0.5μM) [164] **MRP2**
R-Ibuprofen	129 (MTX 1μM) [210]	303 (MTX 1μM) [210] **MRP2**
S-Ibuprofen	267 (MTX 1μM) [210]	139 (MTX 1μM) [210] **MRP2**
R-Ibuprofen glucuronide	3.6 (MTX 1μM) [210]	208 (MTX 1μM) [210] **MRP2**
S-Ibuprofen glucuronide	66.6 (MTX 1μM) [210]	80.9 (MTX 1μM) [210] **MRP2**

(Table 2) contd….

Compound	MRP4	Other MRP
Indomethacin	ND (DHEAS 2μM) [211] ND (E217βG 1μM) [79] ND (LTB4 0.1μM &) [89] 6.1 (MTX 0.5μM) [164] ND (PGE2 0.02μM) [195] ND (PMEA 1μM) [212]	ND (E217βG 1μM) [79] **MRP1** 0.06 (H)/46 (L) (MTX 0.5μM) [164] **MRP2** ND (E217βG 10μM) [211] **MRP3** 22 (cGMP 2μM) [45] **MRP5** ND (MTX 100μM) [213] **MRP5** ND (PMEA 1μM) [212] **MRP5** ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
Indoprofen	ND (E217βG 1μM) [79]	
Ketoprofen	ND (E217βG 1μM) [79] 11.9 (MTX 0.5μM) [164] ND (PGE2 0.02μM) [195]	1.4 (H)/470 (L) (MTX 0.5μM) [164] **MRP2**
Naproxen	42.3 (MTX 0.5μM) [164]	609 (MTX 0.5μM) [164] **MRP2**
R-Naproxen	8.06 (MTX 1μM) [210]	510 (MTX 1μM) [210] **MRP2**
S-Naproxen	49.8 (MTX 1μM) [210]	7.11 (MTX 1μM) [210] **MRP2**
R-Naproxen glucuronide	1.63 (MTX 1μM) [210]	771 (MTX 1μM) [210] **MRP2**
S-Naproxen glucuronide	48.7 (MTX 1μM) [210]	475 (MTX 1μM) [210] **MRP2**
Phenylbutazone	130 (MTX 0.5μM) [164]	605 (MTX 0.5μM)/STIM 1μM [164] **MRP2**
Piroxicam	216 (MTX 0.5μM) [164]	257 (MTX 0.5μM) [164] **MRP2**
Rofecoxib	ND(E217βG 1μM) [79]	
Salicylic acid	ND (DHEAS 2μM) [211] 1500 (MTX 0.5μM) [164] 2.1(H)/1547(L) (Urate 100μM) [214]	1760 (MTX 0.5μM) [164] **MRP2** NI (E217βG 10μM) [211] **MRP3**
Sulfasalazine	ND (DHEAS 2μM) [211]	ND (E217βG 10μM) [211] **MRP3**
Sulindac	ND (DHEAS 2μM) [211] ND (LTB4 0.1μM &) [89] ND (LTC4 0.1μM &) [89] 2.11 (MTX 0.5μM) [164]	38 (MTX 0.5μM) [164] **MRP2** ND (E217βG 10μM) [211] **MRP3**
Tolmetin	20.5 (MTX 0.5μM) [164]	494 (MTX 0.5μM) [164] **MRP2**
PDE inhibitors		
Dipyridamole	5.5 (cAMP) [215] ND (cGMP 1μM) [7] ND (PGE2 0.02μM) [195] 2 (PMEA 1μM) [165] ND (LTB4 0.1μM &) [89] ND (PAH 100μM) [8] ND (TC 5μM) [4]	ND (PAH 100μM) [8] **MRP2** 12; 1.2; 0.35 (cGMP) [45, 206, 215] **MRP5** ND (MTX 100μM) [213] **MRP5** 30 (PMEA 1μM) [165] **MRP5**
IS-39213	0.16 (cAMP 2μM) [134]	0.17 (cGMP 2μM) [134] **MRP5**
IS-60049	0.35 (cAMP 2μM) [134]	0.16 (cGMP 2μM) [134] **MRP5**
PHAR0099048	2 (cAMP 2μM) [134]	0.52 (cGMP 2μM) [134] **MRP5**
Sildenafil	3.8 (cAMP 2μM) [134] ND (E217βG 30μM) [165] ND (MTX 20μM) [106] 20 (PMEA 1μM) [165]	80 (PMEA 1μM) [165] **MRP5** 2.9 (cGMP 2μM) [134] **MRP5** 0.26, 3.6, 1.2 (cGMP) [90, 206, 216] **MRP5** ND (E217βG 1μM) [91] **MRP8** ND (DHEAS 100nM) [91] **MRP8**
Tadalafil	194 (cAMP) [215]	14.1 (cGMP) [215] **MRP5**

(Table 2) contd….

Compound	MRP4	Other MRP
Trequinsin	ND(E217βG 30μM) [165] ND (MTX 20μM) [106] 10 (PMEA 1μM) [165]	30 (PMEA 1μM) [165] **MRP5** 0.24 (cGMP) [90] **MRP5**
Vardenafil	3.4 (cAMP) [215]	0.62 (cGMP) [215] **MRP5**
Zaprinast	2.8 (cAMP) [215] ND (E217βG 30μM) [165] ND (MTX 20μM) [106] 250 (PMEA 1μM) [165]	0.68; 0.35 (cGMP) [206, 215] **MRP5** ND (MTX 100μM) [213] **MRP5** 250 (PMEA 1μM) [165] **MRP5**
Experimental drugs		
Ceefourin 1	ND (E217βG 1μM) [217]	
Ceefourin 2	ND (E217βG 1μM) [217]	
Ceefourin 3	ND (E217βG 1μM) [217]	
IBMX	16.2 (cAMP) [215] ND (PMEA 1μM) [128]	10 (cGMP) [215] **MRP5** NI (PMEA 1μM) [128] **MRP5**
MK571 (Verlukast)	2.1 (cGMP 3.3μM) [207] 2.1 (E217βG 30μM) [218] 9.8 (LTB4 &) [89] ND (LTC4 0.1μM) [89] ND (MTX 20μM) [106] ND (PAH 100μM) [8] 10 (PMEA 1μM) [165] ND (TC 5μM) [4]	1.8 (E217βG 0.4μM) [218] **MRP1** 2.7 (LTC4 0.05μM) [218] **MRP1** 21.2; 1# (E217βG 0.4μM) [218] **MRP2** ND (PAH 100μM)/STIM 10μM [8] **MRP2** ND (E217βG 1μM) [218] **MRP3** 43 (cGMP 2μM) [45] **MRP5** 0.38; 0.41 (cGMP 3.3μM) [111, 207] **MRP5** ND (MTX 100μM) [213] **MRP5** 40 (PMEA 1μM) [165] **MRP5** ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**
Other drugs		
Misoprostol	4.5 (cAMP 2μM) [134]	24.5 (cGMP 2μM) [134] **MRP5**
Probenecid	ND (cGMP 1μM) [7] ND (LTB4 0.1μM &) [89] ND (MTX 20μM) [106] ND (PAH 100μM) [8] 2300 (PMEA 1μM) [165] ND (TC 5μM) [4] 132 (Urate 100μM) [214]	ND; STIM 10μM (PAH 100μM) [8] **MRP2** ND (E217βG 10μM) [207] **MRP3** ND (cGMP 1μM) [90] **MRP5** ND (cGMP 3.3μM) [111] **MRP5** ND (MTX 100μM) [213] **MRP5** 200 (PMEA 1μM) [165] **MRP5** ND (DHEAS 100nM) [91] **MRP8** ND (E217βG 1μM) [91] **MRP8**

Table 2. Records of the inhibitory activity of different chemical substances obtained from *in vitro* experiments. Data was selected from rapid vesicle filtering experiments carried out in MRP-overexpressing systems, with the exception of PMEA transport assays that were performed in whole MRP-overexpressing cells that were incubated with the indicated concentration of its precursor bis-POM-PMEA (bis-pivaloyloxymethyl-PMEA). Numerical values indicate the IC50, Ki (inhibition constant) or SC50 (half-maximal stimulation concentration, tagged with the # character) values expressed in the μM order. The substrate of reference for each value is indicated in parenthesis, followed by its tested concentration with the exception of Ki values. Compounds were classified as not determined (ND) when the latter parameter values were not available, but were shown to inhibit MRP-mediated transport of a given substrate by more than 20% at the tested concentration. Compounds that failed to display inhibitory effects in the transport of a given substrate in dose-response assays were classified as non-inhibitors (NI). Blank cells correspond to cases where a fixed concentration of the inhibitor was tested and no inhibition was observed or where there is no available data. STIM: stimulates transport by more than 20% at the indicated concentration, &: transport experiment performed in the presence of 5mM GSH. IBMX: 3-isobutyl-1-methylxanthine; TC: taurocholate; PAH: p-aminohippurate.

be further analyzed regarding cAMP transport by MRP4 (Fig. **2, 3**).

Another section of the MRP4 TM6 and ICL3 was studied using point mutations and measuring the resulting kinetic parameters over several transported substrates in MRP4 [172, 191]. Additional rows were added in this alignment in order to illustrate the MRP4 mutagenesis studies (Fig. **2**, Block 2), being F368 the most exhaustively investigated residue. Mutations in this site have shown to reduce transport of cGMP, folate, $E_2$17βG, and MTX, but kinetic parameter analysis

has shown that this reduction may be caused by different factors. To start with, mutations on F368 directly affect MRP4 Km for cGMP, *i.e.* they affect the binding of this substrate to the high affinity sites of the inward state. While F368Y reduces the Km and augments cGMP transport, F368W increases the Km and diminishes this substrates' traffic. Regarding other endogenous molecules, mutations on F368 always modified the Vmax without changing the Km. These observations imply that this amino acid intervenes in the first stage binding of certain substrates and is involved in the second or third stage binding of other transported molecules. Also, since F445 contacts with the purine base of cGMP in docking experiments in the outward model [148], the neighboring phenylalanine set of residues in MRP4 (F368 and F369) and in MRP5 (F432 and F433) could be fulfilling the same role regarding cyclic nucleotide transport.

Similarly, R375 (located in ICL3) appears to have a substrate specific effect as well. R375S has practically no effect on cGMP transport, does not change the Km and mildly increases the Vmax, diminishing MTX transport by augmenting the Km without modifying the Vmax. Therefore, MRP4 R375 seems to have a role in MTX binding at the high affinity site in the inward conformation. cGMP and MTX transport have also been tested in MRP4 F369-, E374S, and E378Q and they all showed very little transport activity levels. Forthcoming studies using these mutants should include determining the kinetic parameters for some of the already tested substrates and extending the study to other transported molecules [172]. The replacement of MRP1 S604 in the ECL3 (conserved in all members of the "MRP1 group" and aligned with MRP4 E374) for alanine only showed an increase in $E_2$17βG transport but did not affect GSH and LTC_4 traffic [190]. These evidences, together with the sequence alignment analysis, allowed us to propose F368, F369, E374, E375, and E378 as candidate amino acids that may be involved in cAMP binding and transport by MRP4 (Fig. **2, 3**).

In **TM7** and **ECL4** (Fig. **2**, Block 6), predicted positions of all MRP4, MRP5, and MRP8 models overlap significantly. Almost all sites predicted by the MRP5 outward model and the inward and outward MRP8 models correspond to the intracellular binding pocket. Notably, the MRP8 inward model proposes three amino acids which correspond to the extracellular binding pocket. On the other hand, the compilation of the MRP1 mutagenesis studies did not yield many results regarding the substrate recognition sites [175]. However, one particular MRP1 polymorphism is worth mentioning: A989T inhibits 50% of $E_2$17βG transport and 25% of MTX transport, but maintains LTC_4 transport. Kinetic analysis shows that an increase in the Km is responsible for the reduction of $E_2$17βG transport [171]. Interestingly, MRP4 has a tyrosine in that position and MRP5 and MRP8 also have aromatic residues two amino acids downstream. Another interesting discovery when analyzing TM7 proximity corresponds to the MRP4 extracellular loop (ECL4). A positively charged amino acid is found in both MRP4 (K741) and MRP5 (K885) in this site, whereas the "MRP1 group" and MRP8 (E832) depict a negatively charged residue. Moreover, the MRP8 inward model suggested the proximate L831 as a binding site. Finally, while, MRP4 and MRP5 present a small aliphatic residue in this site (A739 in MRP4 and I884 in MRP5), all the members of the "MRP1 group" have a tyrosine residue.

These aspects, though not experimentally addressed, could be related to differential substrate selectivity between both groups of transporters. We propose Y728, A739, and K741 as candidate sites for cAMP binding although further studies should be performed in order to determine in which binding stage they participate (Fig. **2, 3**).

Unlike the TM7, the candidate residues in **TM8** (Fig. **2**, Block 7) suggested by the homology models do not overlap among the members of the "MRP4 group". Notably, most of all the predicted binding sites locate in the section between L777 and Y788 in MRP4 and correspond to the intracellular binding pocket of MRP5 and MRP8. Additionally, only a few mutagenesis studies have been performed in this particular section of the MRP. Notably, MRP1 R1046D (ICL4) presents similar $E_2$17βG, E_1S0_4, and MTX transport parameters to those of the wild type variant, but has a 40% reduction in LTC_4 transport, while GSH traffic has not been tested yet [185]. Also, a natural occurring polymorphism (C1047S) reduced 10-20% $E_2$17βG and LTC_4 transport, though no changes were observed in MTX or GSH transport [170, 171]. Although information regarding TM8 and the ICL4 adjacent portion is limited, we suggest that the region comprising G779 to R782 in MRP4, as well as the high density of the hydroxyl-group containing residues in ICL4, which is present in all the members of the "MRP4 group", are potential cyclic nucleotide binding sites which need further experimental confirmation (Fig. **2, 3**).

TM9 (Fig. **2**, Block 8) participation on binding pockets has been suggested for MRP1, MRP4, and MRP8 by the presented models, but not for MRP5. Regarding MRP8, amino acids participating in substrate

binding in the outward conformation are located mostly in the intracellular part of TM9 and in its most proximal ICL4 section, while sites involved in binding in the inward conformation are mostly positioned in the extracellular section of TM9. The candidate residues predicted by the MRP8 inward models, the MRP1 inward CEM structure and the MRP4 outward model significantly overlap. When focusing on these sites, one notorious difference between the "MRP1 group" and the "MRP4 group" is in positions L841 and D842 in MRP4 where the "MRP1 group" presents a positively charged (MRP1 and MRP2) or neutral (MRP3) residue, and cyclic nucleotide transporters depict a negatively charged residue. It has been suggested that this negative charge is part of the binding pocket of MRP4 (D842) and the intracellular binding site of MRP8 (E991), while in the bMRP1 CEM structure, M1092 (corresponding to hMRP1 M109) has been proposed to be part of the h-pocket. This positive charged residue is not important for MRP1 substrate recognition, since K1092A, K1092E, and K1092R do not affect GSH, LTC_4, or $E_217\beta G$ transport. However, a negative charged residue could still be key for the cyclic nucleotide MRP transporters binding pocket. Based on all this evidence, together with the sequence alignment analysis, we propose that the MRP4 D842 residue could be an important site for cyclic nucleotide binding (Fig. **2, 3**).

Near the terminal section of the intracellular loop (ICL5) and at the beginning of the **TM11** (Fig. **2**, Block 9), the suggested sites of the MRP5 extracellular binding pocket overlap with those suggested for the MRP4 binding pocket in the outward model. In contrast, the sites suggested for the MRP8 extracellular binding site, both in the inward and the outward models, are located within the TM11. The proposed binding sites A957 of MRP4 and I1107 of MRP5 greatly diverge from the asparagine residues present in all members of the "MRP1 group". Mutation of this residue in MRP1 (N1208A) has shown similar LTC_4, GSH, and $E_217\beta G$ transport levels to those of the wild type variant [192]. The A957 residue may participate in cyclic nucleotide binding, while it may not determine binding of LTC_4, GSH, and $E_217\beta G$, which are all transported by MRP1 and MRP4. Also, D953 of MRP4 and D1103 of MRP5 have been proposed as binding sites in the corresponding homology models. Non-conservative mutations in the equivalent MRP1 residue (E1204L) have shown to diminish LTC_4, $E_217\beta G$, GSH, and E_13SO_4 transport without affecting MTX traffic. MRP1 conservative mutation (E1204D), where the residue was replaced for the one present in MRP4 and MRP5, showed similar or increased levels of $E_217\beta G$, LTC_4, MTX, and E_13SO_4 transport, but significantly reduced the activity of GSH transport. Since photo-labeling with LTC_4 of E1204L was comparable to the wild type variant, E1204 has been postulated as a non-essential residue for LTC_4 first high affinity binding, but could be involved in the transport process subsequent to binding, including, for example, signaling between the TM and the NBD [185].

Among these outward-binding sites, we propose MRP4 C956 as an interesting site to analyze cyclic nucleotide binding involvement, since this position is occupied by S1106 in MRP5 and by T1054 in MRP8 (which are also hydrogen bonding residues), while a glycine is positioned in this site in all members of the "MRP1 group". In fact, C956S is a natural occurring polymorphic variant in MRP4 and shows an increased function regarding 9-(2-phosphonylme-thoxyethyl) adenine (PMEA) transport [195]. Lastly, the only site near this TM proposed by the bMRP1 CEM structure is R1196, which corresponds to R1197 in MRP1 and is thought to participate in the p-pocket. Conservative and non-conservative mutations of this residue have shown to disrupt MRP1 activity for all substrates and to abrogate LTC_4 photo-labeling [185]. Given that this residue is conserved in all MRP members (R946 in MRP4), it is probably essential for the binding of most substrates. Based on this evidence, together with the sequence alignment analysis, we propose that R946, C956, and A957 are interesting residues to analyze cAMP binding (Fig. **2, 3**).

TM12 (Fig. **2**, Block 10) is particularly interesting because many of the mutagenesis experiments have been carried out not only with MRP1, but with MRP3 and MRP4 as well. One interesting site suggested from the MRP8 inward model and mutagenesis studies from MRP1 and MRP3 corresponds to the MRP4 A982 equivalent residue. An alanine residue is conserved on this site, both for MRP5 and MRP8, while a serine is conserved in the "MRP1 group", with the exception of MRP2 that depicts a valine. Mutation on MRP1 S1233A does not present significant differences regarding LTC_4 or $E_217\beta G$ transport [188], while the corresponding S1229A in MRP3 reduces 50% MTX transport without affecting the $E_217\beta G$ and taurocholate traffic.

The MRP8 inward model also proposes T1094 and I1097 as candidate binding residues. Both are located at the end of TM12, right at the beginning of the N-terminal section, and correspond to the intracellular binding pocket. These amino acids are aligned with

C996 and Q999 of MRP4 and with T1146 and L1149 of MRP5. All the members of the "MRP1 group" present a conserved leucine in the first site (except for MRP3) and a methionine in the second site. Despite no mutagenesis studies have been performed for these particular residues, adjacent residues have been replaced and transport was monitored in MRP1-4 [121, 182, 185, 186, 191, 196]. One of these amino acids corresponds to the conserved MRP1 W1246, and together with its matching bMRP1 W1245, it has been suggested to participate in the h-pocket. This residue is aligned with MRP3 W1242 and MRP4 W995 [121, 191] and mutating the site impairs the transport of at least one substrate. When the transport of more than one substrate is compromised, the mutation is thought to affect different steps of the transport mechanism, depending on the substrate. This is well described for MRP4: W995F impairs $E_2$17βG and folic acid transport by an increment in the Km and a decrease in the Vmax, while a reduction in MTX transport is due to a lower Vmax. Finally, this mutation also reduces cGMP transport by a reduction of the Vmax, but as cGMP Km also diminishes, it helps the binding process. W995Y in MRP4 produces a diminished transport of all tested substrates by different modifications of its kinetic constants [191]. This supports the notion that one residue can affect the first stage binding of a certain substrate and also participate in the translocation or release processes of another substrate. In fact, some of the binding sites which are predicted by models in the inward conformation overlap with the binding sites predicted by the outward conformation models. Further mutagenesis studies confirmed that MRP4 R998 site determines the transport of all the tested substrates, including cGMP [191]. Corresponding aligned residue in bMRP1 R1248 has been suggested to be part of the p-pocket and, in this case, this result was supported by mutagenesis experiments performed in MRP1, where R1249D and R1249K diminished $E_2$17βG, LTC_4, MTX, and $E_1$3$S0_4$ transport. Also, R1249K mutant abrogates LTC_4 photolabeling, which suggests that the residue is associated to this substrate binding [185].

Finally, regarding the outward models, we propose that MRP4 F993 and Q994 would be interesting sites to further analyze as probable cyclic nucleotide binding residues. F993 has not been suggested as a MRP4 binding site, but aligned F1091 was suggested for MRP8 as an outward binding site in cGMP-docking experiments. Further, MRP4, MRP5, and MRP8 show a phenylalanine in this position, while a leucine is conserved in all members of the "MRP1 group". Also, MRP4 Q994 and MRP5 Q1144 have been proposed as part of the binding pocket for both transporters. In fact, docking experiments revealed that the cGMP purine interacts with MRP5 Q1144 in several binding sites. This position is occupied by an asparagine in MRP4, MRP5, and MRP8, while its higher order homolog, glutamine, is particularly conserved in the "MRP1 group". In fact, in the MRP1 CEM structure, the corresponding bMRP1 N1244 has been suggested to participate in the p-pocket. Mutation of this residue in MRP1 (N1245A) does not alter LTC_4 transport, but impairs $E_2$17βG traffic by increasing the Km and reducing the Vmax [188]. Also, MRP3 N1241A reduces $E_2$17βG and MTX transport, but has no significant change in taurocholate traffic [196]. Summarizing, candidate amino acids in this block for cAMP binding are A982, F993, Q994, W995, R998, and Q999 (Fig. **2, 3**).

In order to confirm the localization of our MRP4 candidate residues, we constructed two protein homology models in the inward and outward conformation (Fig. **3**). Most of the proposed residues have their side chains oriented towards the cavity of the protein in at least one of the models. This means the amino acids are exposed to the region which allows interaction with potential substrates. However, some residues have their side chains orientated to the alpha helix core, meaning that their participation in substrate binding occurs during an intermediate transition state or that they somehow determine the packing of the alpha helixes which in turn influences the exposition of other residues. Taking in consideration that both MRP5 and MRP8 homology models describe two recognition sites for each transporter [148, 149] and that the lateral side chains of the candidate residues identified in our MRP4 model group in two spatially proximate, yet distant clusters, we propose two distinct MRP4 cAMP binding sites. In (Fig. **3**), recognition site 1 and 2 are represented in green and purple, respectively, within the models and (Fig. **3C**) depicts the potential residues of each site. We included other residues that although were not identified by our analysis, are located in the vicinity of both recognition sites and their residue side chains are exposed in such a way they allow the interaction with potential substrates. Interestingly, as MRP4 passes from the inward to the outward conformation, at least one positively charged residue is lost in each binding site (R946 for binding site 1 and R362 and R998 for binding site 2) due to changes in the orientation of the side chain. Furthermore, H153 present in binding site 1 may also have a positive charge and is available for binding in the inward model and hidden within the alpha helix core in the outward model. In order to transport cAMP in an efficient way,

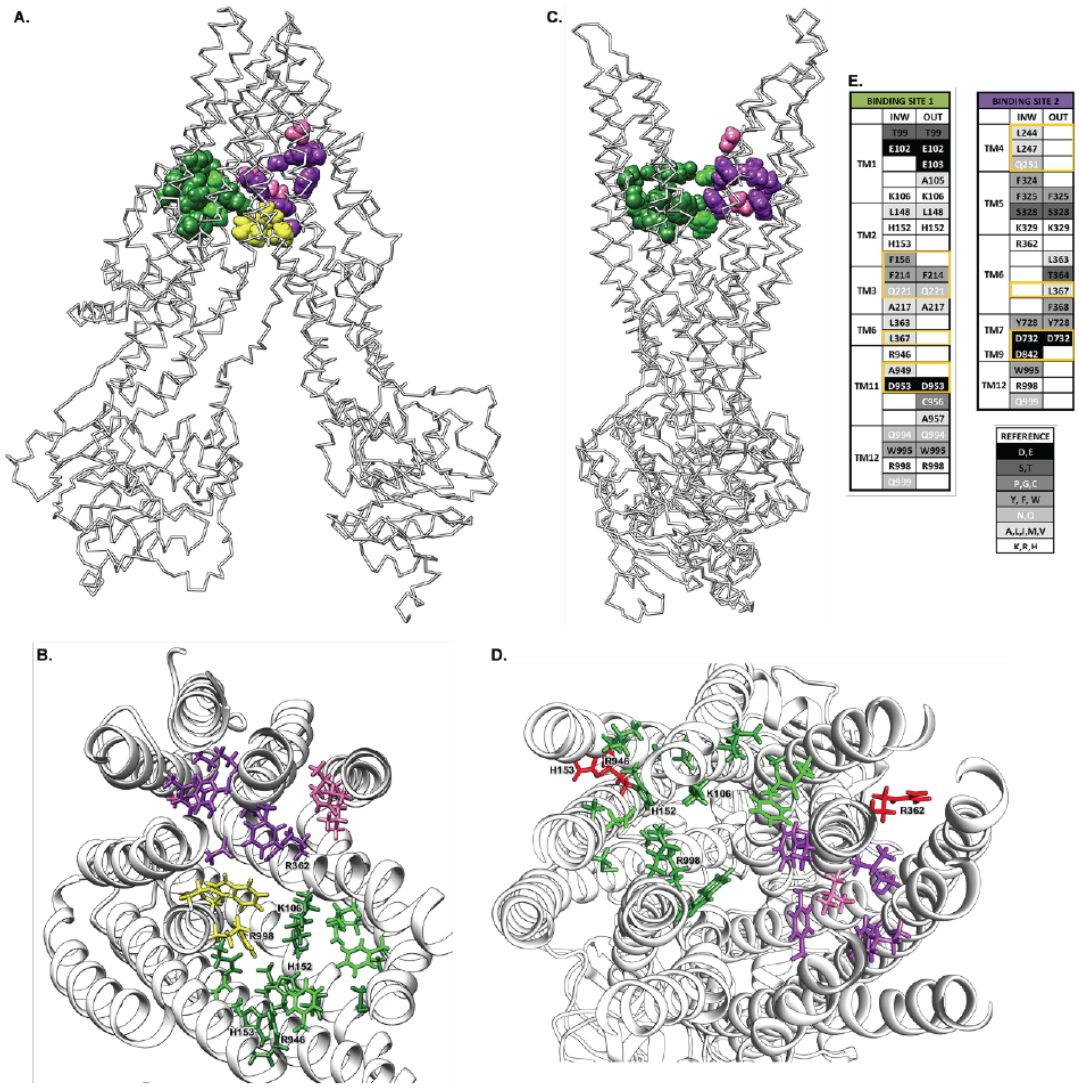


Fig. (3). Proposed cAMP binding sites in MRP4 inward and outward models. (A and B) Side view and cytosolic view of MPR4 inward model. (C and D) Side and extracellular view of MRP4 outward model. Inward model was constructed using Swiss-Model [197] and apo-bMRP1 (INW, #5UJ9) as template [168]. Outward model was kindly provided by Franz Russell [172]. Both models were then refined using the 3D-Refine server [198], followed by energy minimization using CHARMM36 force field implemented in NAMD 2.9 [199, 200]. The latter was performed for the side chains using water as an implicit solvent and leaving the backbone atoms fixed until the total energy of the system was stabilized. Model quality was then assessed by QMEAN [201], DOPE implemented in MODELLER [202] and Ramachandran values analysis [203]. (A-D) The residues which were suggested by the analysis presented in Figure 2 that correspond to binding sites 1 and 2 are represented in dark green and purple, respectively. We included other residues in sites 1 (light green) and 2 (pink) that although were not identified by our analysis, are located in the vicinity of both recognition sites and their residue side chains are exposed in such a way they allow the interaction with potential substrates (E: square in yellow). (A,B) Supplementary residues that could be participating in binding sites 1 and 2 in the inward model (yellow). (B and D) Positive charged residues corresponding to each binding site are labeled. (E) Table representing the proposed residues involved in cAMP binding in sites 1 and 2 in the MRP4 inward and outward conformations.

the cyclic nucleotide should bind to a high affinity site in the inward conformation and be released from a low affinity site in the outward conformation. We propose that, in the inward conformation, the negative charge of the cAMP phosphate group is stabilized by some of these positively charged residues which are later lost in the outward conformation, thus the interaction is destabilized favoring the release of cAMP in the extracellular side. In fact, in MRP4 cGMP docking experiments, K448 has shown an interaction with the

cGMP phosphate group. When analyzing the alignment sequence, the closest positive charge in MRP4 is R362. We recommend that suggested cAMP sites need further confirmation with mutagenesis studies to test both their involvement in cAMP transport and their importance in substrate selectivity.

7. MRP4 SELECTIVE INHIBITORS

The knowledge of the structural characteristics of MRP4 substrates and their specific binding provides a starting point for the design of selective inhibitors. That is, given that MRP4 has multiple substrate binding sites, the possibility of designing inhibitors that might impair its transport activity concerning one or more substrates but do not affect the transport of others would be ideal. At the same time, one of the problems to be faced during drug design is the appearance of undesired off-target effects. Ideally, a selective inhibitor should have activity on MRP4 but not on other ABC transporters or other molecular targets in the organism. The search for ABC transporter inhibitors has especially focused on MDR1, MRP1, and BCRP (ABCB1, ABCC1, ABCG2, respectively), which are the most studied transporters regarding multidrug resistance. The majority of these studies intended to find a co-adjuvant inhibitor which could block the transport of a given drug in order to increase its bioavailability. Unfortunately, these inhibitors were not successful in clinical trials due to their low potency and off-target action on other ABC or molecular targets, such as cytochrome P450 enzymes. Besides, as was reviewed in detail by Yu *et al.* [204], some of these inhibitors show adverse side-effects as they also block the transport of endogenous substrates in sites where this activity is physiologically relevant. It is also important to mention that the possibility of drug-drug interactions between specific MRP4 inhibitors and the primary therapy of a particular disease should also be taken into account, since they may lead to enhanced toxicity and/or unexpected side effects[205].

One of the available approaches that could be useful for the identification of MRP4 inhibitors is to focus on safe drugs that are already in the market but are used for other applications, a strategy also known as drug repositioning. Ideally, the candidate drug should have high specificity and inhibit a particular substrate transport with high efficiency (low IC50), without significantly affecting the traffic of another. As was previously mentioned, many endogenous and exogenous molecules of diverse chemical structure have been demonstrated to inhibit MRP4-dependent transport of a particular substrate (Table **2**). In this section, we will focus on the pharmacological classes that have a high safety profile, present few adverse effects, and show MRP4 inhibitory activity.

In this regard, one of the most studied drug classes in relation to MRP4 transport inhibition are the non-steroidal anti-inflammatory drugs (NSAID). In particular, when analyzing MRP4-mediated MTX transport inhibition, sulindac and indomethacin appeared to be the most potent inhibitors. But if both efficacy and selectivity are taken into account, the best MRP4 inhibitors are ibuprofen and tolmetin due to their low IC50 values for MRP4 MTX transport and high IC50 values for MRP2 MTX transport [164]. In a recent publication, the influence of NSAID stereochemistry and glucuronidation on their ability to inhibit MRP4- and MRP2-mediated MTX transport was studied. Interestingly, the authors showed that the *R*-stereoisomers and the respective glucuronidated derivatives of ibuprofen, flurbiprofen, and naproxen are better inhibitors than their *S*-antipodes or aglycone molecules in a MRP4 selective manner [199]. On the other hand, when NSAID were tested regarding MRP4- and MRP1-mediated $E_2$17βG transport, flurbiprofen, indoprofen and ketoprofen were found to be better at inhibiting MRP4 than MRP1, with indoprofen as the most potent inhibitor of the set. Furthermore, diclofenac, rofecoxib, and celecoxib showed poor inhibition of $E_2$17βG transport by both transporters [79]. Also, inhibition of MRP4-mediated PGE_2 transport was observed in the cases of indomethacin, ibuprofen, ketoprofen, celecoxib [195], and *R*-flurbiprofen [219]. It is worth noting that indomethacin, one of the most tested drugs in the NSAID class regarding MRP, was also shown to inhibit MRP4 transport of DHEAS and LTB_4, the latter in the presence of GSH, as well as other substrates shared with MRP5 and MRP8. Remarkably, the most structurally simple drug within the NSAID class, salicylic acid showed poor MRP4 inhibition of MTX and uric acid transport and no inhibition of $E_2$17βG MRP3-mediated transport. Altogether, these results suggest that NSAID are a promising starting point for the identification and development of potential selective inhibitors.

Another interesting pharmacological class with proven inhibitory efficacy on MRP4 group-dependent transport consists of PDE inhibitors. Among this class, trequinsin showed to be the most active MRP4 inhibitor regarding MTX transport when compared to zaprinast and sildenafil [108]. In another study, trequinsin and sildenafil inhibited PMEA transport medi-

ated by both MRP4 and MRP5 with similar IC50 values, while zaprinast demonstrated to be a weaker inhibitor for both substrates and transporters [79]. As expected, given that the natural substrates of PDE are cyclic nucleotides, the strongest inhibition was observed regarding cAMP and cGMP transport. In these cases, sildenafil and its derivatives, IS-39213 and IS-60049, showed similar inhibitory potency regarding the transport of both cyclic nucleotides. Conversely, sildenafil derivate PHAR0099048, tadalafil, vardenafil, and zaprinast inhibited cGMP efflux more powerfully than cAMP efflux, which indicates their preference for MRP5 [134]. Notably, tadalafil is the weakest inhibitor of MRP4 cAMP transport but still shows selectivity over MRP5 cGMP traffic by MRP5 [215]. Dipyridamole, a rather general PDE inhibitor, was also tested in inhibition experiments of various substrates and demonstrated to inhibit cAMP, PMEA, and cGMP transport with high efficiency [7, 107, 215], while it presented poor inhibition of MRP4 taurocholate co-transport with GSH [4]. Also, high concentrations of dipyridamole (50μM) have been shown to inhibit PGE_2, $PGF_{2\alpha}$, and TXB_2 transport by MRP4 [146]. Overall, the fact that most of the PDE inhibitors studied present selectivity over the PDE5 isoform and have also shown greater selectivity towards the inhibition of cGMP transport suggests that these drugs would be useful for the development of cGMP rather than cAMP transport inhibitors.

Interestingly, misoprostol, a semi-synthetic analogue of PGE_1 used in prevention and treatment of gastric and duodenal ulcers, has shown to inhibit cAMP transport with an IC50 five-fold lower than that for cGMP transport in inside-out vesicles of human erythrocytes [134]. This indicates that this drug could preferentially inhibit MRP4 over MRP5, although it has not been yet tested against other substrates.

Probenecid and MK571 are also MRP4 inhibitors and have been widely used for research purposes. The former is mainly an urate-anion-exchanger blocker, but also acts on several ABC transporters, inhibiting the transport of multiple substrates of MRP1, MRP4, MRP5, and MRP6 with low potency [220]. On the other hand, MK571 is a potent LTD_4 antagonist that also inhibits MRP1, MRP2, MRP3, and MRP5 effectively. Interestingly, both drugs have also been found to inhibit cAMP- and cGMP-PDE activity and, in this regard, MK571 also appears to be more potent than probenecid [139].

Another strategy that could be advantageous for the identification of candidate compounds for MRP4 inhibition is high-throughput screening (HTS), as it provides experimental information regarding a vast number of structurally diverse compounds at the same time and can also be applied in drug repositioning approaches. Three potent MRP4 inhibitors have been identified using this method: ceefourin 1, ceefourin 2, and ceefourin 3. The first two have been deeply characterized regarding selectivity given that they showed to be non-toxic for a broad spectrum of cell types. In subsequent drug resistance assays, ceefourin 1 and 2 showed no inhibition of MDR1, MRP2, MRP1, MRP3, and MRP5, and only a moderate inhibition of ABCG2. Besides, both inhibitors showed different relative potency regarding the tested substrates. However, comparison is challenging because not the same techniques were used to measure substrate transport [217]. Another HTS assayed D-luciferin transport by MRP4 as a tool to identify new specific inhibitors from a library of FDA approved drugs. Numerous compounds were identified, but only four were tested regarding their capability of sensitizing cells to 6-mercaptopurine and SN-38 (an active metabolite of irinotecan), which are both MRP4 substrates. Glafenine, AG1478, prazosin, dantrolene, and nalidixic acid showed distinct degrees of sensitization for each cytotoxic agent, demonstrating some substrate selectivity regarding both tested drugs [221]. A recent patent has identified several compounds capable of inhibiting an Alexa-flour-cAMP derivative transport [222]. The authors claimed that MRP4 inhibition of cAMP transport could be achieved with these compounds although no selectivity studies were performed. These sort of studies are vital because they help to foresee the potential adverse effects that those compounds may cause. In this regard, a study including different bile-salt-export-pump (BSEP) non-inhibitors demonstrated that blocking MRP4-mediated DHEAS transport correlates in most cases with the cholestatic potential of the tested drug [211], thus suggesting that this is one of the aspects that should be analyzed when testing MRP4 inhibitors, especially when HTS or virtual HTS studies are plausible.

A complementary strategy to those previously mentioned for the design of inhibitors in cases where little is known about the three-dimensional structure of a given molecular target and its binding sites is ligand-based drug design. This methodology has been proven successful in the case of drug transporters, where the availability of multiple binding sites and the chemical diversity of their substrates and inhibitors make direct approaches difficult [223].

In this matter, three pharmacophore models regarding MRP4 drug-interaction have been generated to date. The first two were described by Fukuda *et al*. [224] and were developed based on either a set of five HIV protease inhibitors (PI) with nelfinavir as the most active compound, or a set of ten diverse known MRP4 inhibitors including dipyridamole and quercetin as reference structures. While the first approach rendered a model consisting in four hydrogen bond acceptors, one hydrogen bond donor and three hydrophobes, the second exhibited only two hydrogen bond acceptors and one hydrophobe as determinant features for drug recognition. Interestingly, PGE_2 shared most features with the PI based pharmacophore suggesting a shared binding site with nelfinavir, which was proved experimentally. Also, quercetin failed to overlap with this model indicating that it may bind to another site in the transporter.

More recently, Welch *et al.* [225] developed another common feature pharmacophore model for MRP4 inhibition constructed using a set of nine structurally diverse DHEAS transport inhibitors. This model consisted in two hydrophobic features 5.01Å apart from each other, and one hydrogen bond acceptor feature located 4.81Å and 8.86Å away from the neighboring and the distal hydrophobic groups respectively. Implementation of this model for compound screening led to a correct classification of inhibitors and non-inhibitors of most compounds tested by the authors, with the exception of some compounds baring sulfonamides, sulfamides, and positively charged amine groups probably due to a poor match to the model or an incorrect parametrization of their features.

Additionally, Welch *et al.* also developed a Bayesian model for MRP4-mediated DHEAS transport inhibition that led to the identification of structural fingerprints and molecular properties associated with MRP4 inhibition. Among these fingerprints, the presence of three or more aromatic rings, oxygen atoms, and negatively ionized oxygen atoms proved to be favorable features for MRP4 inhibitory activity, while positively charged amines were frequently associated with non-inhibition. Interestingly, the authors found a trend towards a better inhibitory activity with high lipophilicity (>2,92) and molecular weight (≥356Da) values.

In spite of the differences between the available pharmacophore models, they all highlight the presence of hydrogen bond acceptor and hydrophobic groups as structural requirements for MRP4 drug recognition, which could be a valuable tool for virtual screening and compound selection.

Interestingly, most of the inhibitors listed in Table **2** fulfill these requirements. Nonetheless, given that MRP4 presents multiple and functionally different binding sites, the structural requirements for each specific substrate inhibition should be assessed individually.

CONCLUSIONS AND PERSPECTIVES

The development of MRP4 inhibitors represents an interesting pharmacological tool in pathologies where MRP4 is overexpressed or determines a worse outcome of the disease. However, the design of MRP4 specific and selective inhibitors is a complex problem with many edges. First, MRP4 structure has not been yet defined by any of the techniques used to investigate the three-dimensional structure of proteins. In addition, MRP4 presents low sequence homology with ABC transporters whose structure has already been elucidated. In any case, usage of crystallographic structures or homology models in target structure-based drug design for this family of proteins presents an extra challenge given the great conformational change they undergo when they exert their function. Besides, as it was previously mentioned, MRP4 has several endogenous substrates with defined physiological roles and shares most of them with other members of the ABC family. Remarkably, Mrp4-knockout mice models have not shown major physiological alterations [18], indicating that inhibiting MRP4 could be a feasible therapeutic approach. Although, they present different pharmacokinetics regarding kidney drug disposal or brain accumulation of transported drugs [18] which is why drug-drug interactions must be taken into account in a possible therapy combined with inhibitors.

In this regard, the development of both substrate and target selective MRP4 inhibitors or even allosteric modulators, that can regulate MRP4 activity regarding the transport of a specific substrate rather than blocking it, seems imperative. In this matter, ligand-based drug design appears as a good strategy for the design of this kind of pharmacological agents. To this end, there are several reports regarding the inhibitory effects of a large number of compounds on the activity of MRP4 and other MRP (Table **2**). However, a full characterization of the inhibitors of interest by means of biological descriptors, such as Ki or IC50 values, including their selectivity profiles in regard to the available substrates and transporters would be ideal. In view of all this, we consider that given current knowledge an appropriate workflow to achieve the objective would be the use of

a combination of different medicinal chemistry approaches.

On the other hand, a detailed analysis of the MRP amino acid sequence allowed us to identify several residues which could be important for MRP4 cyclic nucleotide transport. The amino acid position and conservation degree among the MRP members were crucial to detect these candidate residues. This exploration has the advantage that as it is based on sequence identity, homology models and mutagenesis experiments, it provides information not only about the residues involved in the substrate initial binding, but also about the subsequent interactions that occur during the transport transition states and during the release of the substrate. All the suggested sites were located in the MRP4 homology models in both their inward and outward conformations (Fig. **3**). To further confirm the significance of each of the proposed sites, future MRP4 mutagenesis and functionality studies, along with specific cAMP-docking assays are required.

Moreover, we compiled information from available MRP4 inhibition experiments (Table **2**). Most of the proven MRP4 inhibitors are clinically approved and have other primal activities acting on alternative targets, which makes them good candidates for drug repositioning. Also, these drugs could serve as starting points in the design of more selective inhibitors, especially when the lead compound has proven to show a high safety profile and presents low adverse effects. Conversely, the recompiled information could be used to generate pharmacophore models for each of MRP4 substrates. These models can be useful as a selection criterion for the subsequent screening of virtual chemical libraries in order to be combined with docking studies using the constructed homology models and also as a pre-selective criterion for later HTS.

Given that MRP4 cAMP transport is extremely relevant in several physio-pathological settings, the design of potent and selective inhibitors for this efflux represents a challenge of clinical need medicinal chemists. This review raises the possible uses and problems to consider when designing an MRP4 inhibitor and sets the basis for a multiple approach design that could serve to overcome this complex problem.

ABBREVIATIONS

15d-PGJ_2	=	15-Deoxy-Delta-12,14-prostaglandin J2
5-FU	=	5-Fluorouracil
ABC	=	ATP Binding Cassette
AML	=	Acute Myeloid Leukemia
ADP	=	Adenosine Diphosphate
ATP	=	Adenosine Triphosphate
bMRP1	=	bovine MRP1
BSEP	=	Bile Salt Export Pump
CEM	=	Cryo-Electron Microscopy
CFTR	=	Cystic Fibrosis Transmembrane conductance Regulator
cAMP	=	cyclic Adenosine Monophosphate
cGMP	=	cyclic Guanosine Monophosphate
cCMP	=	cyclic Cytidine Monophosphate
cUMP	=	cyclic Uridine Monophosphate
DHEAS	=	Dehydroepiandrosterone-3-sulfate
E2-17βG	=	oestradiol-17-beta-D-glucuronide
E_13SO_4	=	Estrone Sulfate
ECL	=	Extracellular Loop
EPAC	=	Exchange Proteins Activated by cAMP
GSSG	=	Glutathione disulfide
GSH	=	Glutathione
hMRP1	=	human MRP1
HTS	=	High Throughput Screening
Ki =		inhibitory *constant*
Km	=	affinity constant
LTB_4	=	Leukotriene B4
LTC_4	=	Leukotriene C4
LTD_4	=	Leukotriene D4
LTE_4	=	Leukotriene E4
IBMX	=	3-isobutyl-1-methylxanthine
IC50	=	half maximal inhibitory concentration
ICL	=	Intracellular Loop
MRP	=	Multidrug Resistance Protein
MSD	=	Membrane Spanning Domains
MTX	=	Methotrexate
NSAID	=	Non-Steroidal Anti Inflammatory Drugs
NBD	=	Nucleotide Binding Domains
PAH	=	p-aminohippurate
PDE	=	Phosphodiesterase E

PGA_1	=	Prostaglandin A1
PGE_1	=	Prostaglandin E1
PGE_2	=	Prostaglandin E2
PGF_1	=	Prostaglandin F1
$PGF_{2\alpha}$	=	Prostaglandin F2α
Pi	=	inorganic phosphate
PI	=	Protease Inhibitor
PMEA	=	9-(2-phosphonylme-thoxyethyl) adenine
siRNA	=	small interfering RNA
shRNA	=	short hairpin RNA
TM	=	Transmembrane Domain
TXB_2	=	Thromboxane B2
Vmax	=	maximum speed of transport

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Dean, M.; Annilo, T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. *Annu. Rev. Genomics Hum. Genet.*, **2005**, 6 (1), 123-142.

[2] Borst, P.; de Wolf, C.; van de Wetering, K. Multidrug resistance-associated proteins 3, 4, and 5. *Pflugers Arch.*, **2007**, 453 (5), 661-673.

[3] Wen, J.; Luo, J.; Huang, W.; Tang, J.; Zhou, H.; Zhang, W. The pharmacological and physiological role of multidrug-resistant protein 4. *J. Pharmacol. Exp. Ther.*, **2015**, 354 (3), 358-375.

[4] Rius, M.; Nies, A.T.; Hummel-Eisenbeiss, J.; Jedlitschky, G.; Keppler, D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology*, **2003**, 38 (2), 374-384.

[5] Lee, K.; Klein-Szanto, A.J.; Kruh, G.D. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. *J. Natl. Cancer Inst.*, **2000**, 92 (23), 1934-1940.

[6] Leggas, M.; Adachi, M.; Scheffer, G.; Sun, D.; Wielinga, P.; Du, G.; Mercer, K.; Zhuang, Y.; Panetta, J.; Johnston, B.; Scheper, R.; Stewart, C.; Schuetz, J. MRP4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol. Cell. Biol.*, **2004**, 24 (17), 7612-7621.

[7] van Aubel, R. a M.H.; Smeets, P.H.E.; Peters, J.G.P.; Bindels, R.J.M.; Russel, F.G.M. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J. Am. Soc. Nephrol.*, **2002**, 13 (3), 595-603.

[8] Smeets, P.H.E.; van Aubel, R.A.M.H.; Wouterse, A.C.; van den Heuvel, J.J.M.W.; Russel, F.G.M. Contribution of multidrug resistance protein 2 (MRP2/ABCC2) to the renal excretion of p-aminohippurate (PAH) and identification of MRP4 (ABCC4) as a novel PAH transporter. *J. Am. Soc. Nephrol.*, **2004**, 15 (11), 2828-2835.

[9] Lye, L.H.; Kench, J.G.; Handelsman, D.J.; Scheffer, G.L.; Stricker, P.D.; Grygiel, J.G.; Sutherland, R.L.; Henshall, S.M.; Allen, J.D.; Horvath, L.G. Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer. *Prostate*, **2008**, 68 (13), 1421-1429.

[10] Cai, C.; Omwancha, J.; Hsieh, C.; Shemshedini, L. Androgen induces expression of the multidrug resistance protein gene MRP4 in prostate cancer cells. *Prostate Cancer Prostatic Dis.*, **2007**, 10 (1), 39-45.

[11] Maher, J.M.; Cheng, X.; Tanaka, Y.; Scheffer, G.L.; Klaassen, C.D. Hormonal regulation of renal multidrug resistance-associated proteins 3 and 4 (Mrp3 and Mrp4) in mice. *Biochem. Pharmacol.*, **2006**, 71 (10), 1470-1478.

[12] Gradhand, U.; Lang, T.; Schaeffeler, E.; Glaeser, H.; Tegude, H.; Klein, K.; Fritz, P.; Jedlitschky, G.; Kroemer, H.K.; Bachmakov, I.; Anwald, B.; Kerb, R.; Zanger, U.M.; Eichelbaum, M.; Schwab, M.; Fromm, M.F. Variability in human hepatic MRP4 expression: influence of cholestasis and genotype. *Pharmacogenomics J.*, **2008**, 8 (1), 42-52.

[13] Maher, J.M.; Dieter, M.Z.; Aleksunes, L.M.; Slitt, A.L.; Guo, G.; Tanaka, Y.; Scheffer, G.L.; Chan, J.Y.; Manautou, J.E.; Chen, Y.; Dalton, T.P.; Yamamoto, M.; Klaassen, C.D. Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. *Hepatology*, **2007**, 46 (5), 1597-1610.

[14] Bröderdorf, S.; Zang, S.; Schaletzki, Y.; Grube, M.; Kroemer, H.K.; Jedlitschky, G. cAMP regulates expression of the cyclic nucleotide transporter MRP4 (ABCC4) through the EPAC pathway. *Pharmacogenet. Genomics*, **2014**, 24 (10), 522-526.

[15] Carozzo, A.; Diez, F.; Gomez, N.; Cabrera, M.; Shayo, C.; Davio, C.; Fernández, N. Dual role of cAMP in the transcriptional regulation of multidrug resistance-associated protein 4 (MRP4) in pancreatic adenocarcinoma cell lines. PLoS One, 2015, 10 (3), e0120651. www.ncbi.nlm.nih.gov/pmc/articles/PMC4366062/

[16] Maeng, H.J.; Lee, W.J.; Jin, Q.R.; Chang, J.E.; Shim, W.S. Upregulation of COX-2 in the lung cancer promotes overexpression of multidrug resistance protein 4 (MRP4) via PGE2-dependent pathway. *Eur. J. Pharm. Sci.*, **2014**, 62 (June), 189-196.

[17] Gu, X.; Manautou, J.E. Regulation of hepatic ABCC transporters by xenobiotics and in disease states. *Drug Metab. Rev.*, **2010**, 42 (3), 482-538.

[18] Russel, F.G.M.; Koenderink, J.B.; Masereeuw, R. Multidrug resistance protein 4 (MRP4/ABCC4): a versatile

efflux transporter for drugs and signalling molecules. *Trends Pharmacol. Sci.*, **2008**, 29 (4), 200-207.

[19] Norris, M.D.; Smith, J.; Tanabe, K.; Tobin, P.; Flemming, C.; Scheffer, G.L.; Wielinga, P.; Cohn, S.L.; London, W.B.; Marshall, G.M.; Allen, J.D.; Haber, M. Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan *in vitro*. *Mol. Cancer Ther.*, **2005**, 4 (4), 547-553.

[20] Copsel, S.; Garcia, C.; Diez, F.; Vermeulem, M.; Baldi, A.; Bianciotti, L.G.; Russel, F.G.M.; Shayo, C.; Davio, C. Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J. Biol. Chem.*, **2011**, 286 (9), 6979-6988.

[21] Sinha, C.; Ren, A.; Arora, K.; Moon, C.S.; Yarlagadda, S.; Zhang, W.; Cheepala, S.B.; Schuetz, J.D.; Naren, A.P. Multi-drug resistance protein 4 (MRP4)-mediated regulation of fibroblast cell migration reflects a dichotomous role of intracellular cyclic nucleotides. *J. Biol. Chem.*, **2013**, 288 (6), 3786-3794.

[22] Jedlitschky, G.; Greinacher, A.; Kroemer, H.K. Transporters in human platelets: physiologic function and impact for pharmacotherapy. *Blood*, **2012**, 119 (15), 3394-3402.

[23] Sodani, K.; Patel, A.; Kathawala, R.J.; Chen, Z.-S. Multidrug resistance associated proteins in multidrug resistance. *Chin. J. Cancer*, **2012**, 31 (2), 58-72.

[24] Li, C.; Krishnamurthy, P.C.; Penmatsa, H.; Marrs, K.L.; Wang, X.Q.; Zaccolo, M.; Jalink, K.; Li, M.; Nelson, D.J.; Schuetz, J.D.; Naren, A.P. Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia. *Cell*, **2007**, 131 (5), 940-951.

[25] Sassi, Y.; Hara, Y.; Lompré, A.M.; Hulot, J.S. Multi-drug Resistance Protein 4 (MRP4/ABCC4) and cyclic nucleotides signaling pathways. *Cell Cycle*, **2009**, 8 (7), 959-964.

[26] Ritter, C.A.; Jedlitschky, G.; Meyer zu Schwabedissen, H.; Grube, M.; Köck, K.; Kroemer, H.K. Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). *Drug Metab. Rev.*, **2005**, 37 (1), 253-278.

[27] Sager, G.; Ravna, A.W. Cellular efflux of cAMP and cGMP - a question about selectivity. *Mini Rev. Med. Chem.*, **2009**, 9 (8), 1009-1013.

[28] Buffone, M.G.; Wertheimer, E. V.; Visconti, P.E.; Krapf, D. Central role of soluble adenylyl cyclase and cAMP in sperm physiology. *Biochim. Biophys. Acta*, **2014**, 1842 (12 Pt B), 2610-2620.

[29] Alonso, C.A.I.; Osycka-Salut, C.E.; Castellano, L.; Cesari, A.; Di Siervi, N.; Mutto, A.; Johannisson, A.; Morrell, J.M.; Davio, C.; Perez-Martinez, S. Extracellular cAMP activates molecular signalling pathways associated with sperm capacitation in bovines. *Mol. Hum. Reprod.*, **2017**, 23 (8), 521-534.

[30] Osycka-Salut, C.; Diez, F.; Burdet, J.; Gervasi, M.G.; Franchi, A.; Bianciotti, L.G.; Davio, C.; Perez-Martinez, S. Cyclic AMP efflux, via MRPs and A1 adenosine receptors, is critical for bovine sperm capacitation. *Mol. Hum. Reprod.*, **2014**, 20 (1), 89-99.

[31] Tagami, M.; Kusuhara, S.; Imai, H.; Uemura, A.; Honda, S.; Tsukahara, Y.; Negi, A. MRP4 knockdown enhances migration, suppresses apoptosis, and produces aggregated morphology in human retinal vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **2010**, 400 (4), 593-598.

[32] van de Ven, R.; Scheffer, G.L.; Reurs, A.W.; Lindenberg, J.J.; Oerlemans, R.; Jansen, G.; Gillet, J.; Glasgow, J.N.; Pereboev, A.; Curiel, D.T.; Scheper, R.J.; de Gruijl, T.D. A role for multidrug resistance protein 4 (MRP4; ABCC4) in human dendritic cell migration. *Blood*, **2008**, 112 (6), 2353-2359.

[33] Zelcer, N.; Reid, G.; Wielinga, P.; Kuil, A.; van der Heijden, I.; Schuetz, J.D.; Borst, P. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem. J.*, **2003**, 371 (Pt 2), 361-367.

[34] Denk, G.U.; Soroka, C.J.; Takeyama, Y.; Chen, W.; Schuetz, J.D.; Boyer, J.L. Multidrug resistance-associated protein 4 is up-regulated in liver but down-regulated in kidney in obstructive cholestasis in the rat. *J. Hepatol.*, **2004**, 40 (4), 585-591.

[35] Sassi, Y.; Lipskaia, L.; Vandecasteele, G.; Nikolaev, V.O.; Hatem, S.N.; Aubart, F.C.; Russel, F.G.; Mougenot, N.; Vrignaud, C.; Lechat, P.; Lompré, A.M.; Hulot, J.S. Multidrug resistance-associated protein 4 regulates cAMP-dependent signaling pathways and controls human and rat SMC proliferation. *J. Clin. Invest.*, **2008**, 118 (8), 2747-2757.

[36] Chen, Z.S.; Lee, K.; Kruh, G.D. Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J. Biol. Chem.*, **2001**, 276 (36), 33747-33754.

[37] Fletcher, J.I.; Haber, M.; Henderson, M.J.; Norris, M.D. ABC transporters in cancer: more than just drug efflux pumps. *Nat. Rev. Cancer*, **2010**, 10 (2), 147-156.

[38] Schuetz, J.; Connelly, M.; Sun, D.; Paibir, S.; Flynn, P.; Srinivas, R.; Kumar, A.; Fridland, A. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat. Med.*, **1999**, 5 (9), 1048-1051.

[39] Imaoka, T.; Kusuhara, H.; Adachi, M.; Schuetz, J.D.; Takeuchi, K.; Sugiyama, Y. Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Mol Pharmacol*, **2007**, 71 (2), 619-627.

[40] Sassi, Y.; Abi-Gerges, A.; Fauconnier, J.; Mougenot, N.; Reiken, S.; Haghighi, K.; Kranias, E.G.; Marks, A.R.; Lacampagne, A.; Engelhardt, S.; Hatem, S.N.; Lompre, A.M.; Hulot, J.S. Regulation of cAMP homeostasis by the efflux protein MRP4 in cardiac myocytes. *FASEB J.*, **2012**, 26 (3), 1009-1017.

[41] Belleville-rolland, T.; Sassi, Y.; Decouture, B.; Dreano, E.; Hulot, J.; Gaussem, P.; Bachelot-loza, C. MRP4 (ABCC4) as a potential pharmacologic target for cardiovascular disease. *Pharmacol. Res.*, **2016**, 107, 381-389.

[42] Rybalkin, S.D.; Yan, C.; Bornfeldt, K.E.; Beavo, J.A. Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ. Res.*, **2003**, 93 (4), 280-291.

[43] Hara, Y.; Sassi, Y.; Guibert, C.; Gambaryan, N.; Dorfmüller, P.; Eddahibi, S.; Lompré, A.M.; Humbert, M.; Hulot, J.S. Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice. *J. Clin. Invest.*, **2011**, 121 (7), 2888-2897.

[44] Sassi, Y.; Ahles, A.; Truong, D.J.J.; Baqi, Y.; Lee, S.Y.; Husse, B.; Hulot, J.S.; Foinquinos, A.; Thum, T.; Müller, C.E.; Dendorfer, A.; Laggerbauer, B.; Engelhardt, S. Cardiac myocyte-secreted cAMP exerts paracrine action via adenosine receptor activation. *J. Clin. Invest.*, **2014**, 124 (12), 5385-5397.

[45] Jedlitschky, G.; Tirschmann, K.; Lubenow, L.E.; Nieuwenhuis, H.K.; Akkerman, J.W.N.; Greinacher, A.; Kroemer, H.K. The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage. *Blood*, **2004**, 104 (12), 3603-3610.

[46] Decouture, B.; Dreano, E.; Belleville-Rolland, T.; Kuci, O.; Dizier, B.; Bazaa, A.; Coqueran, B.; Lompre, A.-M.; Denis,

C. V.; Hulot, J.-S.; Bachelot-Loza, C.; Gaussem, P. Impaired platelet activation and cAMP homeostasis in MRP4-deficient mice. *Blood*, **2015**, 126 (15), 1823-1830.

[47] Cheepala, S.B.; Pitre, A.; Fukuda, Y.; Takenaka, K.; Zhang, Y.; Wang, Y.; Frase, S.; Pestina, T.; Gartner, T.K.; Jackson, C.; Schuetz, J.D. The ABCC4 membrane transporter modulates platelet aggregation. *Blood*, **2015**, 126 (20), 2307-2319.

[48] Borgognone, A.; Pulcinelli, F.M. Reduction of cAMP and cGMP inhibitory effects in human platelets by MRP4-mediated transport. *Thromb. Haemost.*, **2012**, 108 (5), 955-962.

[49] Barnes, S.N.; Aleksunes, L.M.; Augustine, L.; Scheffer, G.L.; Goedken, M.J.; Jakowski, A.B.; Pruimboom-Brees, I.M.; Cherrington, N.J.; Manautou, J.E. Induction of hepatobiliary efflux transporters in acetaminophen-induced acute liver failure cases. *Drug Metab. Dispos.*, **2007**, 35 (10), 1963-1969.

[50] Zollner, G.; Wagner, M.; Fickert, P.; Silbert, D.; Gumhold, J.; Zatloukal, K.; Denk, H.; Trauner, M. Expression of bile acid synthesis and detoxification enzymes and the alternative bile acid efflux pump MRP4 in patients with primary biliary cirrhosis. *Liver Int.*, **2007**, 27 (7), 920-929.

[51] Mennone, A.; Soroka, C.J.; Cai, S.-Y.; Harry, K.; Adachi, M.; Hagey, L.; Schuetz, J.D.; Boyer, J.L. Mrp4-/- mice have an impaired cytoprotective response in obstructive cholestasis. *Hepatology*, **2006**, 43 (5), 1013-1021.

[52] Marschall, H.U.; Wagner, M.; Zollner, G.; Fickert, P.; Diczfalusy, U.; Gumhold, J.; Silbert, D.; Fuchsbichler, A.; Benthin, L.; Grundström, R.; Gustafsson, U.; Sahlin, S.; Einarsson, C.; Trauner, M. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. *Gastroenterology*, **2005**, 129 (2), 476-485.

[53] Aleksunes, L.M.; Slitt, A.M.; Cherrington, N.J.; Thibodeau, M.S.; Klaassen, C.D.; Manautou, J.E. Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. *Toxicol. Sci.*, **2005**, 83 (1), 44-52.

[54] Aleksunes, L.M.; Scheffer, G.L.; Jakowski, A.B.; Pruimboom-Brees, I.M.; Manautou, J.E. Coordinated expression of multidrug resistance-associated proteins (Mrps) in mouse liver during toxicant-induced injury. *Toxicol. Sci.*, **2006**, 89 (2), 370-379.

[55] Maher, J.M.; Aleksunes, L.M.; Dieter, M.Z.; Tanaka, Y.; Peters, J.M.; Manautou, J.E.; Klaassen, C.D. Nrf2- and PPARα-mediated regulation of hepatic mrp transporters after exposure to perfluorooctanoic acid and perfluorodecanoic acid. *Toxicol. Sci.*, **2008**, 106 (2), 319-328.

[56] Zhang, Z.; Wang, J.; Shen, B.; Peng, C.; Zheng, M. The ABCC4 gene is a promising target for pancreatic cancer therapy. *Gene*, **2012**, 491 (2), 194-199.

[57] Sun, Y.; Shi, N.; Lu, H.; Zhang, J.; Ma, Y.; Qiao, Y.; Mao, Y.; Jia, K.; Han, L.; Liu, F.; Li, H.; Lin, Z.; Li, X.; Zhao, X. ABCC4 copy number variation is associated with susceptibility to esophageal squamous cell carcinoma. *Carcinogenesis*, **2014**, 35 (9), 1941-1950.

[58] Pereira, C.; Queirós, S.; Galaghar, A.; Sousa, H.; Marcos-Pinto, R.; Pimentel-Nunes, P.; Brandão, C.; Medeiros, R.; Dinis-Ribeiro, M. Influence of genetic polymorphisms in prostaglandin E2 pathway (COX-2/HPGD/SLCO2A1/ABCC4) on the risk for colorectal adenoma development and recurrence after polypectomy. Clin. Transl. Gastroenterol., 2016, 7 (9), e191. www.ncbi.nlm.nih.gov/pmc/articles/PMC5288593/

[59] Leonard, G.D.; Fojo, T.; Bates, S.E. The role of ABC transporters in clinical practice. *Oncologist*, **2003**, 8 (5), 411-424.

[60] Copsel, S.; Bruzzone, A.; May, M.; Beyrath, J.; Wargon, V.; Cany, J.; Russel, F.G.; Shayo, C.; Davio, C. Multidrug resistance protein 4/ ATP binding cassette transporter 4: a new potential therapeutic target for acute myeloid leukemia. *Oncotarget*, **2014**, 5 (19), 9308-9321.

[61] Zhao, X.; Guo, Y.; Yue, W.; Zhang, L.; Gu, M.; Wang, Y. ABCC4 is required for cell proliferation and tumorigenesis in non-small cell lung cancer. *Onco. Targets. Ther.*, **2014**, 7, 343-351.

[62] Moustafa, M.A.; Ogino, D.; Nishimura, M.; Ueda, N.; Naito, S.; Furukawa, M.; Uchida, T.; Ikai, I.; Sawada, H.; Fukumoto, M. Comparative analysis of ATP-binding cassette (ABC) transporter gene expression levels in peripheral blood leukocytes and in liver with hepatocellular carcinoma. *Cancer Sci.*, **2004**, 95 (6), 530-536.

[63] Holla, V.R.; Backlund, M.G.; Yang, P.; Newman, R. a.; DuBois, R.N. Regulation of prostaglandin transporters in colorectal neoplasia. *Cancer Prev. Res.*, **2008**, 1 (2), 93-99.

[64] Beretta, G.L.; Benedetti, V.; Cossa, G.; Assaraf, Y.G.A.; Bram, E.; Gatti, L.; Corna, E.; Carenini, N.; Colangelo, D.; Howell, S.B.; Zunino, F.; Perego, P. Increased levels and defective glycosylation of MRPs in ovarian carcinoma cells resistant to oxaliplatin. *Biochem. Pharmacol.*, **2010**, 79 (8), 1108-1117.

[65] Bagnoli, M.; Beretta, G.L.; Gatti, L.; Pilotti, S.; Alberti, P.; Tarantino, E.; Barbareschi, M.; Canevari, S.; Mezzanzanica, D.; Perego, P. Clinicopathological impact of ABCC1/MRP1 and ABCC4/MRP4 in epithelial ovarian carcinoma. *Biomed Res. Int.*, **2013**, 2013, 1-7.

[66] Montani, M.; Hermanns, T.; Herrmanns, T.; Müntener, M.; Wild, P.; Sulser, T.; Kristiansen, G. Multidrug resistance protein 4 (MRP4) expression in prostate cancer is associated with androgen signaling and decreases with tumor progression. *Virchows Arch.*, **2013**, 462 (4), 437-443.

[67] Huynh, T.; Norris, M.D.; Haber, M.; Henderson, M.J. ABCC4/MRP4: a MYCN-regulated transporter and potential therapeutic target in neuroblastoma. *Front. Oncol.*, **2012**, 2, 1-7.

[68] Borst, P.; Schinkel, A.H. Genetic dissection of the function of mammalian P-glycoproteins. *Trends Genet.*, **1997**, 13 (6), 217-222.

[69] Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. A family of drug transporters: the multidrug resistance-associated proteins. *J. Natl. Cancer Inst.*, **2000**, 92 (16), 1295-1302.

[70] He, Z.; Hu, B.; Tang, L.; Zheng, S.; Sun, Y.; Sheng, Z.; Yao, Y.; Lin, F. The overexpression of MRP4 is related to multidrug resistance in osteosarcoma cells. *J. Cancer Res. Ther.*, **2015**, 11 (1), 18-23.

[71] Dean, M.; Fojo, T.; Bates, S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer*, **2005**, 5 (4), 275-284.

[72] Zhang, Y.H.; Wu, Q.; Xiao, X.Y.; Li, D.W.; Wang, X.P. Silencing MRP4 by small interfering RNA reverses acquired DDP resistance of gastric cancer cell. *Cancer Lett.*, **2010**, 291 (1), 76-82.

[73] Yu, Z.; Zhang, C.; Chai, R.; Du, Y.; Gao, X.; Xing, J.; Yu, E.; Zhang, W.; Zhang, X.; Cao, G.; Fu, C. Prognostic significance and molecular mechanism of ATP-Binding cassette subfamily C member 4 in resistance to neoadjuvant radiotherapy of locally advanced rectal carcinoma. PLoS One, 2014, 9 (1), e85446. journals.plos.org/plosone/article?id=10.1371/journal.pone.0085446

[74] Henderson, M.J.; Haber, M.; Porro, A.; Munoz, M.A.; Iraci, N.; Xue, C.; Murray, J.; Flemming, C.L.; Smith, J.; Fletcher, J.I.; Gherardi, S.; Kwek, C.K.; Russell, A.J.; Valli,

E.; London, W.B.; Buxton, A.B.; Ashton, L.J.; Sartorelli, A.C. Cohn, S.L.; Schwab, M.; Marshall, G.M.; Perini, G; Norris, M.D. ABCC multidrug transporters in childhood neuroblastoma: Clinical and biological effects independent of cytotoxic drug efflux. *J. Natl. Cancer Inst.*, **2011**, 103 (16), 1236-1251.

[75] Kochel, T.J.; Reader, J.C.; Ma, X.; Kundu, N.; Fulton, A.M. Multiple drug resistance-associated protein (MRP4) exports prostaglandin E2 (PGE) and contributes to metastasis in basal/triple negative breast cancer. *Oncotarget*, **2017**, 8 (4), 6540-6554.

[76] Hagmann, W.; Jesnowski, R.; Faissner, R.; Guo, C.; Löhr, J.M. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. *Pancreatology*, **2009**, 9 (1-2), 136-144.

[77] Wang, D.; DuBois, R.N. Eicosanoids and cancer. *Nat. Rev. Cancer*, **2010**, 10 (3), 181-193.

[78] Yu, D.M.T.; Huynh, T.; Truong, A.M.; Haber, M.; Norris, M.D. ABC transporters and neuroblastoma. *Adv. Cancer Res.*, **2015**, 125, 139-170.

[79] Reid, G.; Wielinga, P.; Zelcer, N.; van der Heijden, I.; Kuil, A.; de Haas, M.; Wijnholds, J.; Borst, P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc. Natl. Acad. Sci. U. S. A.*, **2003**, 100 (16), 9244-9249.

[80] Wang, D.; DuBois, R.N. The Role of Prostaglandin E(2) in Tumor-Associated Immunosuppression. *Trends Mol. Med.*, **2016**, 22 (1), 1-3.

[81] Wang, D.; Dubois, R.N. Prostaglandins and cancer. *Gut*, **2006**, 55 (1), 115-122.

[82] Mauritz, I.; Westermayer, S.; Marian, B.; Erlach, N.; Grusch, M.; Holzmann, K. Prostaglandin E(2) stimulates progression-related gene expression in early colorectal adenoma cells. *Br. J. Cancer*, **2006**, 94 (11), 1718-1725.

[83] Pitre, A.; Ge, Y.; Lin, W.; Wang, Y.; Fukuda, Y.; Temirov, J.; Phillips, A.H.; Peters, J.L.; Fan, Y.; Ma, J.; Nourse, A.; Sinha, C.; Lin, H.; Kriwacki, R.; Downing, J.R.; Gruber, T.A.; Centonze, V.E.; Naren, A.P.; Chen, T.; Schuetz, J.D. An unexpected protein interaction promotes drug resistance in leukemia. *Nat. Commun.*, **2017**, 8 (1547), 1-14.

[84] Cui, Y.; König, J.; Buchholz, J.K.; Spring, H.; Leier, I.; Keppler, D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol. Pharmacol.*, **1999**, 55 (5), 929-937.

[85] Zeng, H.; Liu, G.; Rea, P.A.; Kruh, G.D. Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res.*, **2000**, 60 (17), 4779-4784.

[86] Iliás, A.; Urbán, Z.; Seidl, T.L.; Saux, O. Le; Sinkó, E.; Boyd, C.D.; Sarkadi, B.; Váradi, A. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). *J. Biol. Chem.*, **2002**, 277 (19), 16860-16867.

[87] Belinsky, M.G.; Chen, Z.S.; Shchaveleva, I.; Zeng, H.; Kruh, G.D. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res.*, **2002**, 62 (21), 6172-6177.

[88] Chen, Z.S.; Hopper-Borge, E.; Belinsky, M.G.; Shchaveleva, I.; Kotova, E.; Kruh, G.D. Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Mol. Pharmacol.*, **2003**, 63 (0026-895X), 351-358.

[89] Rius, M.; Hummel-Eisenbeiss, J.; Keppler, D. ATP-dependent transport of leukotrienes B4 and C4 by the multidrug resistance protein ABCC4 (MRP4). *J. Pharmacol. Exp. Ther.*, **2008**, 324 (1), 86-94.

[90] Jedlitschky, G.; Burchell, B.; Keppler, D. The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J. Biol. Chem.*, **2000**, 275 (39), 30069-30074.

[91] Chen, Z.S.; Guo, Y.; Belinsky, M.G.; Kotova, E.; Kruh, G.D. Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C4 by human multidrug resistance protein 8 (ABCC11). *Mol. Pharmacol.*, **2005**, 67 (2), 545-557.

[92] Kruh, G.D.; Guo, Y.; Hopper-Borge, E.; Belinsky, M.G.; Chen, Z.-S. ABCC10, ABCC11, and ABCC12. *Pflugers Arch.*, **2007**, 453 (5), 675-684.

[93] Jedlitschky, G.; Leier, I.; Buchholz, U.; Barnouin, K.; Kurz, G.; Keppler, D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res.*, **1996**, 56 (5), 988-994.

[94] Rius, M.; Thon, W.F.; Keppler, D.; Nies, A.T. Prostanoid transport by multidrug resistance protein 4 (MRP4/ABCC4) localized in tissues of the human urogenital tract. *J. Urol.*, **2005**, 174 (6), 2409-2414.

[95] Evers, R.; Cnubben, N.H.P.; Wijnholds, J.; Deemter, L. Van; Bladeren, P.J. Van; Borst, P. Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Lett.*, **1997**, 419 (1), 112-116.

[96] Paumi, C.M.; Wright, M.; Townsend, A.J.; Morrow, C.S. Multidrug resistance protein (MRP) 1 and MRP3 attenuate cytotoxic and transactivating effects of the cyclopentenone prostaglandin, 15-deoxy-Δ12,14prostaglandin J2 in MCF7 breast cancer cells. *Biochemistry*, **2003**, 42 (18), 5429-5437.

[97] Jedlitschky, G.; Leier, I.; Buchholz, U.; Hummel-Eisenbeiss, J.; Burchell, B.; Keppler, D. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochem. J.*, **1997**, 327 (1) (12), 305-310.

[98] Kamisako, T.; Leier, I.; Gui, Y.; König, J.; Buchholz, U.; Hummel-Eisenbeiss, J.; Keppler, D. Transport of monoglucuronosyl and bisglucuronosyl bilirubin by recombinant human and rat multidrug resistance protein 2. *Hepatology*, **1999**, 30 (2), 485-490.

[99] Lee, Y.A.; Cui, Y.; König, J.; Risch, A.; Jäger, B.; Drings, P.; Bartsch, H.; Keppler, D.; Nies, A.T. Identification and functional characterization of the natural variant MRP3-Arg1297His of human multidrug resistance protein 3 (MRP3/ABCC3). *Pharmacogenetics*, **2004**, 14 (4), 213-223.

[100] Akita, H.; Suzuki, H.; Hirohashi, T.; Takikawa, H.; Sugiyama, Y. Transport activity of human MRP3 expressed in Sf9 cells: comparative studies with rat MRP3. *Pharm. Res.*, **2002**, 19 (1), 34-41.

[101] Rius, M.; Hummel-Eisenbeiss, J.; Hofmann, A.F.; Keppler, D. Substrate specificity of human ABCC4 (MRP4)-mediated cotransport of bile acids and reduced glutathione. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **2006**, 290 (4), 640-649.

[102] Zelcer, N.; Saeki, T.; Bot, I.; Kuil, A.; Borst, P. Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na+-dependent bile-acid transporter. *Biochem. J.*, **2003**, 369 (Pt 1), 23-30.

[103] Sasaki, M.; Suzuki, H.; Ito, K.; Abe, T.; Sugiyama, Y. Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and Multidrug resistance-associated protein 2 (MRP2/ABCC2). *J. Biol. Chem.*, **2002**, 277 (8), 6497-6503.

[104] Zeng, H.; Chen, Z.S.; Belinsky, M.G.; Rea, P.A.; Kruh, G.D. Transport of methotrexate (MTX) and folates by

multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res.*, **2001**, 61 (19), 7225-7232.

[105] Hooijberg, J.H.; Peters, G.J.; Assaraf, Y.G.; Kathmann, I.; Priest, D.G.; Bunni, M.A.; Veerman, A.J.P.; Scheffer, G.L.; Kaspers, G.J.L.; Jansen, G. The role of multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular folate homeostasis. *Biochem. Pharmacol.*, **2003**, 65 (5), 765-771.

[106] Chen, Z.S.; Lee, K.; Walther, S.; Raftogianis, R.B.; Zeng, H.; Kruh, G.D.; Kuwano, M. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. *Cancer Res.*, **2002**, 62 (11), 3144-3150.

[107] Wielinga, P.; Hooijberg, J.H.; Gunnarsdottir, S.; Kathmann, I.; Reid, G.; Zelcer, N.; Van Der Born, K.; De Haas, M.; Van Der Heijden, I.; Kaspers, G.; Wijnholds, J.; Jansen, G.; Peters, G.; Borst, P. The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. *Cancer Res.*, **2005**, 65 (10), 4425-4430.

[108] Reichel, V.; Masereeuw, R.; van den Heuvel, J.J.M.W.; Miller, D.S.; Fricker, G. Transport of a fluorescent cAMP analog in teleost proximal tubules. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **2007**, 293 (6), R2382-R2389.

[109] van Aubel, R. a M.H.; Smeets, P.H.E.; van den Heuvel, J.J.M.W.; Russel, F.G.M. Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites. *Am. J. Physiol. Renal Physiol.*, **2005**, 288 (2), 327-333.

[110] Guo, Y.; Kotova, E.; Chen, Z.-S.; Lee, K.; Hopper-Borge, E.; Belinsky, M.G.; Kruh, G.D. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *J. Biol. Chem.*, **2003**, 278 (32), 29509-29514.

[111] Klokouzast, A.; Wu, C.P.; Van Veen, H.W.; Barrand, M.A.; Hladky, S.B. cGMP and glutathione-conjugate transport in human erythrocytes: The roles of the multidrug resistance-associated proteins, MRP1, MRP4 and MRP5. *Eur. J. Biochem.*, **2003**, 270 (18), 3696-3708.

[112] Laue, S.; Winterhoff, M.; Kaever, V.; Van Den Heuvel, J.J.; Russel, F.G.; Seifert, R. cCMP is a substrate for MRP5. *Naunyn. Schmiedebergs. Arch. Pharmacol.*, **2014**, 387 (9), 893-895.

[113] Leslie, E.M.; Bowers, R.J.; Deeley, R.G.; Cole, S.P.C. Structural requirements for functional interaction of glutathione tripeptide analogs with the human multidrug resistance protein 1 (MRP1). *J. Pharmacol. Exp. Ther.*, **2003**, 304 (2), 643-653.

[114] Kopplow, K.; Letschert, K.; König, J.; Walter, B.; Keppler, D. Human hepatobiliary transport of organic anions analyzed by quadruple-transfected cells. *Mol. Pharmacol.*, **2005**, 68 (4), 1031-1038.

[115] Bortfeld, M.; Rius, M.; König, J.; Herold-Mende, C.; Nies, A.T.; Keppler, D. Human multidrug resistance protein 8 (MRP8/ABCC11), an apical efflux pump for steroid sulfates, is an axonal protein of the CNS and peripheral nervous system. *Neuroscience*, **2006**, 137 (4), 1247-1257.

[116] Leier, I.; Jedlitschky, G.; Buchholz, U.; Center, M.; Cole, S.P.; Deeley, R.G.; Keppler, D. ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. *Biochem. J.*, **1996**, 314 (2), 433-437.

[117] Paulusma, C.C.; van Geer, M. a; Evers, R.; Heijn, M.; Ottenhoff, R.; Borst, P.; Oude Elferink, R.P. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. *Biochem. J.*, **1999**, 338 (2), 393-401.

[118] Wijnholds, J.; Mol, C.A.; van Deemter, L.; de Haas, M.; Scheffer, G.L.; Baas, F.; Beijnen, J.H.; Scheper, R.J.; Hatse, S.; De Clercq, E.; Balzarini, J.; Borst, P. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97 (13), 7476-7481.

[119] Leslie, E.M.; Deeley, R.G.; Cole, S.P.C. Bioflavonoid stimulation of glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). *Drug Metab. Dispos.*, **2003**, 31 (1), 11-15.

[120] Zelcer, N.; Saeki, T.; Reid, G.; Beijnen, J.H.; Borst, P. Characterization of Drug Transport by the Human Multidrug Resistance Protein 3 (ABCC3). *J. Biol. Chem.*, **2001**, 276 (49), 46400-46407.

[121] Oleschuk, C.J.; Deeley, R.G.; Cole, S.P.C. Substitution of Trp1242 of TM17 alters substrate specificity of human multidrug resistance protein 3. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **2003**, 284 (2), 280-289.

[122] Bakos, E.; Evers, R.; Szakacs, G.; Tusnady, G.E.; Welker, E.; Szabo, K.; de Haas, M.; van Deemter, L.; Borst, P.; Varadi, A.; Sarkadi, B. Functional multidrug resistance protein (MRP1) lacking the N- terminal transmembrane domain. *J. Biol. Chem.*, **1998**, 273 (48), 32167-32175.

[123] Slot, A.J.; Molinski, S. V; Cole, S.P.C. Mammalian multidrug-resistance proteins (MRPs). *Essays Biochem.*, **2011**, 50 (1), 179-207.

[124] Kool, M.; van der Linden, M.; de Haas, M.; Scheffer, G.L.; de Vree, J.M.; Smith, A.J.; Jansen, G.; Peters, G.J.; Ponne, N.; Scheper, R.J.; Elferink, R.P.; Baas, F.; Borst, P. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc. Natl. Acad. Sci. U. S. A.*, **1999**, 96 (12), 6914-6919.

[125] Annilo, T.; Chen, Z.; Shulenin, S.; Costantino, J.; Thomas, L.; Lou, H.; Stefanov, S.; Dean, M. Evolution of the vertebrate ABC gene family: Analysis of gene birth and death. *Genomics*, **2006**, 88 (1), 1-11.

[126] Hopper, E.; Belinsky, M.G.; Zeng, H.; Tosolini, A.; Testa, J.R.; Kruh, G.D. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. *Cancer Lett.*, **2001**, 162 (2), 181-191.

[127] Kruh, G.D.; Belinsky, M.G.; Gallo, J.M.; Lee, K. Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. *Cancer Metastasis Rev.*, **2007**, 26 (1), 5-14.

[128] Wielinga, P.R.; Van der Heijden, I.; Reid, G.; Beijnen, J.H.; Wijnholds, J.; Borst, P. Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. *J. Biol. Chem.*, **2003**, 278 (20), 17664-17671.

[129] Davoren, P.R.; Sutherland, E.W. The effect of L-epinephrine and other agents on the synthesis and release of adenosine 3',5'- phosphate by whole pigeon erythrocytes. *J. Biol. Chem.*, **1963**, 238 (9), 3009-3015.

[130] Brunton, L.L.; Heasley, L.E. cAMP export and its regulation by prostaglandin A1. *Methods Enzymol.*, **1988**, 159, 83-93.

[131] Fehr, T.F.; Dickinson, E.S.; Goldman, S.J.; Slakey, L.L. Cyclic AMP efflux is regulated by occupancy of the adenosine receptor in pig aortic smooth muscle cells. *J. Biol. Chem.*, **1990**, 265 (19), 10974-10980.

[132] Billiar, T.R.; Curran, R.D.; Harbrecht, B.G.; Stadler, J.; Williams, D.L.; Ochoa, J.B.; Di Silvio, M.; Simmons, R.L.; Murray, S.A. Association between synthesis and release of cGMP and nitric oxide biosynthesis by hepatocytes. *Am. J. Physiol.*, **1992**, 262 (4 (1)), 1077-1082.

[133] Scavennec, J.; Carcassonne, Y.; Gastaut, J.A.; Blanc, A.; Cailla, H.L. Relationship between the levels of cyclic

cytidine 3':5'-monophosphate, cyclic guanosine 3':5'-monophosphate, and cyclic adenosine 3':5'-monophosphate in urines and leukocytes and the type of human leukemias. *Cancer Res.*, **1981**, 41 (8), 3222-3227.

[134] Ørvoll, E.; Lysaa, R.A.; Ravna, A.W.; Sager, G. Misoprostol and the sildenafil analog (PHAR-0099048) modulate cellular efflux of cAMP and cGMP differently. *Pharmacol. & Pharm.*, **2013**, 4 (1), 104-109.

[135] Lin, Z.P.; Zhu, Y.L.; Johnson, D.R.; Rice, K.P.; Nottoli, T.; Hains, B.C.; McGrath, J.; Waxman, S.G.; Sartorelli, A.C. Disruption of cAMP and prostaglandin E2 transport by multidrug resistance protein 4 deficiency alters cAMP-mediated signaling and nociceptive response. *Mol. Pharmacol.*, **2008**, 73 (1), 243-251.

[136] Chiavegatti, T.; Costa, V.; Araújo, M.; Godinho, R. Skeletal muscle expresses the extracellular cyclic AMP-adenosine pathway. *Br. J. Pharmacol.*, **2008**, 153 (December 2007), 1331-1340.

[137] Godinho, R.O.; Duarte, T.; Pacini, E.S.A. New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP efflux and extracellular cAMP-adenosine pathway. *Front. Pharmacol.*, **2015**, 6 (58), 1-9.

[138] Adachi, M.; Sampath, J.; Lan, L. Bin; Sun, D.; Hargrove, P.; Flatley, R.; Tatum, A.; Edwards, M.Z.; Wezeman, M.; Matherly, L.; Drake, R.; Schuetz, J. Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. *J. Biol. Chem.*, **2002**, 277 (41), 38998-39004.

[139] Xie, M.; Rich, T.C.; Scheitrum, C.; Conti, M.; Richter, W. Inactivation of multidrug resistance proteins disrupts both cellular extrusion and intracellular degradation of cAMP. *Mol. Pharmacol.*, **2011**, 80 (2), 281-293.

[140] Sellers, Z.M.; Naren, A.P.; Xiang, Y.; Best, P.M. MRP4 and CFTR in the regulation of cAMP and β-adrenergic contraction in cardiac myocytes. *Eur. J. Pharmacol.*, **2012**, 681 (1-3), 80-87.

[141] Hofer, A.M.; Lefkimmiatis, K. Extracellular calcium and cAMP: second messengers as 'third messengers'? *Physiology*, **2007**, 22, 320-327.

[142] Milatovic, D.; Montine, T.J.; Aschner, M. Prostanoid signaling: Dual role for prostaglandin E2 in neurotoxicity. *Neurotoxicology*, **2011**, 32 (3), 312-319.

[143] Narumiya, S.; FitzGerald, G.A. Genetic and pharmacological analysis of prostanoid receptor function. *J. Clin. Invest.*, **2001**, 108 (1), 25-30.

[144] Hao, C.M.; Breyer, M.D. Physiologic and pathophysiologic roles of lipid mediators in the kidney. *Kidney Int.*, **2007**, 71 (11), 1105-1115.

[145] Harris, S.G.; Padilla, J.; Koumas, L.; Ray, D.; Phipps, R.P. Prostaglandins as modulators of immunity. *Trends Immunol.*, **2002**, 23 (3), 144-150.

[146] Furugen, A.; Yamaguchi, H.; Tanaka, N.; Shiida, N.; Ogura, J.; Kobayashi, M.; Iseki, K. Contribution of multidrug resistance-associated proteins (MRPs) to the release of prostanoids from A549 cells. *Prostaglandins Other Lipid Mediat.*, **2013**, 106 (1), 37-44.

[147] Tanaka, N.; Yamaguchi, H.; Mano, N. Transport of eicosapentaenoic acid-derived PGE3, PGF(3α), and TXB3 by ABCC4. PLoS One, 2014, 9 (10), e109270. journals.plos.org/plosone/article?id=10.1371/journal.pone.0109270

[148] Ravna, A.W.; Sylte, I.; Sager, G. A molecular model of a putative substrate releasing conformation of multidrug resistance protein 5 (MRP5). *Eur. J. Med. Chem.*, **2008**, 43 (11), 2557-2567.

[149] Honorat, M.; Terreux, R.; Falson, P.; Di Pietro, A.; Dumontet, C.; Payen, L. Localization of putative binding sites for cyclic guanosine monophosphate and the anti-cancer drug 5-fluoro-2'-deoxyuridine-5'-monophosphate on ABCC11 in silico models. *BMC Struct. Biol.*, **2013**, 13 (7), 1-12.

[150] Haimeur, A.; Deeley, R.G.; Cole, S.P.C. Charged amino acids in the sixth transmembrane helix of multidrug resistance protein 1 (MRP1/ABCC1) are critical determinants of transport activity. *J. Biol. Chem.*, **2002**, 277 (44), 41326-41333.

[151] Deeley, R.G.; Westlake, C.; Cole, S.P.C. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol. Rev.*, **2006**, 86 (3), 849-899.

[152] Oswald, C.; Holland, I.B.; Schmitt, L. The motor domains of ABC-transporters. What can structures tell us? *Naunyn. Schmiedebergs. Arch. Pharmacol.*, **2006**, 372 (6), 385-399.

[153] Safa, A.R. Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. *Curr. Med. Chem.*, **2004**, 4 (1), 1-17.

[154] Frelet, A.; Klein, M. Insight in eukaryotic ABC transporter function by mutation analysis. *FEBS Lett.*, **2006**, 580 (4), 1064-1084.

[155] George, A.M.; Jones, P.M. Perspectives on the structure-function of ABC transporters: the Switch and Constant Contact models. *Prog. Biophys. Mol. Biol.*, **2012**, 109 (3), 95-107.

[156] Jones, P.M.; George, A.M. Opening of the ADP-bound active site in the ABC transporter ATPase dimer: Evidence for a constant contact, alternating sites model for the catalytic cycle. *Proteins Struct. Funct. Bioinforma.*, **2009**, 75 (2), 387-396.

[157] Jones, P.M.; George, A.M. A reciprocating twin-channel model for ABC transporters. *Q. Rev. Biophys.*, **2014**, 3, 1-32.

[158] Higgins, C.F.; Linton, K.J. The ATP switch model for ABC transporters. *Nat. Struct. Mol. Biol.*, **2004**, 11 (10), 918-926.

[159] Rothnie, A.; Callaghan, R.; Deeley, R.G.; Cole, S.P.C. Role of GSH in estrone sulfate binding and translocation by the multidrug resistance protein 1 (MRP1/ABCC1). *J. Biol. Chem.*, **2006**, 281 (20), 13906-13914.

[160] Wang, E.J.; Casciano, C.N.; Clement, R.P.; Johnson, W.W. Cooperativity in the inhibition of P-glycoprotein-mediated daunorubicin transport: evidence for half-of-the-sites reactivity. *Arch. Biochem. Biophys.*, **2000**, 383 (1), 91-98.

[161] Linton, K.J.; Higgins, C.F. Structure and function of ABC transporters: The ATP switch provides flexible control. *Pflugers Arch. Eur. J. Physiol.*, **2007**, 453 (5), 555-567.

[162] Ravna, A.W.; Sager, G. Molecular model of the outward facing state of the human multidrug resistance protein 4 (MRP4/ABCC4). *Bioorg. Med. Chem. Lett.*, **2008**, 18 (12), 3481-3483.

[163] Bodo, A.; Bakos, E.; Szeri, F.; Varadi, A.; Sarkadi, B. Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. *J. Biol. Chem.*, **2003**, 278 (26), 23529-23537.

[164] Azza A. K. El-Sheikh, Jeroen J. M. W. van den Heuvel, J.B.K.; Russel, and F.G.M. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2-and MRP4/ABCC4-mediated methotrexate transport. *J. Pharmacol. Exp. Ther.*, **2007**, 320 (1), 229-235.

[165] Reid, G.; Wielinga, P.; Zelcer, N.; De Haas, M.; Van Deemter, L.; Wijnholds, J.; Balzarini, J.; Borst, P. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol. Pharmacol.*, **2003**, 63 (5), 1094-1103.

[166] Zelcer, N.; Huisman, M.T.; Reid, G.; Wielinga, P.; Breedveld, P.; Kuil, A.; Knipscheer, P.; Schellens, J.H.M.;

Schinkel, A.H.; Borst, P. Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). *J. Biol. Chem.*, **2003**, 278 (26), 23538-23544.

[167] Zehnpfennig, B.; Urbatsch, I.L.; Galla, H.J. Functional reconstitution of human ABCC3 into proteoliposomes reveals a transport mechanism with positive cooperativity. *Biochemistry*, **2009**, 48 (20), 4423-4430.

[168] Johnson, Z.L.; Chen, J. Structural basis of substrate recognition by the multidrug resistance protein MRP1. *Cell*, **2017**, 168 (6), 1075-1085.

[169] Grant, C.E.; Gao, M.; DeGorter, M.K.; Cole, S.P.C.; Deeley, R.G. Structural determinants of substrate specificity differences between human multidrug resistance protein MRP1 (ABCC1) and MRP3 (ABCC3). *Drug Metab. Dispos.*, **2008**, 36 (12), 2571-2581.

[170] Conseil, G.; Cole, S.P.C. Two polymorphic variants of ABCC1 selectively alter drug resistance and inhibitor sensitivity of the multidrug and organic anion transporter multidrug resistance protein 1. *Drug Metab. Dispos.*, **2013**, 41 (12), 2187-2196.

[171] Létourneau, I.J.; Deeley, R.G.; Cole, S.P.C. Functional characterization of non-synonymous single nucleotide polymorphisms in the gene encoding human multidrug resistance protein 1 (MRP1/ABCC1). *Pharmacogenet. Genomics*, **2005**, 15 (9), 647-657.

[172] El-Sheikh, A.A.K.; van den Heuvel, J.J.M.W.; Krieger, E.; Russel, F.G.M.; Koenderink, J.B. Functional role of arginine 375 in transmembrane helix 6 of multidrug resistance protein 4 (MRP4/ABCC4). *Mol. Pharmacol.*, **2008**, 74 (4), 964-971.

[173] Yoshiura, K.; Kinoshita, A.; Ishida, T.; Ninokata, A.; Ishikawa, T.; Kaname, T.; Bannai, M.; Tokunaga, K.; Sonoda, S.; Komaki, R.; Ihara, M.; Saenko, V.A.; Alipov, G.K.; Sekine, I.; Komatsu, K.; Takahashi, H.; Nakashima, M.; Sosonkina, N.; Mapendano, C.K.; Ghadami, M.; Nomura, M.; Liang, D.S.; Miwa, N.; Kim, D.K.; Garidkhuu, A.; Natsume, N.; Ohta, T.; Tomita, H.; Kaneko, A.; Kikuchi, M.; Russomando, G.; Hirayama, K.; Ishibashi, M.; Takahashi, A.; Saitou, N.; Murray, J.C.; Saito, S.; Nakamura, Y.; Niikawa, N. A SNP in the ABCC11 gene is the determinant of human earwax type. *Nat. Genet.*, **2006**, 38 (3), 324-330.

[174] Koike, K.; Oleschuk, C.J.; Haimeur, A.; Olsen, S.L.; Deeley, R.G.; Cole, S.P.C. Multiple membrane-associated tryptophan residues contribute to the transport activity and substrate specificity of the human multidrug resistance protein, MRP1. *J. Biol. Chem.*, **2002**, 277 (51), 49495-49503.

[175] Koike, K.; Conseil, G.; Leslie, E.M.; Deeley, R.G.; Cole, S.P.C. Identification of proline residues in the core cytoplasmic and transmembrane regions of multidrug resistance protein 1 (MRP1/ABCC1) important for transport function, substrate specificity, and nucleotide interactions. *J. Biol. Chem.*, **2004**, 279 (13), 12325-12336.

[176] Campbell, J.D.; Koike, K.; Moreau, C.; Sansom, M.S.P.; Deeley, R.G.; Cole, S.P.C. Molecular modeling correctly predicts the functional importance of Phe594 in transmembrane helix 11 of the multidrug resistance protein, MRP1 (ABCC1). *J. Biol. Chem.*, **2004**, 279 (1), 463-468.

[177] Conseil, G.; Deeley, R.G.; Cole, S.P.C. Role of two adjacent cytoplasmic tyrosine residues in MRP1 (ABCC1) transport activity and sensitivity to sulfonylureas. *Biochem. Pharmacol.*, **2005**, 69 (3), 451-461.

[178] Conseil, G.; Deeley, R.G.; Cole, S.P.C. Functional importance of three basic residues clustered at the cytosolic interface of transmembrane helix 15 in the multidrug and organic anion transporter MRP1 (ABCC1). *J. Biol. Chem.*, **2006**, 281 (1), 43-50.

[179] Conseil, G.; Rothnie, A.J.; Deeley, R.G.; Cole, S.P. Multiple roles of charged amino acids in cytoplasmic loop 7 for expression and function of the multidrug and organic anion transporter MRP1 (ABCC1). *Mol. Pharmacol.*, **2009**, 75 (2), 397-406.

[180] DeGorter, M.K.; Conseil, G.; Deeley, R.G.; Campbell, R.L.; Cole, S.P.C. Molecular modeling of the human multidrug resistance protein 1 (MRP1/ABCC1). *Biochem. Biophys. Res. Commun.*, **2008**, 365 (1), 29-34.

[181] Haimeur, A.; Conseil, G.; Deeley, R.G.; Cole, S.P.C. Mutations of charged amino acids in or near the transmembrane helices of the second membrane spanning domain differentially affect the substrate specificity and transport activity of the multidrug resistance protein MRP1 (ABCC1). *Mol. Pharmacol.*, **2004**, 65 (6), 1375-1385.

[182] Ito, K.; Olsen, S.L.; Qiu, W.; Deeley, R.G.; Cole, S.P. Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport. *J. Biol. Chem.*, **2001**, 276 (19), 15616-15624.

[183] Ryu, S.; Kawabe, T.; Nada, S.; Yamaguchi, A. Identification of basic residues involved in drug export function of human multidrug resistance-associated protein 2. *J. Biol. Chem.*, **2000**, 275 (50), 39617-39624.

[184] Maeno, K.; Nakajima, A.; Conseil, G.; Rothnie, A.; Deeley, R.G.; Cole, S.P.C. Molecular basis for reduced estrone sulfate transport and altered modulator sensitivity of transmembrane helix (TM) 6 and TM17 mutants of multidrug resistance protein 1 (ABCC1). *Drug Metab. Dispos.*, **2009**, 37 (7), 1411-1420.

[185] Situ, D.; Haimeur, A.; Conseil, G.; Sparks, K.E.; Zhang, D.; Deeley, R.G.; Cole, S.P.C. Mutational analysis of ionizable residues proximal to the cytoplasmic interface of membrane spanning domain 3 of the multidrug resistance protein, MRP1 (ABCC1): glutamate 1204 is important for both the expression and catalytic activity of the transporter. *J. Biol. Chem.*, **2004**, 279 (37), 38871-38880.

[186] Zhang, D.W.; Cole, S.P.C.; Deeley, R.G. Identification of a nonconserved amino acid residue in multidrug resistance protein 1 important for determining substrate specificity: Evidence for functional interaction between transmembrane helices 14 and 17. *J. Biol. Chem.*, **2001**, 276 (37), 34966-34974.

[187] Zhang, D.W.; Cole, S.P.; Deeley, R.G. Identification of an amino acid residue in multidrug resistance protein 1 critical for conferring resistance to anthracyclines. *J. Biol. Chem.*, **2001**, 276 (16), 13231-13239.

[188] Zhang, D.; Cole, S.P.C.; Deeley, R.G. Determinants of the substrate specificity of multidrug resistance protein 1: role of amino acid residues with hydrogen bonding potential in predicted transmembrane helix 17. *J. Biol. Chem.*, **2002**, 277 (23), 20934-20941.

[189] Zhang, D.W.; Gu, H.M.; Situ, D.; Haimeur, A.; Cole, S.P.C.; Deeley, R.G. Functional importance of polar and charged amino acid residues in transmembrane helix 14 of multidrug resistance protein 1 (MRP1/ABCC1): Identification of an aspartate residue critical for conversion from a high to low affinity substrate binding state. *J. Biol. Chem.*, **2003**, 278 (46), 46052-46063.

[190] Zhang, D.; Nunoya, K.; Vasa, M.; Gu, H.; Theis, A.; Cole, S.P.C.; Deeley, R.G. Transmembrane helix 11 of multidrug resistance protein 1 (MRP1/ABCC1): identification of polar amino acids important for substrate specificity and binding of ATP at nucleotide binding domain 1. *Biochemistry*, **2004**, 43 (29), 9413-9425.

[191] Wittgen, H.G.M.; Van Den Heuvel, J.J.M.W.; Krieger, E.; Schaftenaar, G.; Russel, F.G.M.; Koenderink, J.B. Phenylalanine 368 of multidrug resistance-associated protein 4 (MRP4/ABCC4) plays a crucial role in substrate-specific transport activity. *Biochem. Pharmacol.*, **2012**, 84 (3), 366-373.

[192] Zhang, D.; Nunoya, K.; Vasa, M.; Gu, H.; Cole, S.P.C.; Deeley, R.G. Mutational analysis of polar amino acid residues within predicted transmembrane helices 10 and 16 of multidrug resistance protein 1 (ABCC1): effect on substrate specificity. *Drug Metab. Dispos.*, **2006**, 34 (4), 539-546.

[193] Banerjee, M.; Marensi, V.; Conseil, G.; Le, X.C.; Cole, S.P.C.; Leslie, E.M. Polymorphic variants of MRP4/ABCC4 differentially modulate the transport of methylated arsenic metabolites and physiological organic anions. *Biochem. Pharmacol.*, **2016**, 120, 72-82.

[194] Notredame, C., Higgins, D. G., & Heringa, J.; Notredame, C.; Higgins, D.G.; Heringa, J. T-coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.*, **2000**, 302 (1), 205-217.

[195] Akanuma, S. i.; Hosoya, K. i.; Ito, S.; Tachikawa, M.; Terasaki, T.; Ohtsuki, S. Involvement of multidrug resistance-associated protein 4 in efflux transport of prostaglandin E2 across mouse blood-brain barrier and Its inhibition by intravenous administration of cephalosporins. *J. Pharmacol. Exp. Ther.*, **2010**, 333 (3), 912-919.

[196] Zhang, D.; Gu, H.; Vasa, M.; Muredda, M.; Cole, S.P.C.; Deeley, R.G. Characterization of the role of polar amino acid residues within predicted transmembrane helix 17 in determining the substrate specificity of multidrug resistance protein 3. *Biochemistry*, **2003**, 42 (33), 9989-10000.

[197] Biasini, M.; Bienert, S.; Waterhouse, A.; Arnold, K.; Studer, G.; Schmidt, T.; Kiefer, F.; Gallo Cassarino, T.; Bertoni, M.; Bordoli, L.; Schwede, T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res., 2014, 42 (Web Server issue), W252-258. www.ncbi.nlm.nih.gov/pmc/articles/PMC4086089/

[198] Bhattacharya, D.; Nowotny, J.; Cao, R.; Cheng, J. 3Drefine: an interactive web server for efficient protein structure refinement. *Nucleic Acids Res.*, **2016**, 44 (W1), W406-409. www.ncbi.nlm.nih.gov/pmc/articles/PMC4987902/

[199] Best, R.B.; Zhu, X.; Shim, J.; Lopes, P.E.M.; Mittal, J.; Feig, M.; Mackerell, A.D. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone φ, ψ and side-chain χ(1) and χ(2) dihedral angles. *J. Chem. Theory Comput.*, **2012**, 8 (9), 3257-3273.

[200] Phillips, J.C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R.D.; Kalé, L.; Schulten, K. Scalable molecular dynamics with NAMD. *J. Comput. Chem.*, **2005**, 26 (16), 1781-1802.

[201] Benkert, P.; Biasini, M.; Schwede, T. Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, **2011**, 27 (3), 343-350.

[202] Webb, B.; Sali, A. Protein Structure Modeling with MODELLER. *Methods Mol. Biol.*, **2017**, 1654, 39-54.

[203] Lovell, S.C.; Davis, I.W.; Arendall, W.B.; de Bakker, P.I.W.; Word, J.M.; Prisant, M.G.; Richardson, J.S.; Richardson, D.C. Structure validation by Calpha geometry: phi,psi and Cbeta deviation. *Proteins*, **2003**, 50 (3), 437-450.

[204] Yu, M.; Ocana, A.; Tannock, I.F. Reversal of ATP-binding cassette drug transporter activity to modulate chemoresistance: why has it failed to provide clinical benefit? *Cancer Metastasis Rev.*, **2013**, 32 (1-2), 211-227.

[205] Yin, J.; Wang, J. Renal drug transporters and their significance in drug-drug interactions. *Acta Pharm. Sin. B*, **2016**, 6 (5), 363-373.

[206] Sundkvist, E.; Jaeger, R.; Sager, G. Pharmacological characterization of the ATP-dependent low K(m) guanosine 3',5'-cyclic monophosphate (cGMP) transporter in human erythrocytes. *Biochem. Pharmacol.*, **2002**, 63 (5), 945-949.

[207] Wu, C.P.; Klokouzas, A.; Hladky, S.B.; Ambudkar, S. V; Barrand, M.A. Interactions of mefloquine with ABC proteins, MRP1 (ABCC1) and MRP4 (ABCC4) that are present in human red cell membranes. *Biochem. Pharmacol.*, **2005**, 70 (4), 500-510.

[208] El-Sheikh, A.A.K.; Greupink, R.; Wortelboer, H.M.; van den Heuvel, J.J.M.W.; Schreurs, M.; Koenderink, J.B.; Masereeuw, R.; Russel, F.G.M. Interaction of immunosuppressive drugs with human organic anion transporter (OAT) 1 and OAT3, and multidrug resistance-associated protein (MRP) 2 and MRP4. *Transl. Res.*, **2013**, 162 (6), 398-409.

[209] Mutsaers, H.A.M.; van den Heuvel, L.P.; Ringens, L.H.J.; Dankers, A.C.A.; Russel, F.G.M.; Wetzels, J.F.M.; Hoenderop, J.G.; Masereeuw, R. Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. *PLoS One*, **2011**, 6 (4), e18438. journals.plos.org/plosone/article?id=10.1371/journal.pone.0018438

[210] Kawase, A.; Yamamoto, T.; Egashira, S.; Iwaki, M. Stereoselective inhibition of methotrexate excretion by glucuronides of nonsteroidal anti-inflammatory drugs via multidrug resistance proteins 2 and 4. *J. Pharmacol. Exp. Ther.*, **2016**, 356 (2), 366-374.

[211] Köck, K.; Ferslew, B.C.; Netterberg, I.; Yang, K.; Urban, T.J.; Swaan, P.W.; Stewart, P.W.; Brouwer, K.L.R. Risk factors for development of cholestatic drug-induced liver injury: Inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab. Dispos.*, **2014**, 42 (4), 665-674.

[212] Dallas, S.; Schlichter, L.; Bendayan, R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia. *J. Pharmacol. Exp. Ther.*, **2004**, 309 (3), 1221-1229.

[213] Pratt, S.; Chen, V.; Perry, W.I.; Starling, J.J.; Dantzig, A.H. Kinetic validation of the use of carboxydichlorofluorescein as a drug surrogate for MRP5-mediated transport. *Eur. J. Pharm. Sci.*, **2006**, 27 (5), 524-532.

[214] El-Sheikh, A.A.K.; Van Den Heuvel, J.J.M.W.; Koenderink, J.B.; Russel, F.G.M. Effect of hypouricaemic and hyperuricaemic drugs on the renal urate efflux transporter, multidrug resistance protein 4. *Br. J. Pharmacol.*, **2009**, 155 (7), 1066-1075.

[215] Aronsen, L.; Orvoll, E.; Lysaa, R.; Ravna, A.W.; Sager, G. Modulation of high affinity ATP-dependent cyclic nucleotide transporters by specific and non-specific cyclic nucleotide phosphodiesterase inhibitors. *Eur. J. Pharmacol.*, **2014**, 745, 249-253.

[216] Sager, G.; Ørvoll, E.Ø.; Lysaa, R.A.; Kufareva, I.; Abagyan, R.; Ravna, A.W. Novel cGMP efflux inhibitors identified by virtual ligand screening (VLS) and confirmed by experimental studies. *J. Med. Chem.*, **2012**, 55 (7), 3049-3057.

[217] Cheung, L.; Flemming, C.L.; Watt, F.; Masada, N.; Yu, D.M.T.; Huynh, T.; Conseil, G.; Tivnan, A.; Polinsky, A.; Gudkov, A. V; Munoz, M.A.; Vishvanath, A.; Cooper, D.M.F.; Henderson, M.J.; Cole, S.P.C.; Fletcher, J.I.; Haber, M.; Norris, M.D. High-throughput screening identifies Ceefourin 1 and Ceefourin 2 as highly selective

inhibitors of multidrug resistance protein 4 (MRP4). *Biochem. Pharmacol.*, **2014**, 91 (1), 97-108.

[218] Csandl, M.A.; Conseil, G.; Cole, S.P.C. Cysteinyl Leukotriene Receptor 1/2 Antagonists Nonselectively Modulate Organic Anion Transport by Multidrug Resistance Proteins (MRP1-4). *Drug Metab. Dispos.*, **2016**, 44 (6), 857-866.

[219] Wobst, I.; Ebert, L.; Birod, K.; Wegner, M.S.; Hoffmann, M.; Thomas, D.; Angioni, C.; Parnham, M.J.; Steinhilber, D.; Tegeder, I.; Geisslinger, G.; Grösch, S. R-flurbiprofen traps prostaglandins within cells by inhibition of multidrug resistance-associated protein-4. *Int. J. Mol. Sci.*, **2017**, 18 (1), 1-16.

[220] Zhou, S.; Wang, L.; Di, Y.M.; Xue, C.C.; Duan, W. Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. *Curr. Med. Chem.*, **2008**, 15 (20), 1981-2039.

[221] Cheung, L.; Yu, D.M.T.; Neiron, Z.; Failes, T.W.; Arndt, G.M.; Fletcher, J.I. Identification of new MRP4 inhibitors from a library of FDA approved drugs using a high-throughput bioluminescence screen. *Biochem. Pharmacol.*, **2015**, 93 (3), 380-388.

[222] Alexandre, C.; Larry A., S.; Dominique, P. Method for cancer cell reprograming. US Patent 9,314,460 B. April 9, 2014.

[223] Montanari, F.; Ecker, G.F. Prediction of drug-ABC-transporter interaction — Recent advances and future challenges. *Adv. Drug Deliv. Rev.*, **2015**, 86, 17-26.

[224] Fukuda, Y.; Takenaka, K.; Sparreboom, A.; Cheepala, S.B.; Wu, C.-P.; Ekins, S.; Ambudkar, S. V.; Schuetz, J.D. Human immunodeficiency virus protease inhibitors interact with ATP binding cassette transporter 4/multidrug resistance protein 4: a basis for unanticipated enhanced cytotoxicity. *Mol. Pharmacol.*, **2013**, 84 (3), 361-371.

[225] Welch, M.A.; Köck, K.; Urban, T.J.; Brouwer, K.L.R.; Swaan, P.W. Toward predicting drug-induced liver injury: parallel computational approaches to identify multidrug resistance protein 4 and bile salt export pump inhibitors. *Drug Metab. Dispos.*, **2015**, 43 (5), 725-734.

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