



Review

ABC transporters and their role in nucleoside and nucleotide drug resistance

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ABSTRACT

ATP-binding cassette (ABC) transporters confer drug resistance against a wide range of chemotherapeutic agents, including nucleoside and nucleotide based drugs. While nucleoside based drugs have been used for many years in the treatment of solid and hematological malignancies as well as viral and autoimmune diseases, the potential contribution of ABC transporters has only recently been recognized. This neglect is likely because activation of nucleoside derivatives require an initial carrier-mediated uptake step followed by phosphorylation by nucleoside kinases, and defects in uptake or kinase activation were considered the primary mechanisms of nucleoside drug resistance. However, recent studies demonstrate that members of the ABCC transporter subfamily reduce the intracellular concentration of monophosphorylated nucleoside drugs. In addition to the ABCC subfamily members, ABCG2 has been shown to transport nucleoside drugs and nucleoside-monophosphate derivatives of clinically relevant nucleoside drugs such as cytarabine, cladribine, and clofarabine to name a few. This review will discuss ABC transporters and how they interact with other processes affecting the efficacy of nucleoside based drugs.

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1. ABC transporters—historical background and transport mechanisms

Successful chemotherapy depends on achieving an adequate amount of a drug at an intracellular target. However, intracellular drug accumulation is the product of import and export processes that we now recognize occurs by families of membrane transporters. However, the importance of drug export was not

recognized as a process affecting drug accumulation at first, and in cancer cells, reduced drug accumulation was mostly attributed to decreased uptake rather than export as a mechanism of drug resistance [1–3]. However, direct evidence for active export as a means of drug resistance was first demonstrated by Dano in 1973. Dano observed that “in resistant cells there is an outward flow of daunomycin against an electrochemical gradient that is carrier mediated, and dependent on the energy metabolism” [4]. This early theory accurately presaged the properties of the yet to be identified ATP-binding cassette (ABC) transporters. ABC transporters mediate the ATP-dependent, and unidirectional transmembrane extrusion of various compounds, both endogenous and exogenous (Table 1). Among the 48 ABC transporters identified in humans, those localized to the plasma membrane reduce intracellular drug concentrations by export [5,6]. While ABC

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Table 1

Function and diseases/phenotypes caused by variation or deficiency of ABC genes.

Gene	Alias	Location	Mendelian disorder/phenotype	Function/drugs
ABCA1	ABC1	9q31.1	Tangier disease, FHLDL	Cholesterol efflux
ABCA2	ABC2	9q34	Early onset of Alzheimer disease?	Drug resistance
ABCA3	ABC3	16p13.3	Lung surfactant deficiency-NRD, ILD	Homeostasis of pulmonary surfactant
ABCA4	ABCR	1p22.1-p21	Stargards/FFM, RP, CRD	Processing or transport of all <i>trans</i> -retinal in retinoid cycle
ABCA5		17q24		
ABCA6		17q24		Putative role in lipid homeostasis
ABCA7		19p13.3		
ABCA8		17q24		
ABCA9		17q24		Putative role in lipid homeostasis
ABCA10		17q24		Lipid transport
ABCA12		2q34	Lamellar and harlequin ichthyosis	Lipid trafficking in keratinocytes
ABCA13		7p11-q11		
ABCB1	P-GP, MDR	7p21		Multidrug resistance
ABCB2	TAP1	6p21	Immune deficiency	Peptide transport
ABCB3	TAP2	6p21	Immune deficiency	Peptide transport
ABCB4	MDR3	7q21.1	PFIC-3	PC transport
ABCB5		7p14		Putative role in melanocytes and melanoma
ABCB6	MTABC3, UMAT, PRP	2q36		Porphyrin transport
ABCB7	ABC7	Xq12-q13	XLSA/A	Fe-S cluster
ABCB8	MABC1	7q36		Protection against oxidative stress
ABCB9		12q24		
ABCB10	MTABC2, ABC-me	1q42		Putative heme transport
ABCB11	SPGP, BSEP	2q24	PFIC-2	Bile salt transport
ABCC1	MRP1	16p13.1		Multidrug resistance
ABCC2	MRP2	10q24	Dubin-Johnson syndrome	Organic anion transport
ABCC3	MRP3	17q21.3		Drug resistance
ABCC4	MRP4	13q32		Nucleoside/nucleotide transport
ABCC5	MRP5	3q27		Nucleoside/nucleotide transport
ABCC6	MRP6	16p13.1	Pseudoxanthoma elasticum	
ABCC7	CFTR	7q31.2	Cystic fibrosis	Chloride ion channel
ABCC8	SUR1	11p15.1	FPHHI	Sulfonylurea receptor, ion channel regulator
ABCC9	SUR2	12p12.1	DCVT	Sulfonylurea receptor ion channel regulator
ABCC10	MRP7	6p21		
ABCC11	MRP8	16q11-q12	Dry ear wax	Nucleoside/nucleotide transport
ABCC12	MRP9	16q11-q12		
ABCD1	ALD	Xq28	ALD	VLCHA
ABCD2	ALDL1, ALDR	12q11-q12		
ABCD3	PXMP1, PMP70	1p22-p21		Pristanic acid, DHCA/THCA
ABCD4	PMP69, P70R	14q24.3		
ABCE1	OABP	4q31		
ABCF1	ABC50	6p21.33		
ABCF2		7q36		
ABCF3		3q25		
ABCG1	ABC8, white	21q22.3		Cholesterol transport
ABCG2	BCRP, MXR, ABCP	4q22	Porphyria (in mouse)	Multidrug resistance, protective role (hypoxia, etc.)
ABCG4	White2	11q23		Cholesterol transport
ABCG5	White3	2p21	Sitosterolemia	Sterol transport
ABCG8		2p21	Sitosterolemia	Sterol transport

FHLDL, familial hypoapoptroteinemia; NRD, neonatal respiratory distress; ILD, pediatric interstitial lung disease; FFM, fundus flavimaculatus; RP, retinitis pigmentosa 19; CRD, cone-rod dystrophy; PFIC, progressive familial intrahepatic cholestasis; XLSA/A X-linked sideroblastic anemia and ataxia; FPHHI, familial persistent hyperinsulinemic hypoglycemia of infancy; DCVT, dilated cardiomyopathy with ventricular tachycardia; ALD, adrenoleukodystrophy; VLCHA, very long-chain fatty acids; DHCA, dihydroxycholestanic acid; THCA, trihydroxycholestanic acid [5,40,128–136].

transporters interact with an array of structurally diverse chemotherapeutic agents and their metabolites to produce a multidrug resistance (MDR) phenotype, this review will primarily focus on nucleoside-based drugs and their relationship with ABC transporters.

The first member of the ABC transporter superfamily was identified in 1976 by Ling and colleagues as a 170-kDa membrane glycoprotein overexpressed in colchicine resistant cell lines and referred to as a *glycoprotein* that reduces drug permeability (P-gp) [7]. Subsequently multiple laboratories cloned and identified the gene that encoded P-gp (*P-gp/multidrug resistance (Mdr)* aka ABCB1) which was often amplified in drug resistant cells [8–13]. For more details the reader is referred to recent reviews on P-gp [14–18]. The 48 human ABC proteins are separated into seven subfamilies (ABCA-to-ABCG) based on their gene structure, amino acid sequence, domain organization, and phylogenetic analysis [19]. All members except for ABCE and ABCF are localized to

various membranes of cellular organelles (referred to ABC transporters hereafter). Insights into the biological function and endogenous substrates of many ABC transporters has been revealed by genetic deficiencies (Table 1). While some ABC transporters have trivial names that reflect, in some cases, their tissue of origin a new nomenclature was established by the Human Genome Organization guidelines in 1999 and P-gp for example is now referred to as ABCB1 (<http://www.genenames.org/genefamily/abc.php>).

Many years after the initial characterization of P-gp, two additional ABC transporters were identified by screening anthracycline resistant cell lines for drug resistance that was independent of functional P-gp. The first was identified by the laboratories of Cole and Deeley in 1991 and is referred to as multidrug resistance protein 1 (MRP1/ABCC1) [20]. The second breast cancer resistance protein (BCRP/ABCG2) was independently and almost concurrently identified by three laboratories each employing different

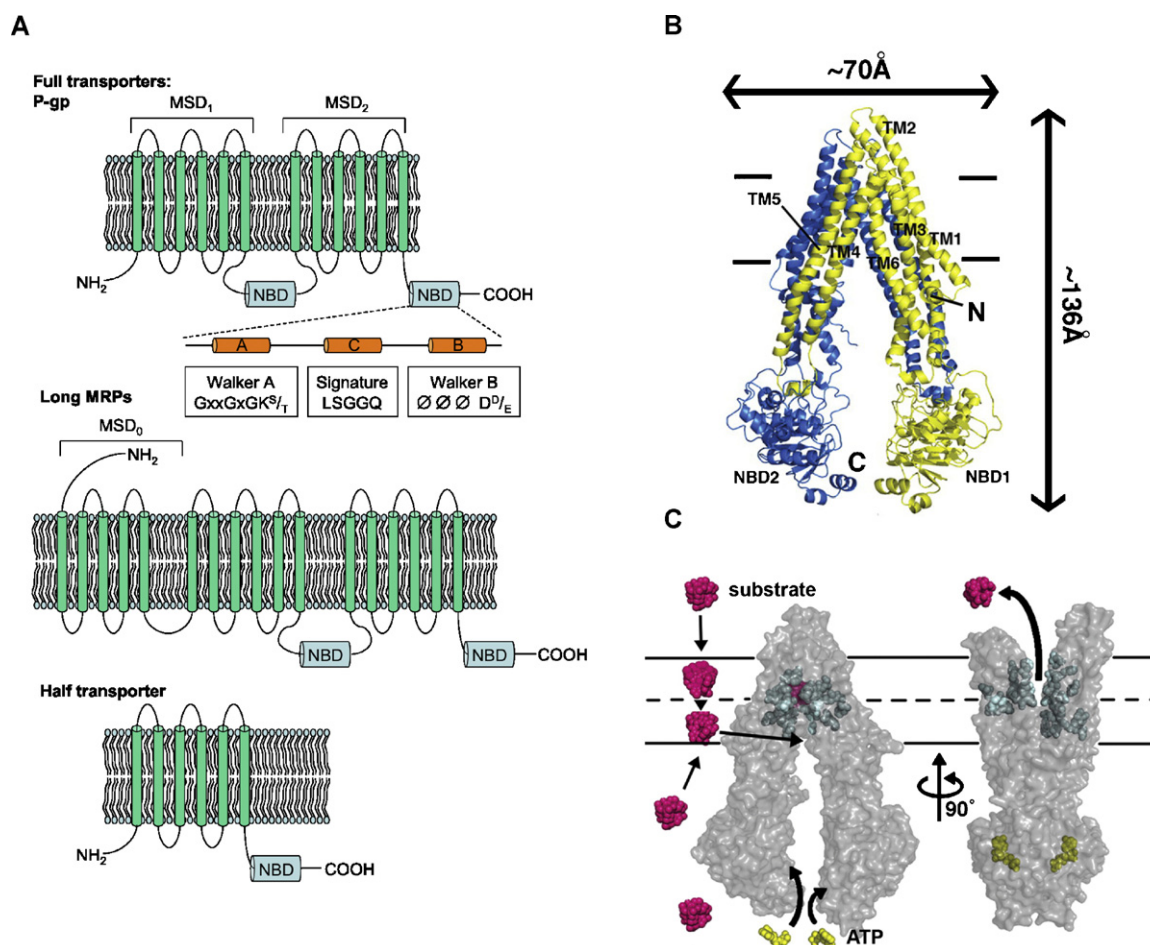


Fig. 1. Predicted two-dimensional topology of ABC transporters and model of ABC transporter mechanism. (A) The two membrane spanning domains (MSDs) and nucleotide binding domains (NBDs) are represented by P-gp and some MRP (ABCC) family members with some MRPs containing an additional N-terminal extension (MSD₀), while some ABC transporters have only one MSD and NBD. (B) A structure of P-gp in the inward facing conformation showing the transmembrane domains (TMs) and the NBDs. (C) Transporter mechanism showing a large conformational change as a result of ATP binding. Substrates can enter by either the cytoplasmic face or extracellular face of an ABC transporter. Adopted from [43]. Reprinted with permission from AAAS.

strategies and assigning their own unique names (e.g., MXR, ABCP) [21–23]. Currently, P-gp (ABCB1), MRP1 (ABCC1) and ABCG2 are considered to be the “major” drug transporters for their roles in MDR phenotypes seen in many tumors and cell lines [24].

ABC proteins are characterized by Walker A and B motifs as well as a signature motif, which is unique to this superfamily, within the nucleotide-binding domain (NBD) (see Fig. 1A) [11,25–29]. A core unit of ABC transporters contains six or more α -helices in a membrane-spanning domain (MSD) [30,31]. A functional ABC transporter requires two core units comprised of two NBDs and two MSDs [32–35]. A simple classification of ABC transporters is based upon the number of core units encoded in a single polypeptide (Fig. 1A). ABC transporters are classified as “full” transporters if they contain two core units in tandem (MSD-NBD-MSD-NBD) that are active alone, whereas “half” transporters have only one core unit (an NBD and MSD), and therefore require to form either obligate homo- or hetero-dimers [36–40]. ABCD and ABCG subfamilies consist of half transporters, whereas both full and half transporters can be found in the ABCB subfamily. A subset of the ABCC subfamily members contains an amino terminal extension comprised of five additional transmembrane α -helices (termed MSD₀). Recent electron cryomicroscopy studies of MRP1 suggested that MSD₀ interacts with transmembrane helices from MSD₁ and MSD₂. These interactions might play a role in substrate recognition [41].

Recent structural studies of ABC transporters based on a bacterial protein, Sav1866 and a murine P-gp provided insights into the topology of the ABC transporter drug-binding site [42,43]. The MSDs appear to form an internal cavity that binds substrates through multiple interactions (Fig. 1B). For instance, studies with Sav1866 suggest that aromatic residues as well as charged amino acid residues provide an internal binding cavity that neutralizes charged compounds. This “drug-binding” pocket is thought to be highly flexible containing several “mini-pockets” that can accommodate not just structurally diverse classes of drugs, but more than one drug [44]. However, in the absence of additional ABC transporter structures, predicting whether a drug is a substrate for an ABC transporter is daunting and requires rigorous concurrent use of homology models, *in silico* prediction and biochemical studies.

The structures of the ABC transporters coupled with our knowledge of their transport biochemistry provide the following insights into the ABC transport mechanism [42,43,45–47]. The MSDs create an inverted “V” shape to accept substrates from either the cytoplasmic side or extracellular space with substrate binding increasing the affinity of the NBDs for ATP (see Fig. 1B) [48,49]. The binding and hydrolysis of ATP, by the NBDs, induces a conformational change in the MSDs yielding an outward opening permitting substrate/drug release into the extracellular milieu [50] (Fig. 1C). Upon ATP hydrolysis, the NBD dimer disengages and the

conformation of the transporter is restored to its original inward facing orientation possessing a high affinity for substrate. Further structural studies reveal interactions between the NBDs as well as the cytoplasmic loops. These interactions may be a key to understanding the transmission of conformational changes from MSDs to NBDs and vice versa.

In addition to their roles in the MDR phenotype, ABC transporters have “natural” roles in restricting drug and toxic compounds penetration by creating barriers between systemic blood circulation and organs such as: brain, gastrointestinal tract, and placenta [51–55]. This “protective role” is not their only function. ABC transporters have diverse physiological roles in regulating endogenous molecules affecting multiple pathways such as lipid and bile acid synthesis, antigen presentation, heme and iron homeostasis and signaling molecules such as cyclic nucleotides and ions, etc. [137]. The crucial role that ABC transporters play in maintaining cellular homeostasis is in part illustrated by the fact that almost a third of the defective human ABC genes are associated with a disease phenotype [5]. Therefore, an understanding of their normal biological roles and structures will minimize unexpected adverse effects when designing inhibitors to overcome the MDR caused by transporters such as P-gp, MRP1 or ABCG2.

2. Nucleoside-based drugs-transporters and therapeutics

Structural analogs of natural nucleosides effectively insinuate themselves into cells by using the normal transport routes of endogenous nucleosides [56]. Subsequently, these nucleosides are activated by phosphorylation through endogenous nucleoside and nucleotide kinases. Mechanistically these nucleoside analogs are considered “antimetabolites” because they disrupt biosynthetic steps in DNA or RNA synthesis. Nucleoside-derived drugs are not only used as cytotoxic agents in the treatment of solid and hematological malignancies, but are also widely used as antivirals and immunosuppressants [57–59]. For example, nucleoside-derivatives used in the antiviral therapy typically inhibit reverse transcriptase by incorporation into the DNA chain, and due to their unnatural structure, terminate further chain elongation.

The biochemical properties of uptake carriers for nucleosides were well established prior to their identification and cloning [60,61,56]. While the solute carrier (SLC) superfamily consists of more than 300 members assigned to 51 subfamilies (SLC1–51), only two structurally unrelated but well defined nucleoside subfamily members exist, SLC28 and SLC29 [62]. These carriers mediate the uptake of natural nucleosides with their alternative names indicating their function: the cation ion symporter known as a concentrative nucleoside transporter (CNT, aka SLC28) [63,64] and the facilitated diffusion carrier known as the equilibrative nucleoside transporter (ENT, aka SLC29) [65,66], respectively. The three CNT members have different substrate specificity where CNT1 prefers pyrimidines, CNT2 accepts purine, and CNT3 transports both pyrimidine and purine molecules [64,67–69]. The transport of nucleosides by CNTs is coupled to a sodium - gradient and is thereby electrogenic. Members of the ENT subfamily exhibit broad substrate specificity for nucleoside-derived molecules with ENT2 also transporting the precursors of nucleosides, the nucleobases [66,70–73]. The ENT mediated direction of transport is dictated by nucleoside concentration. Therefore a high extracellular nucleoside concentration favors movement into the cell, conversely a large concentration of intracellular nucleoside switches transport to an outward direction, thus demonstrating ENTs are bidirectional.

The mRNA levels of ENT1 are highly variable among leukemic patients suggesting transcriptional regulation. The few polymorphic genetic variants reported for ENT1 do not affect transport [74]

or require further investigation [75]. Both CNTs and ENTs exhibit a wide tissue distribution with an expression at the apical surface of epithelia, suggesting that they play an important role in uptake of nucleoside-derivatives administered orally [64,66].

Although SLC22 transporters do not typically transport natural nucleosides, some family members especially organic anion and organic cation transporters (OAT, OCT, respectively) appear to transport nucleoside analogs such as thiopurines, 5-FU and many nucleoside-based antivirals [76–78].

Nucleoside drugs require metabolic activation by phosphorylation before interacting with their intracellular targets [79]. Some purine and pyrimidine-based drugs are phosphorylated by deoxycytidine kinase (dCK), whereby nucleobase drugs such as the thiopurines and 5-fluorouracil (5-FU) can be converted to nucleoside monophosphates by specific phosphoribosyltransferases that add a ribosyl phosphate group [80,81]. Subsequently, additional phosphorylation by kinases yields the nucleoside triphosphates. The nucleoside triphosphate form can be incorporated into growing DNA or RNA chains leading to inhibition of DNA polymerases, ligases, and/or endonucleases [82–85]. The nucleotide-derivatives can also interfere with de novo nucleotide biosynthesis (e.g., the 5-FU nucleotide, 5-FdUMP inhibits thymidylate synthase) or through other modes of action such as inhibiting ribonucleotide reductase or depleting endogenous nucleotide pools by feedback mechanisms [86,87]. Analogs of natural purines and pyrimidines are used as templates to design chemotherapeutic drugs (Table 2). Many of these drugs have been very successful in treating or managing a variety of hematological malignancies as well as solid tumors and viral diseases. Specifically, 6-MP is effective in pediatric acute lymphoblastic leukemia (ALL) [88], and cytarabine and clofarabine are used as a frontline therapy for acute megakaryoblastic leukemia (AML) (Table 2) [89,90].

3. Classical nucleoside resistance mechanisms

Tumors acquire resistance to nucleoside-derivatives by multiple mechanisms with many studies showing reduction in the intracellular concentration of phosphorylated metabolites. To achieve this, two classical mechanisms have been reported: defective nucleoside kinase activity that occurs secondary to mutation or impaired uptake. While other potential mechanisms of chemotherapeutic resistance such as alterations in amounts of a drug's target protein or induction of drug metabolism pathways have been identified, these will not be discussed in detail here.

Several enzymes are responsible for the intracellular phosphorylation of nucleoside drugs (nucleoside kinases). One example is deoxycytidine kinase (dCK), which phosphorylates clinically important nucleosides such as cytarabine, gemcitabine, cladribine, fludarabine, and clofarabine (see Table 2). Phosphorylation of nucleosides to their monophosphates is often a rate-limiting step in the activation of these drugs. Due to its important role in activation of the aforementioned drugs, the genetics of dCK has been extensively studied. The expression of dCK shows tissue specific variation, which could impact host toxicity. Further, dCK expression in tumor cells also varies [91,92]. While transcription factors regulating dCK can impact dCK expression, genomic variation in dCK has revealed more than 60 single nucleotide polymorphisms (SNP). However, among these only three SNP's produce amino acid changes (nonsynonymous SNP's) [93]. Notably, these nonsynonymous SNP's in dCK produce proteins with reduced kinase activity compared to the wild type protein. These findings suggest that ethnic differences in variant dCK might produce different responses to chemotherapy relying on dCK [93]. In addition, acquired resistance to cytarabine is

Table 2
Relationship between ABC transporters and nucleoside cancer drugs and their metabolites.

Drugs	Transporters MRP4		MRP5		MRP7		MRP8		ABCG2		Other factors	
	Resistance	Transport	Resistance	Transport	Resistance	Transport	Resistance	Transport	Resistance	Transport	Uptake	Kinase
Purine												
Mercaptopurine	X ^a	X ^a	X	X	NC ^b				X		CNT3, ENT2, OAT1,3	Phosphoribosyl-transferase
Thioguanine	X		X		NC		NC		X			
Thiopurine metabolites		X		X					X			
Cladribine	X						NC		X	X	CNT2,3 ENT1,2	dCK
Cladribine-MP										X		
Fludarabine	NC		NC		NC						CNT2, 3 ENT1,2	dCK
Clofarabine									X	X	CNT2,3 ENT1,2	dCK
Clofarabine-MP										NT ^c		
PMEA (antiviral)	X	X	X	X	X	X	X	X	X	X		
Pyrimidine												
Cytarabine	NC		NC		X	X	NC, X				CNT1,3 ENT1,2	dCK
Cytarabine-MP								X				
Gemcitabine	NC		NC, X		X		NC, X		NC		CNT1,3 ENT1,2,3	dCK
Fluorouridine												
5-Fluorouracil	NC			NT	NC		X	NT, X			OAT2,3	Phosphoribosyl-transferase
5-FdUrd				NT			X	NT				
5-dFUrd					NC		X					
5-FdUMP				X				X				
Reference	[99,105,106,109]		[105,108–111]		[115]		[112–114]		[117–119]			

AZT, azathiopurine; MP, monophosphate; 5-FdUrd, 5'-fluoro-2'-deoxyuridine; 5-dFUrd, 5'-deoxy-5'-fluorouridine; 5-FdUMP, 5'-fluoro-2'-deoxyuridine monophosphate; PMEA, 9-(2-phosphonylmethoxyethyl)-adenine.

^a Resistance determined by cytotoxicity assay or transport determined by cellular uptake or membrane vesicles.

^b No resistance observed.

^c No transport detected.

associated with loss of dCK expression or activity thus providing evidence that lack of dCK activity is a *bona fide* mechanism for drug resistance [94–96].

Nucleoside resistance can arise by concurrent changes in more than one cellular factor [97]. For instance, HL-60 cell lines selected for resistance to the nucleosides cladribine or fludarabine exhibited differential changes in dCK activity depending upon which drug they were exposed to. In cells selected for cladribine resistance dCK activity was substantially reduced to ~10% of non-resistant cells whereas fludarabine resistant cells dCK activity was ~60% of non-resistant cells [95]. A logical explanation for the differential effect of these drugs on dCK was that cladribine has a 10-fold greater affinity for dCK than fludarabine. Therefore, the selective pressure to reduce dCK activity was greater for cladribine exposed cells than those exposed to fludarabine. Beside altered dCK, the uptake of radiolabeled nucleosides was also reduced in the cladribine-resistant cells, suggesting an alteration in drug transport as a concurrent mechanism of resistance. Further studies explored the relationship between uptake carriers and dCK using cell lines that acquired either high or low cytarabine resistance [96]. In cells with low resistance, a mutation in the uptake carrier, ENT1 was identified. These cells with mutant ENT1 showed reduced transport activity, but no alteration in dCK activity; therefore, it is likely that the reduced uptake mediated the resistance. In contrast, a cell line that was highly resistant to cytarabine showed no defect in uptake, but a strong reduction in dCK activity, which was due to a mutation in *dCK* gene. One explanation for the resistance by defective uptake being less profound is that at high concentrations of cytarabine the rate of passive diffusion of cytarabine exceeds that of the carrier-mediated uptake [98]. Therefore, resistance mediated by defects in nucleoside uptake carriers might be less effective because an alternate route of drug uptake (passive diffusion) is available.

4. Role of ABC transporters in nucleotide/nucleoside efflux

Initial studies of nucleoside resistance mechanisms might have overlooked the contribution of ABC transporters (Fig. 2). One explanation is that export of nucleosides or nucleotide derivatives cannot be experimentally revealed by simply evaluating uptake or profiling the intracellular nucleotide drug metabolites. Another is that conventional wisdom believed phosphorylated metabolites

were not exported, but were retained in the cell. Furthermore, export of nucleosides or nucleotides would resemble a defect in either nucleoside uptake or phosphorylation; therefore, specific strategies are required to discern their contribution (Fig. 2). Because ABC transporters require cellular energy to export drugs, one approach to uncover the contribution of an ABC transporter to nucleotide or nucleoside export is to evaluate their accumulation after reducing a cell's ATP by treatment with sodium azide and/or deoxyglucose [99]. Under these conditions, a defect in a nucleoside kinase will result in reduced levels of phosphorylated nucleosides, whereas drug accumulation will increase if export occurs by an ABC transporter.

Members of the MRP subfamily (ABCC) are typically described as transporters of organic anionic conjugates such as glucuronide and glutathione. The first evidence that nucleotides could also be substrates for an ABC family member was provided in 1999. MRP4 (ABCC4) was identified as the first ABC transporter capable of exporting nucleoside-monophosphates from mammalian cells [99]. The genesis of this discovery was using cell lines with acquired resistance to the cytotoxic effects of a purine nucleoside-monophosphate derivative (PMEA; 9-(2-phosphonylmethoxyethyl) adenine) that were developed by Fridland and colleagues [100]. These cell lines had an impaired ability to accumulate monophosphorylated nucleosides, and the accumulation defect was reversed by reduction in intracellular ATP. Furthermore, the stability of drug resistance during culture for many generations in the absence of drug suggested an amplification of a gene conferring PMEPA resistance. The mechanistic basis of heritable and stable drug resistance due to amplification of a single gene that was a drug target was elucidated by Schimke and colleagues [101–103]. Typically, in gene amplification the number of copies of the target gene increases at its normal chromosomal address. While Southern blot screening of genomic DNA from PMEPA sensitive and resistant cells revealed selective *MRP4* gene amplification (compared to other ABC transporters), fluorescent in situ hybridization demonstrated that the *MRP4* gene was focally amplified at its chromosomal address of 13q32 [99]. Further studies showing somatic cell-fusions confirmed that resistance to PMEPA was dominant. These results implicated *MRP4* as a *bona fide* drug resistance protein conferring nucleoside resistance, and accordingly, it was demonstrated that *MRP4* exported not only PMEPA, but also azidothymidine monophosphate (AZTMP).

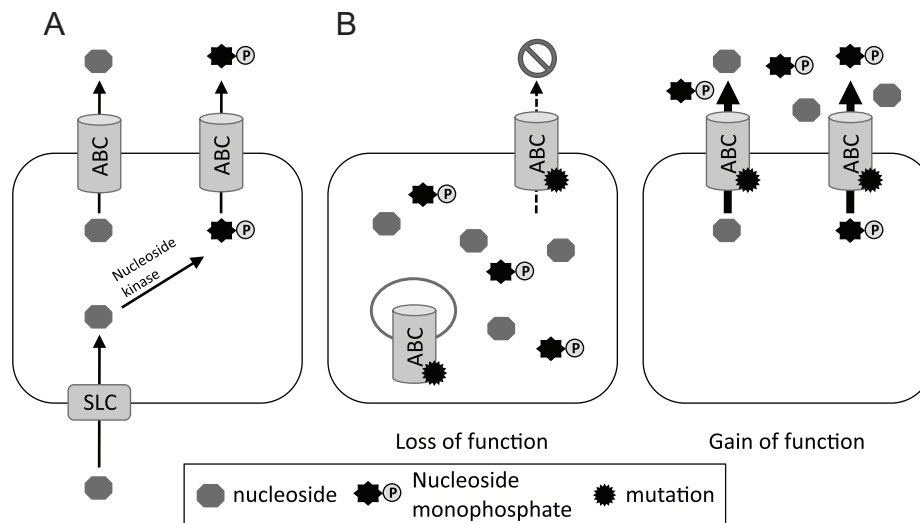


Fig. 2. ABC transporters can confer resistance to nucleosides and single-nucleotide polymorphisms (SNP's) can modify ABC transporter function. (A) The pathway of nucleoside uptake and activation by a nucleoside kinase can be disrupted by an ABC transporter. (B) Single-nucleotide polymorphisms (SNP's) can result in loss of function through mechanisms affecting protein targeting, stability, etc. resulting in "loss of function" but SNP's can also result in enhanced transport activity: "gain of function." The arrow size indicates transport activity.

Subsequent studies confirmed MRP4 role in resistance to nucleoside chemotherapeutic drugs such as the thiopurines, 6-mercaptopurine (6-MP) and 6-thioguanine (TG) by transfecting cells with expression constructs overexpressing MRP4 [104–106]. Mechanistically, thiopurine resistance was attributed to MRP4 mediated export of the thiopurine monophosphate derivatives (thioinosine-monophosphate and thioguanosine-monophosphate). This export was specific for mono-phosphates because di- and tri-phosphates were not exported and indicated that thiopurine monophosphates are major substrates of MRP4 [105]. While it has not been established that MRP4 overexpression contributes to impaired patient response in leukemia therapy, it is worth noting that MRP4 expression is highly variable among pediatric leukemia's at diagnosis [107]. Noteworthy, a T-cell leukemia cell line (CEM-MPS) cultured in increasing concentrations of 6-mercaptopurine overexpressed MRP4 that was associated with acquired resistance to 6-MP and 6 TG. While this cell line overexpressed MRP4, the resistance appeared attributable to additional factors such as reduced expression of the equilibrative transporter (SLC28 family) and the concentrative transporters (SLC29 family) [106]. Notably expression of other candidate nucleoside/nucleotide exporters MRP5 and MRP8 were unchanged.

Other ABC family members such as MRP5, MRP7, and MRP8 may also impact nucleoside-mediated resistance. Overexpression of MRP5 by expression vectors revealed that this closely related transporter conferred resistance to various nucleotide derivatives of thiopurines and PMEA [105,108,109]. To date, however it is unknown if cells that acquire resistance to thiopurines PMEA or other related nucleosides overexpress MRP5. Nonetheless, drug accumulation studies in cells engineered to overexpress MRP5 demonstrated lower levels of PMEA and thiopurine nucleotides, and MRP5 conferred resistance to 6-MP, 6-TG, and PMEA but not to other nucleoside derivatives such as 5-fluorouracil (5-FU) and azathiopurine [108]. Accordingly, HPLC analysis of the intracellular concentrations of thiopurine metabolites revealed reduced concentrations of monophosphate forms. Furthermore, an analysis of the extracellular media for cells exposed to 6-MP showed that MRP5 exported 6-thio-IMP into the medium. Like MRP4, cells overexpressing MRP5 effluxed thiopurine metabolites such as thioinosine monophosphate. It is notable that thioxanthosine monophosphate was only exported by MRP5 and not MRP4. Although MRP4 and MRP5 appear to have differential affinity for these thiopurine metabolites, combined expression of these transporters would be very effective in exporting thiopurine monophosphates out of the cells.

It is notable that in addition to purine analogs, MRP4 and MRP5 also export pyrimidine nucleotides (MRP4 = azidothymidine-monophosphate; MRP5 = 5-fluorodeoxyuridine monophosphate). Although overexpression of MRP5 did not confer resistance to 5-FU in initial reports [108], recent studies demonstrated that cell lines engineered to overexpress MRP5 exports 5-FU monophosphorylated metabolites [110]. Intriguingly, a natural metabolite in pyrimidine biosynthesis, 5-dUMP accumulated in an ATP-dependent manner into inside-out membrane vesicles prepared from the cells expressing MRP5. This suggests that high levels of MRP5 might regulate normal pyrimidine biosynthesis by exporting 5-dUMP. In addition, MRP5 has been implicated in resistance to the deoxycytidine analog, gemcitabine, because MRP5 overexpression confers resistance to gemcitabine. For instance, the relationship between expression of MRP5 and gemcitabine cytotoxicity was inverse in non-small cell lung cancer cells, because siRNA against MRP5 increased sensitivity to the drug [111].

Other MRP family members that have been shown to extrude nucleotides are MRP7 and MRP8. Cell lines transfected with an MRP8 expression vector have been shown to export pyrimidine-based

nucleotides such as 5-fluorodeoxyuridine monophosphate (5-FdUMP) and this is associated with resistance to 5-FU derivatives [112]. These results suggested that MRP8 confers resistance to 5-FU by transporting its active metabolite, 5-FdUMP out of the cells rather than 5-FU itself. Furthermore, in a 5-FU resistant human small-cell lung cancer line, MRP8 mRNA but not MRP1-5 or MRP9 was upregulated [113]. This cell line showed cross-resistance with methotrexate, and reduction of MRP8 by siRNA sensitized the cells to 5-FU. Recent studies have shown that high expression of MRP8 but not MRP4 or MRP5 was associated with a poor treatment outcome for AML patients treated with cytarabine [114], a finding that is associated with reduced cytarabine intracellular metabolites.

Interestingly, MRP family members with three membrane-spanning domains (MSD): MRP1, as well as MRP2 and 3 have yet to be shown to transport nucleoside cancer drugs. However, a recent study suggested that cells transfected with MRP7 expression vectors have modest reductions in cytarabine accumulation and increased cytarabine resistance [115]. While these studies demonstrate that engineered overexpression of some MRP's confers drug resistance, it is unknown if cells acquire MRP7 or MRP8 overexpression as drug resistance develops in therapy.

Another ABC transporter implicated in nucleoside transport is the "half transporter," ABCG2. ABCG2 had been demonstrated to export the natural nucleotide, cyclic GMP [116], however recent studies indicate it transports a wide variety of therapeutically important nucleoside-derivatives used in the treatment of both cancer and viral infections [117–119]. The antiviral PMEA and the chemotherapeutic agent cladribine were recently shown to be ABCG2 substrates. These studies illustrated the substrate overlap between MRP4 and ABCG2, but more importantly showed how endogenous differences in tissue distribution of these transporters affected drug accumulation by using different combinations of ABCG2 and MRP4 knockout mice [117]. For example, lack of MRP4 affected PMEA accumulation in spleen suggesting the low contribution of ABCG2 in spleen but efficient efflux at other tissues such as kidney and liver. Intriguingly, ABCG2 expression in polarized cells cultured in a Transwell culture system (polarized cells are cultured on permeable membrane bathed in medium on their basal and apical surface [118]) export 6-MP from the basal compartment to the apical compartment. These findings appear relevant to chemotherapeutic response because a number of studies have found an association between ABCG2 overexpression and poor overall survival and relapse in AML patients [120–123]. Because nucleoside derivatives are frontline therapy for AML, knowledge of ABCG2 role in transporting nucleosides analogs and their derivatives will facilitate not just drug development, but increase therapeutic efficacy.

5. Transporter interaction with intracellular metabolic pathways

As mentioned above, intracellular concentrations of chemotherapeutic nucleosides- and their nucleotide-derivatives can be modulated by processes including uptake carriers, nucleoside, kinases and efflux transporters. The intracellular accumulation of phosphorylated nucleosides is essential for cytotoxicity and a reduction in nucleoside-monophosphate concentration might impair therapy (Fig. 2A). For example, if drug uptake is markedly reduced, the intracellular concentration of nucleoside may be below the affinity of the nucleoside kinase thereby leading to inadequate levels of active nucleotides an outcome associated with concomitant treatment failure. In addition, natural variation in nucleoside kinase levels or functionality (due to mutation) may affect therapeutic efficacy.

The possibility that nucleoside resistance mediated by ABCG2 was affected by intracellular metabolism was evaluated when

two-independent cell lines, engineered for comparable ABCG2 expression and resistance to a prototypical ABCG2 substrate (mitoxantrone), had marked difference in resistance to clofarabine [119]. The ABCG2 resistance to clofarabine was strongly modulated by either up or down-regulation of dCK, the enzyme that activates clofarabine. Reducing dCK levels with a dCK siRNA enhanced ABCG2 mediated clofarabine resistance. In contrast, dCK overexpression reduced clofarabine resistance because the abundance of dCK resulted in a faster rate of clofarabine conversion to clofarabine-monophosphate. It was reasoned that if clofarabine-monophosphate was an ABCG2 substrate then greater resistance would have been expected. These studies suggested clofarabine-monophosphate was not an ABCG2 substrate, but clofarabine was. Based on this, membrane vesicle transport studies determined that clofarabine was an ABCG2 substrate. These studies showed that an ABC transporter could effectively derail the nucleoside phosphorylation pathway by directly exporting the nucleoside. Because ABCG2 has been shown to extrude nucleoside-monophosphates too, it suggests for some nucleosides, ABCG2 can have a powerful effect on response to nucleoside drugs. However, given that several ABC transporters export nucleotide-monophosphates, this finding suggests their role in affecting nucleoside activation and intracellular phosphorylation should also be considered.

6. SNPs in ABC transporters and the double-edge sword in therapeutic response

The function of ABC transporters can be modified by variation in the DNA sequence encoding the polypeptide. These SNP's can produce changes in the amino-acid composition of the polypeptide. One such non-synonymous change is well known for ABCG2, the Arg482 to Gly substitution [124]. While this change alters substrate specificity, its therapeutic relevance is dubious considering it has yet to be identified in patient samples. In contrast, ethnic variation in non-synonymous SNP's can have a profound effect if the allele is frequent and alters function. For instance, the ABCG2 Gln141K reduces the protein level and activity but is important because its frequency varies by ethnic group (35% in Asians and < 1% in African Americans (Fig. 2B) [125].

While recent studies have suggested that variation in MRP4 can affect therapeutic response by affecting host toxicity, it is well established that the lack of a thiopurine inactivating enzyme TPMT produces greater 6-MP toxicity. However, there are patients who are highly sensitive to 6-MP toxicity without the typical reason for sensitivity: a non-functional TPMT. MRP4 is highly expressed in myeloid progenitors, and in a murine model mice lacking MRP4 exhibit high hematopoietic toxicity [126]. These studies identified a human MRP4 SNP that was associated with accumulation of thiopurine nucleotides at a high frequency among the Japanese population (>18%) and Asians (~4%) that resulted in impaired targeting to the plasma membrane producing a loss of MRP4 function. The contribution of this SNP to hematopoietic toxicity was confirmed in Japanese inflammatory bowel disease patients receiving the 6-MP prodrug, azathiopurine [127]. These results illustrate the delicate balance where too little of the ABC transporter function results in the in drug toxicity, whereas too much of it causes MDR.

7. Conclusion and perspective

The efficacy of the nucleoside derivatives relies on the formation and accumulation of phosphorylated metabolites. Nucleoside analogs are widely used in the treatment of both solid and hematologic malignancies with new nucleoside derivatives being developed to increase treatment efficacy. Although uptake

carriers facilitate the movement of nucleosides into the cells, at a high plasma concentration, passive diffusion moves drugs into cells down their concentration gradient. However, the conversion of nucleosides to monophosphates is a rate-limiting step in drug activation. Thus a reduced or a loss in activity of the kinase reduces therapeutic efficacy. A newer concept reveals that impaired response to nucleoside drugs occurs by active efflux due to ABC transporters. Recent studies demonstrate that both ABC family members and ABCG2 play an important role in intracellular accumulation of nucleotide metabolites. For drugs where both the nucleoside and its monophosphate are substrates of ABC transporters, drug efficacy will be strongly reduced and will appear independent of nucleoside kinase activity. However, if the ABC transporter has only affinity for the nucleoside and not the monophosphate form, a high rate of conversion to the monophosphate will result in accumulation of active metabolites with a very little contribution from ABC transporters. As presented here, an intricate cellular balance between uptake carriers, kinases, and ABC transporters determine the resistance to the nucleoside derivatives, and therefore, an understanding of substrate specificity for ABC transporters will be important in predicting drug efficacy.

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