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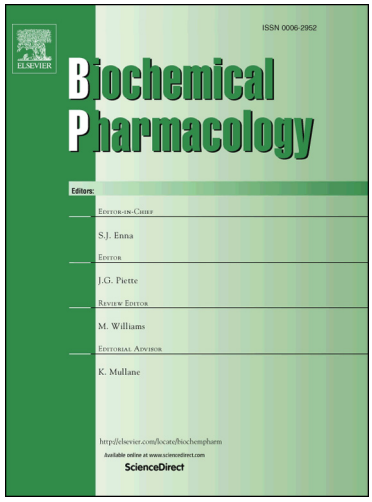

Commentary

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Targeted Pharmacotherapies for Defective ABC Transporters

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ABSTRACT

Human ABC (ATP Binding Cassette) transporters form a superfamily of forty-eight transmembrane proteins, which transport their substrates across biological membranes against important concentration gradients, in an energy-dependent manner. Gene variations in approximately half of these transporters have been identified in subjects with rare and often severe genetic diseases, highlighting the importance of their biological function. For missense variations leading to defects in ABC transporters, the current challenge is to identify new molecules with therapeutic potential able to rescue the induced molecular deficiency. In this review, we first address the progress provided by emerging pharmacotherapies in cystic fibrosis, the most frequent monogenic disease caused by variations of an ABC transporter, *i.e.* ABCC7/CFTR. Then, we enlarge the topic to the other ABC transporters, more notably to canalicular ABC transporters, the variations of which cause rare hepatobiliary diseases, and we discuss the first promising attempts aiming to correct molecular defects of these proteins.

KEYWORDS

Bile secretion; Chaperones; Correctors; Missense variations; Rare diseases.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

Corr-3A (PubChem CID: 1386410); Corr-4A (PubChem CID: 1144671); Curcumin (PubChem CID: 969516); Cyclosporin A (PubChem CID: 5284373); Glycerol (PubChem CID: 753); Sodium 4-phenylbutyrate (PubChem CID: 5258); VRT-325 (PubChem CID: 11957831); VX-770/Ivacaftor (PubChem CID: 16220172); VX-809/Lumacaftor (PubChem CID: 16678941).

1. INTRODUCTION

The superfamily of ABC (ATP-Binding Cassette) transporters is composed of 48 members in human and subdivided in 7 subfamilies (from ABCA to ABCG) based on sequence homologies (for reviews, see [1, 2]). They transport a broad variety of substrates across biological membranes. Full ABC transporters are proteins composed of two modules each formed by a TransMembrane Domain (TMD) with 6 α-helices and a Nucleotide Binding Domain (NBD) which contains highly conserved motifs such as the canonical LSGGQ signature sequence [3]. Their transport activity is catalyzed by their ability to bind and hydrolyze ATP through their NBDs, thus allowing structural modifications and substrate translocation across membrane bilayers [3]. Some of these proteins are hemi-transporters with only one TMD and one NBD which then need to dimerize to be functional, while others contain additional domains regulating their activity [4].

Remarkably, genetic alterations in almost half of ABC transporters have been identified in subjects with a wide range of diseases such as Tangier disease (ABCA1), Stargardt disease (ABCA4), Harlequin and lamellar ichthyosis (ABCA12), hereditary biliary diseases (ABCB4, ABCB11, ABCC2), pseudoxanthoma elasticum (ABCC6), cystic fibrosis (ABCC7), type II diabetes (ABCC8, ABCC9), X-linked adrenoleukodystrophy (ABCD1), gout (ABCG2) and sitosterolemia (ABCG5, ABCG8) (reviewed in [5]). All are rare diseases, and the most common of them is cystic fibrosis (CF) with an estimated mean prevalence of ~0.7/10,000 [6, 7]. CF is caused by variations in *ABCC7/CFTR* (Cystic Fibrosis Transmembrane conductance Regulator) gene, which encodes a channel normally located at the plasma membrane, responsible for chloride and bicarbonate secretion in epithelial cells. Variations identified in CF patients have been classified according to their molecular defects, as follows: absence of protein expression

(class I), improper targeting (class II), impaired gating or conductance (classes III and IV), low protein abundance (class V), or instability (class VI) [8] (Fig. 1). However, this classification tends to be more complicated since subcategories are being proposed [9, 10] and also because multiple defects of single variants make them belonging to several classes [11]. In this review, we will address pharmacotherapies that have been tested, mostly in preclinical models but also in clinical trials, to rescue CFTR variants and other defective ABC transporters, with a particular focus on canalicular transporters involved in rare biliary diseases. All drugs with rescuing effects in preclinical and clinical studies are summarized in Table 1.

2. CFTR MODULATORS

The prevalence of CF – more than 85,000 CF patients worldwide (http://cftr2.org/) – has a significant impact on the population, which could explain why so many efforts have been deployed towards therapies for this disease compared to other rare diseases. Since the discovery of the *CFTR* gene in 1989 [12] until recently, the only treatments for CF patients were symptomatic, aiming to restore airway clearance, reduce lung inflammation and fight bacterial infection without directly targeting CFTR molecular defects (reviewed in [9]). However, these treatments were not sufficient to ensure a good quality of life and additional pharmacological treatments were eagerly needed in order to directly rescue, at least partially, the molecular defects of CFTR variants. Indeed, it has been shown that only 5-25% of CFTR expression compared to normal levels could be sufficient to significantly improve the condition of CF patients [13-15]. Although more than 2000 different *CFTR* variations have been identified so far (http://www.genet.sickkids.on.ca/; https://www.cff.org/), most attention has been paid to the

CFTR-ΔF508 variant, which is found in approximately 70% of CF patients [16, 17] (http://cftr2.org/). This ΔF508 variant consists in a deletion of the phenylalanine residue in position 508 and belongs to the class II of CFTR variants that are characterized by maturation defects and ER retention (Fig. 1A-B). In this section, we provide a brief overview of CFTR targeted pharmacotherapies, notably on CFTR-ΔF508. More details can be found in excellent recent reviews [7, 9, 18, 19].

The proof-of-concept for CFTR-ΔF508 rescue was provided by the observation of a partial restoration of its maturation (assessed by gel migration shift of the fully glycosylated protein), and subsequent targeting to the plasma membrane in cell models cultured at temperatures below 30°C [20]. A rescue of CFTR-ΔF508 was also obtained in cell models using chemical chaperones such as dimethylsulfoxide (DMSO), glycerol or trimethylamine-N-oxide [21-23] but at very high concentrations, incompatible with their use *in vivo*. Other molecules called pharmacological chaperones – by opposition to the non-specific chemical chaperones – were also shown to rescue the maturation of CFTR-ΔF508 both *in vitro* (cell models) and *in vivo* (mouse models). These include the calcium pump inhibitors thapsigargin [24] and curcumin [25], even if conflicting results were obtained with these compounds [26-28]. The α-1,2-glucosidase inhibitor miglustat (or N-ButylDeoxyNoJirimycin, NB-DNJ) also showed ability to partially rescue maturation, localization and function of CFTR-ΔF508 [29], as did the histone deacetylase inhibitor 4-phenylbutyrate (4-PBA) [30]. These results were encouraging and led to several clinical trials in which, however, limited or no improvement in the outcome of CF patients and significant adverse effects were demonstrated [31, 32]. These disappointing results of the first clinical trials might be explained by the complexity of molecular defects induced by the deletion of phenylalanine at position 508 in CFTR. Indeed, it has been proposed that this

deletion induces both misfolding of its NBD1 and loss of crucial interactions between intracellular loops [33]. Moreover, rescued CFTR-ΔF508 is more susceptible to internalisation and ESCRT-mediated lysosomal degradation, which can be related to its weaker interaction with plasma membrane stabilizing partners containing PDZ domains [34, 35]. Thus, at one stage, rescuing sustained CFTR function in CF patients seemed more complicated than what was first anticipated. Thereafter, combinations of pharmacotherapies (e.g. Orkambi®, see below) instead of single molecule-based ones were envisioned in order to rescue CFTR function.

Many other variations with a lower frequency than ΔF508 have been identified in CF patients, such as the class I G452X synthesis-defective or the class III gating-defective G551D variants (Fig. 1B), with allele frequencies of 2.5% and 2.1% in CF patients, respectively (https://www.cff.org/). Pharmacotherapies targeting these variants are also under investigation. They include bypass strategies and the search for potentiators, *i.e.* compounds able to stabilize and/or rescue channel opening of activity-defective variants, as opposed to correctors which rescue traffic-defective variants (for reviews, see [9, 19]).

In addition to candidate approaches, the emergence of miniaturized and automated High-Throughput Screening (HTS) technologies allowed the screening of large chemical libraries containing several tens of thousands of compounds (for a review, see [36]). A first set of HTS allowed the identification of five potential correctors of CFTR-ΔF508 from different chemical subfamilies [37, 38]. In parallel, the company Vertex Pharmaceuticals also launched its own HTS campaign, allowing the identification of the CFTR-ΔF508 correctors VRT-325 and VRT-422 as well as the CFTR-G551D potentiator VRT-532 [39]. The company ultimately developed the CFTR potentiator VX-770, also able to rescue the channel activity of the gating-defective CFTR-G551D variant [40] (Fig. 1B). The results obtained in cell models [40, 41] prompted several

clinical trials in patients carrying the CFTR-G551D variation as well as other gating-defective missense variations. Thus, the VX-770 molecule was shown to significantly improve the outcome of CF patients who carried such variations [42-45]. VX-770 (also called Ivacaftor) is now approved by both FDA (US Food and Drug Administration) and EMA (European Medicines Agency) (NDA203188 and EMEA/H/C/002494, respectively), commercialized under the name Kalydeco®, and indicated in patients aged 2 years and older who carry gating-defective variations of CFTR (R117H, G178R, S549N, S549R, G551D, G551S, G1244E, S1251N, S1255P, G1349D; see http://www.vrtx.com/our-medicines/our-approved-medicines).

Regarding CFTR-ΔF508 correctors, VX-809 (also called Lumacaftor; Fig. 1B), also identified by HTS, proved to be more potent than VRT-325 in cell models [46], possibly through stabilization of the immature form of the variant [47]. However, VX-809 treatment did not significantly improve the outcome of CF patients in a phase II clinical trial [48], which might be due to the complexity of the molecular defects induced by the Phe508 deletion (see above). As suggested by *in vitro* studies [46, 49], another phase II clinical trial showed that the corrector VX-809 combined with the potentiator VX-770 improved the status of CF patients who were homozygous for the CFTR-Δ508 variation [50], which led to a successful phase III trial in these patients [51]. The combination of the corrector VX-809/Lumacaftor with the potentiator VX-770/Ivacaftor is now FDA- and EMA-approved (NDA206038 and EMEA/H/C/003954, respectively), commercialized under the name Orkambi® and indicated only for homozygous CFTR-ΔF508 patients above 12 years old (http://www.vrtx.com/our-medicines/our-approved-medicines). To improve the efficiency of CFTR correcting therapies, Vertex Pharmaceuticals recently initiated new clinical trials based on the administration of one or two correctors different from VX-809/Lumacaftor, as VX-661/Tezacaftor, VX-152, VX-440 and VX-659, still in

combination with the potentiator VX-770/Ivacaftor (for details, see https://www.cff.org/trials/pipeline; http://www.vrtx.com/research-development/pipeline).

Even if these new treatments represent an important breakthrough in the field of CF, they are only indicated for patients with selected variations and cannot be used for those with other CFTR disease-causing variations. Moreover, these treatments are very expensive, which led other companies to undergo clinical trials with alternative pharmacotherapies, such as regulators of proteostasis, *i.e.* the homeostasis of protein expression levels [9, 52].

3. CORRECTION OF OTHER DEFECTIVE ABC TRANSPORTERS

The important progress in understanding the molecular mechanisms leading to CF as well as the emergence of new therapeutic approaches can serve as a basis for the development of new pharmacotherapies for diseases caused by defects in other ABC transporters. In this section, we will review attempts to correct the molecular defects identified in ABC transporters other than CFTR, first by chemical and pharmacological chaperones and second by drug repositioning. Finally, we will focus on correction of canalicular ABC transporters involved in the secretion of bile components.

3.1. Chemical and pharmacological chaperones

As for CFTR, studies aimed at restoring cell surface expression of misfolded and ER-retained variants of other ABC transporters started with the use of non-specific chemical chaperones such as glycerol or DMSO. These compounds could act by different mechanisms during the biosynthesis of the mutated protein in the ER: *i)* through a direct interaction with the

variant which may decrease its degradation and increase its folding rate; *ii)* alternatively, chemical chaperones may interact with endogenous ER protein chaperones with an indirect stimulating effect on protein synthesis (for a review, see [53]). In this context, glycerol, DMSO and polyethylene glycol treatments were shown to partially but significantly rescue the expression of mature forms of misfolded variants of ABCB1/MDR1/P-gp (MultiDrug Resistance protein 1 / P-glycoprotein) [54], ABCB11/BSEP (Bile Salt Export Pump) [55, 56], ABCC1 [57, 58], ABCC4 [59] and ABCC8/SUR1 (SulfonylUrea Receptor 1) [60, 61] in cell models (Table 1). ER-stress inducers such as thapsigargin, dithiothreitol or curcumin have also been used to promote the maturation and trafficking rescue of misfolded ABC transporters (Table 1). This is the case of the Q579L variant of ABCA1 identified in patients with Tangier disease, even if it remained inactive after its plasma membrane targeting [62]. Importantly, these chemical chaperones are not specific and modify the expression levels of many proteins [53]. Moreover, they failed to efficiently rescue the cell surface expression of ER-retained ABCB1/MDR1 and ABCB4/MDR3 (MultiDrug Resistance protein 3) variants [26, 54, 63, 64] (Table 1), which will limit their development as potential new pharmacotherapies.

As opposed to the non-specific chemical chaperones, pharmacological chaperones (also called pharmacochaperones) are defined as small molecules that specifically bind target proteins, then inducing or promoting their proper folding and trafficking [65]. Many ER-retained variants of ABCB1 have been identified [66], so that this transporter, structurally similar to other ABC transporters such as CFTR or ABCB4 [54, 67], has been widely used as a model system. A large variety of ABCB1 substrates and modulators appeared to be acting as powerful chaperones for processing misfolded variants [68]. Thus, vinblastine, verapamil, capsaicin, nonylphenolethoxylates, tariquidar, nilotinib, valinomycin and paclitaxel were all able to correct

the localization of misprocessed ABCB1 variants in a dose-dependent manner, resulting in the expression of functional protein at the cell surface [63, 66, 69, 70] (Table 1). Interestingly, when experiments were performed with molecules which are not ABCB1 substrates or modulators such as cisplatin or camptothecin, plasma membrane targeting of ER-retained ABCB1 variants was not rescued [63].

Cyclosporin A (CsA) is an immunosuppressive compound known to be a substrate and a competitive inhibitor of ABCB1 [71]. Numerous studies have evaluated the effect of CsA on different misprocessed ABCB1 variants. Regardless of the location of the variations involved, CsA was able to convert a large majority of these folding-defective variants into a fully mature protein properly addressed to the cell surface [54, 63, 66, 68, 72, 73]. CsA has also been successfully used to rescue the cell surface targeting of ER-retained forms of ABCB4 [54, 74] (see next section).

ABCC8/SUR1 is a subunit of the K_{ATP} channel, which plays a critical role in glucose-induced insulin secretion (for a review, see [53]). Missense variations of SUR1 (e.g. A116P and V187D) identified in patients with congenital hyperinsulinism, a disease characterized by persistent insulin secretion even under severe hypoglycemia, cause an impairment of its traffic and prevent the normal cell surface expression of the K_{ATP} channel [53]. Glibenclamide and tolbutamide, two sulfonylureas that bind and inhibit SUR1, as well as glinides (a class of hypoglycemic drugs), corrected trafficking defects of SUR1 variants identified in patients with congenital hyperinsulinism, probably by a direct binding of the small compounds to the nascent protein in the ER, thus favoring its proper folding [60, 61, 75-80] (Table 1). Although sulfonylureas are efficient pharmacological agents able to correct the cell surface expression of SUR1 trafficking variants, it is important to note that only variations in the TMD0 (additional

regulatory multispanning domain at the N-terminus of the transporter) were corrected by these compounds. Indeed, variations within the NBD2 of SUR1 were not responsive to the rescue effect of glibenclamide [81-85]. Moreover, variations in TMD0 of SUR1 may also be more complex and impact both protein synthesis and gating activity, as exemplified by the two missense variations R74W and E128K [77]. Finally, the anticancer drug mitoxantrone, which is also a substrate of the drug and urate transporter ABCG2/BCRP (Breast Cancer Related Protein), allowed the rescue of the frequent Q141K variant [86].

3.2. Drug repositioning strategies

New drug development is a long process which can take up to 16 years and requires huge costs for drug discovery, development and preclinical stages [87]. Therefore, finding new applications for already available FDA-approved drugs, a strategy called “drug repositioning” (or “drug repurposing”), would greatly reduce the development and approval times as well as the relative costs. Another advantage of this strategy is to significantly increase the success rates of the repositioned drugs: 25% of them from phase II and 65% from phase III clinical trials make it to market compared to only 10% and 50% for new molecules, respectively [88].

3.2.1. Sodium phenylbutyrate

Sodium phenylbutyrate or 4-phenylbutyrate (4-PBA) is an aromatic fatty acid which has been used as a therapeutic agent since 1975 when Brossmer and colleagues reported its role in the inhibition of platelet aggregation [89]. Then, the FDA approved this well-tolerated agent for clinical applications in urea cycle disorders and β-thalassemia under the trade name Buphenyl

[90-92]. Although the molecular mechanisms involved in the therapeutic actions of 4-PBA are still not completely understood, several evidences suggest that this compound acts *via* non-exclusive modes of action: *i)* chaperone activity and transcriptional upregulation of chaperone proteins; *ii)* ammonia scavenger properties; *iii)* inhibition of histone deacetylase; *iv)* inhibition of ER stress activity; *v)* modification of mitochondrial and peroxisomal biogenesis [93-95]. Recently, a growing number of studies have demonstrated the therapeutic potential of 4-PBA in attenuating diseases in a variety of model systems. Its beneficial action has been observed in neurodegenerative diseases (Huntington and Alzheimer's diseases), diseases related to metabolic syndrome (diabetes), hemoglobinopathies (sickle-cell disease and β-thalassemia), motor neuron disorders (spinal muscular atrophy and amyotrophic lateral sclerosis), fragile X mental retardation, ischemia and certain cancers [96, 97].

Treatment by 4-PBA has been successfully used to improve the folding and the intracellular traffic of several defective ABC transporters involved in human diseases (Table 1). ABCA1 (cholesterol efflux onto apoA-I to form high-density lipoprotein) is mutated in patients with Tangier disease which is characterized by the absence of plasma high-density lipoprotein, thus giving rise to an increased risk of cardiovascular disease. In cell models, 4-PBA treatment led to the plasma membrane relocalization of misfolded ABCA1 variants, concomitantly with a restored cholesterol efflux function [98]. The immature Q141K variant of ABCG2, frequently identified in patients with gout, was also rescued by 4-PBA and also by other histone deacetylase inhibitors such as panobinostat, romidepsin and vorinostat (Table 1), demonstrating the therapeutic potential of this category of small molecules for treating ABCG2-related diseases [86, 99, 100]. Variations of the lipid transporter ABCA3 (e.g. L101P and G1221S) are associated with lung surfactant deficiency in newborn infants, and 4-PBA partially restored trafficking of the

G1221S variant but not of the L101P variant [101] (Table 1). X-linked adrenoleukodystrophy is an inherited disorder characterized by progressive demyelination of the central nervous system and adrenal insufficiency. This disease is caused by variations of the half-transporter ABCD1 which homo- or heterodimerize with ABCD2, ABCD3, or ABCD4. Interestingly, 4-PBA treatment allowed an increased ABCD2 expression when ABCD1 was down-expressed in cultured glial cells [102] (Table 1).

Successful correction of the localization of mistargeted variants by 4-PBA has also been shown *in vivo*. Pseudoxanthoma elasticum is characterized by dystrophic calcification affecting primarily elastic fibers in skin, arteries and the Bruch's membrane of the eye and is caused by loss-of-function variations of ABCC6 – more than 300 disease-causing variants identified so far [103]. 4-PBA treatment of transfected MDCK cells restored the plasma membrane localization of ER-retained ABCC6 variants (Table 1). Interestingly, these intracellular variants have also been expressed in mouse liver by hydrodynamic tail vein injection and their relocalization at the cell surface was observed in liver cells of 4-PBA-treated mice [103, 104].

Finally, 4-PBA has also been used to rescue artificial (*i.e.* not associated with pathologies) misfolded variants in order to dissect the structure-function relationship of ABC transporters. Thus, the chaperone activity of 4-PBA was able to correct some variants of ABCC1 [57, 58], ABCC4 [59] and ABCG2 [105] (Table 1), although further investigation will be necessary to determine the therapeutic benefit of this molecule. Even though 4-PBA appeared to be a good candidate for the treatment of sickle-cell disease [106], α-1 antitrypsin deficiency [107], β-thalassemia [108] and CF [109], enthusiasm for this small molecule waned because of the lack of significant beneficial effects in clinical trials. Nevertheless, interest for 4-PBA therapy rekindled

when potential therapeutic benefits in the treatment of intrahepatic cholestasis caused by defective ABCB11 variants have been demonstrated in patients [110-112] – see next section.

3.2.2. CFTR correctors

Some newly identified CFTR correctors have been predicted to act on both CFTR and structurally similar proteins. Thus, these small molecules have been used in order to correct processing variants of other ABC transporters (Table 1).

Evidence suggested that the CFTR corrector VRT-325 may directly bind to the channel but also to ABCB1 and may thus stabilize the highly conserved NBDs of multiple ABC proteins [72, 113]. In this context, the two CFTR correctors VRT-325 and Corr-4a have been successfully used to partially rescue the gout-associated ABCG2-Q141K variant [100], opening new therapeutic strategies for gout and hyperuricemia (Table 1). Two ABCA4 variations implicated in Stargardt disease have also been partially rescued by VRT-325, Corr-4a, CF-106951 and VX-809 (Table 1) but without evidence of the functionality of the rescued variants [114]. However, the SUR1-A116P variation, found in patients with congenital hyperinsulinism, was not rescued by VRT-325 [61], suggesting that this compound may not be considered as a general ABC transporter corrector. We can hypothesize that the absence of correction of SUR1 variants by VRT-325 may be caused by the inaccessibility of this ABC transporter by VRT-325 due to its co-assembly with Kir6.2, the other subunit of the K_{ATP} channel.

Other CFTR correctors were predicted to rescue several protein trafficking diseases by targeting cellular pathways and acting through regulation of proteostasis [61] (Table 1). This kind

of molecules have been used to correct trafficking defects of the F27S, A116P and V187D variants of SUR1 [78, 79]. For example, the sodium channel blocker carbamazepine, known to promote proteasomal and autophagic degradation of mutated proteins, is an interesting candidate since it is FDA-approved for treatment of epilepsy and bipolar disorder [78]. Although carbamazepine was first thought to improve the processing of mutated K_{ATP} channel by stimulating autophagy [115, 116], it seems more likely that it acts as a small chaperone, like sulfonylureas, to overcome the folding/processing defects of SUR1 variants [78]. Other proteostasis regulators include the cardiac glycoside ouabain, the phosphodiesterase inhibitors KM11057 and KM11060, the chemical chaperone RDR1, the poly-ADP-ribose-polymerase inhibitors latonduine and ABT-888, as well as the cyclooxygenase inhibitor glafenine [61]. These molecules have also been shown to rescue the maturation of the A116P and V187D variants of SUR1 [61], suggesting that CFTR correctors might have broader applications to other diseases implicating ABC transporter trafficking defects such as congenital hyperinsulinism.

These recent studies indicate that disease-causing variations of ABC transporters can be corrected by some CFTR correctors, at least *in vitro*. Even if these studies provide hope for treatment of diseases such as Stargardt disease, gout or congenital hyperinsulinism, further *in vivo* investigations and clinical trials will be essential to demonstrate the real therapeutic potential of these repositioned CFTR correctors.

4. FOCUS ON CANALICULAR ABC TRANSPORTERS

Bile formation is a fine tune process which occurs at the canalicular membrane of hepatocytes. Main components of bile are cholesterol, phosphatidylcholine (PC) and bile salts which form mixed micelles, as well as organic anions [117]. Their amount in bile is tightly controlled by their active secretion into canaliculi, a process ensured by ABC transporters: ABCB11/BSEP, ABCB4/MDR3, ABCG5/G8 and ABCC2 secrete bile salts, PC, cholesterol and organic anions, respectively (for a review, see [118]). In addition, ATP8B1 (FIC1), a type IV P-type ATPase, has been proposed to be essential for a proper composition of the canalicular membrane, and thus for optimal function of these transporters and bile flow [119]. Any modification of the secretion of one of these bile constituents may lead to the development of serious liver diseases [118, 120]. Thus, Progressive Familial Intrahepatic Cholestasis (PFIC) type 1, 2 and 3 are due to variations in ATP8B1, ABCB11, and ABCB4, respectively; ABCC2 deficiency causes Dubin-Johnson syndrome and variations in either ABCG5 or ABCG8 are associated with sitosterolemia [118, 120]. In this section, we will focus on ABCB11 and ABCB4 for which molecular correction of defective variants have been recently developed.

4.1. ABCB11/BSEP

ABCB11 is an ABC transporter expressed at the canalicular membrane of hepatocytes which allows the export of primary bile acids into bile canaliculi against extreme concentration gradients. Human ABCB11 variations result in at least three clinical forms of liver diseases: PFIC2 and two milder phenotypes, Benign Recurrent Intrahepatic Cholestasis type 2 (BRIC2), and Intrahepatic Cholestasis of Pregnancy (ICP) (reviewed in [121]). PFIC2, initially reported as “Byler syndrome”, is an autosomal recessive disease for which impaired biliary secretion of bile

acids leads to decreased bile flow, bile salt accumulation in hepatocytes, hepatocellular damage and increased risk of hepatocellular carcinoma [122, 123]. Jaundice and pruritus, two clinical signs of cholestasis, usually appear during the first months of life. Then the disease progresses to fibrosis and end-stage liver failure before adulthood. Medical therapy with UDCA, rifampicin, and surgical therapy, such as biliary diversion, may provide some symptomatic relief. Nevertheless, in the majority of cases, liver transplantation is required because of unremitting pruritus, hepatic failure, or hepatocellular carcinoma [112, 124].

A comprehensive analysis of 80% of ABCB11 missense variations and single nucleotide polymorphisms at pre-mRNA splicing, protein processing and functional levels has been performed by Byrne and colleagues [56]. This work led to a classification of ABCB11 variants according to their defects (abnormal ABCB11 pre-messenger RNA splicing, alteration of protein processing or function). This study showed that 61% of ABCB11 variants were rescued by glycerol treatment suggesting that these variations result in misfolded proteins and may be rescued by chemical and pharmacological chaperones. Interestingly, the rescued variants included the PFIC2-associated variations G238V, E297G, D482G R1128C, R1231Q and R1268Q, the BRIC2-associated variations R1128H, R1050C and E297K, as well as A570T (Table 1), which could be associated with either form of disease [55, 56].

The potential correcting effect of ABCB11 substrates on the folding-defective ABCB11-E297G variant, known to be fully functional if correctly rescued at the plasma membrane, has been evaluated by Misawa and coworkers [125]. In this study, the authors showed that several bile acids such as cholic acid, chenodeoxycholic acid, deoxycholic acid, and UrsoDeoxyCholic Acid (UDCA) decreased in a dose-dependent manner the intracellular accumulation of taurocholate, suggesting that these substrates were able to promote ABCB11 transport activity.

However, a direct effect of these bile acids on the relocalization of the E297G variant of ABCB11 at the cell surface has not been investigated by the authors in their cell models [125].

Considering that liver transplantation is the only therapy to cure PFIC2 patients, many efforts have been done to identify compounds that may offer a new medical alternative for these patients. Recently, different research teams have demonstrated a pharmacological effect of 4-PBA, which is also used to treat patients with ornithine transcarbamylase deficiency, a disease associated with urea cycle disorders. An increase of ABCB11 expression at bile canaliculi and of biliary excretion capacity of bile salts were observed *in vitro*, in animal models and in PFIC2 patients. *In vitro* studies demonstrated that the E297G and D482G variants of ABCB11, each present in 30% of European PFIC2 families, were correctly addressed at the cell surface in HEK293 cells and in polarized MDCK cells after 4-PBA treatment [122, 126] (Table 1). The increased expression of these two ABCB11 variants at the canalicular membrane was associated with the inhibition of their internalization from the cell surface and their subsequent degradation, and then exhibited normal transport function [122, 126]. Moreover, treatment of Sprague-Dawley rats with clinically-relevant dosage of 4-PBA resulted in an increase of functional ABCB11 hepatocanalicular expression, and consequently enhanced bile acid transport *via* the canalicular membrane [126] (Table 1). The authors of this study also demonstrated that 4-PBA acts at a post-translational level through the stabilization of the mature form of ABCB11 variants since the drug induced a prolonged half-time residence at the plasma membrane of both WT and variant ABCB11 [126]. To explain this observation, they proposed that the increased cell surface expression of ABCB11 variants after 4-PBA treatment may be due to: *i)* a decrease of ubiquitin-dependent degradation of ABCB11 variants [127], and/or *ii)* a decrease of their AP2-mediated endocytosis from the cell surface *en route* for lysosomal degradation [128]. In patients with

ornithine transcarbamylase deficiency, 4-PBA treatment led to a significant increase of ABCB11 expression at the canalicular membrane without important adverse effects [128] (Table 1). These information allow a better understanding of the regulation of ABCB11 expression at bile canaliculi and would help the development of new treatments for ABCB11-related severe liver diseases.

A further step has been taken when Gonzales and colleagues reported the first successful 4-PBA treatment of a PFIC2 patient carrying the homozygous ABCB11-T1210P missense variation leading to the ER-retention of the transporter (Table 1). The authors treated the patient with 4-PBA at 200 to 500 mg/kg/day during 5 months, which led to a marked decrease of serum bile acid concentrations, correlated with increased biliary bile acid concentrations, decreased pruritus, improvement of serum liver function tests and proper canalicular localization of ABCB11 [110]. Importantly, improvement of the medical condition of the patient was stable after a follow-up of 19 months and no severe side effects were reported [110]. This work was followed by other successful 4-PBA pharmacotherapies (always combined with UDCA) of selected PFIC2 patients homozygous for G982R, R1128C, T1210P or R1231Q missense variations of ABCB11, and the rescue of the canalicular targeting of these variants has been validated on liver sections from patients and in cell models [111, 112] (Table 1). 4-PBA also gave encouraging results in a patient with BRIC2 compound heterozygous for ABCB11 with D404G and V444A variations [129] and in a preterm infant with cholestasis and liver fibrosis [130]. These studies provide evidence that 4-PBA treatment improves the quality of life of PFIC2 patients and may thus represent an alternative to, or at least delay, liver transplantation. They also established a proof-of-concept that a variation-specific pharmacotherapy aiming to increase the

canalicular expression of a mistargeted ABCB11 variant may be offered to patients after *in vitro* validation in relevant cell models.

4-PBA opens new perspectives of personalized therapies for selected PFIC2 patients and its use should also be considered for other liver diseases in which missense variations result in mistrafficking proteins such as BRIC2, PFIC1 or Wilson's disease [112]. In this regard, a 4-PBA therapy has been successfully applied to a PFIC1 patient showing stage II/III fibrosis [131]. However, this study pointed out a real problem of compliance since the patient refused to take the treatment anymore after 5 months, due the emetic effect of the medication presumably related to its poor palatability. He was then switched to glycerol-phenylbutyrate concomitantly with discontinuation of rifampicin, which led to a dramatic worsening of the patient's condition who suffered from an acute and severe hepatotoxicity, possibly related to increased circulating phenylacetate concentrations [131]. Sodium-2,2-dimethylbutyrate (also named HQK-1001), a 4-PBA derivate with greater potency and bioavailability than 4-PBA, has been used in phases I and II clinical trial for patients with β-thalassemia and sickle-cell disease. This compound induced fetal hemoglobin production and concomitantly reduced anemia without strong adverse events, even if discrepancies between studies were observed [132-135]. Therefore, it would be interesting to determine if 4-PBA derivatives have the same correction properties than 4-PBA for misfolded ABC transporter variants and might be a good alternative to bypass compliance issues reported for 4-PBA-treated patients.

4.2. ABCB4/MDR3

ABCB4 is exclusively expressed at the canalicular membrane of hepatocytes and is responsible for PC secretion into bile canaliculi. Dysfunctions of this ABC transporter result in destabilization of mixed micelles. Thus, cholesterol is less efficiently solubilized, promoting cholesterol crystallization and resulting in increased biliary lithogenicity, and free hydrophobic bile salts exert their detergent effect on biliary epithelia. Homozygous or compound heterozygous variations of ABCB4 are associated with PFIC3. Like the other PFICs, PFIC3 is an inherited autosomal recessive liver disease and leads to cirrhosis and death from liver failure, usually ranging from infancy to adolescence, in the absence of liver transplantation. Other less severe biliary diseases are associated with monoallelic variations of *ABCB4*, including Low Phospholipid-Associated Cholelithiasis (LPAC) syndrome as well as ICP, both affecting young adults (for a review, see [118] and references therein). Today, UDCA remains the only treatment for ABCB4-related liver diseases. This hydrophilic bile acid partly replaces the endogenous cytotoxic pool of bile salts and induces expression of ABCB11 and ABCB4, then stimulating hepatobiliary secretion of bile salts and protective phospholipids, respectively. Thus, UDCA confers a lower toxicity of bile towards the biological membranes of the biliary tree, as well as a lower lithogenicity [136]. Although LPAC and ICP patients respond relatively well to UDCA therapy, approximately 50% of PFIC3 patients do not respond to this treatment and require liver transplantation [137-139].

We recently proposed a classification of ABCB4 variations identified in PFIC3 patients according to their functional defects (expression, traffic, activity or stability) [74] (Fig. 1C and D). The class II misfolded variants are retained in the ER and constitute good candidates for correction by pharmacological chaperones. In this regard, several studies have attempted to

correct the retention of misfolded ABCB4 variants identified in patients using cyclosporins, thapsigargin, curcumin and 4-PBA. The effect of 4-PBA and curcumin seemed to be variant-specific since these drugs were able to efficiently target functional G228R and A934T variants of ABCB4 to the plasma membrane but failed to rescue G68R, D459H and I541F variants, which may adopt a conformation refractory to the correction by these two chaperones [54, 64] (Table 1). Although ABCB4-I541F variant remained largely intracellular after 4-PBA, curcumin and thapsigargin treatments [54], the possibility of a pharmacological rescue of this variant was shown by testing cyclosporins. Indeed, we and others showed that CsA and other cyclosporin analogs (B, C, D and H) rescued class II variants of ABCB4, including I541F, L556R, Q855L [74], S320F and A953D [140], and A364V [141] (Table 1 and Fig. 1D).

As discussed above, CsA is an inhibitor of ABCB1 and probably also of ABCB4 and would act as a pharmacological chaperone by constraining the misfolded protein to adopt a conformation close to its native state during biosynthesis. In this regard, CsA has been reported to inhibit the PC floppase activity of ABCB4 [140], although another group recently reported activity (indirect measurement of ATPase activity) of CsA-rescued ER-retained variants (G228R and A934T) of ABCB4 [141]. Thus, future work is required to determine if CsA and its analogs really inhibit ABCB4-mediated PC efflux or if these compounds may be considered as suitable pharmacotherapies for liver diseases related to ER-retained forms of ABCB4.

Finally, five activity-defective missense variations located in the two NBDs of ABCB4 have been recently identified (G535D, G536R, S1076C, S1176L and G1178S; Fig. 1D, class III). Interestingly, these variations are homologous to activity-defective variations of CFTR identified in patients with CF (Fig. 1B, classes III/IV). In this context, the CFTR potentiator VX-770/Ivacaftor has been shown to significantly increase the PC secretion activity of these class III

variants of ABCB4 [142] (Table 1), paving the way for repositioning of this small molecule in the frame of ABCB4-related biliary diseases.

5. CONCLUSIONS

The important progress that has been made in the development of new pharmacotherapies for traffic- or activity-defective variants of CFTR opens new perspectives for the treatment of rare diseases related to variations in other ABC transporters. The identification of such therapies will largely benefit from HTS and drug repositioning strategies. However, upstream the identification of new treatments, the variants of ABC transporters identified in patients need to be characterized and classified according to their molecular defect, as done for CFTR [8] and more recently for ABCB11 [56] and ABCB4 [74] (Fig. 1B, D). Thus, as we proposed for ABCB4 [74], a general and simplified nomenclature (using only five categories; see Fig. 1D) may be generalized to all ABC transporters in order to classify the defective variants and to ameliorate the implementation of personalized pharmacotherapies. For specific ABC transporters, subclassifications could also be proposed such as IIIa, b… (e.g. a, gating defect; b, conductance defect, in the case of CFTR) for different types of functional defects, and IVa, b… (e.g. a, low abundance; b, low stability…) for low abundance/stability defects.

ABBREVIATIONS

4-PBA, 4-phenylbutyrate; ABC, ATP-Binding Cassette; BRIC2, Benign Recurrent Intrahepatic Cholestasis type 2; BSEP, Bile Salt Export Pump; CF, Cystic Fibrosis; CFTR, Cystic Fibrosis Transmembrane conductance Regulator; CsA, Cyclosporin A; DMSO, DiMethylSulfOxyde; EMA, European Medicines Agency; FDA, Food and Drug Administration (US); HTS, High-Throughput Screening; ICP, Intrahepatic Cholestasis of Pregnancy; LPAC, Low-Phospholipid Associated Cholelithiasis; MDR1/3,MultiDrug Resistance Proteins 1/3; NBD, Nucleotide Binding Domain; PC, PhosphatidylCholine; PFIC, Progressive Familial Intrahepatic Cholestasis; SUR1, SulfonylUrea Receptor 1; TMD, TransMembrane Domain; UDCA, UrsoDeoxyCholic Acid.

CONFLICT OF INTEREST

The authors have no conflict to declare.

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FIGURE LEGEND

Fig. 1. Classification of molecular defects of ABCC7/CFTR and ABCB4/MDR3 variants, and targeted pharmacotherapies. **(A)** Membrane topology and main domains of ABCC7/CFTR. **(B)** The six different classes of CFTR variants based on their molecular defects are indicated (yellow boxes) and exemplified by variants (red text). The proposed pharmacotherapies are also shown (white boxes). For clarity, only variants and therapies cited in the text are shown in this panel. Underscored compounds indicate FDA- and EMA-approved treatments for CF. **(C)** Membrane topology and main domains of ABCB4/MDR3. **(D)** Same as B for ABCB4/MDR3, except that only five classes of variants have been proposed, according to Delaunay and coworkers [74]. Abbreviations: 4-PBA, 4-phenylbutyrate; DMSO, DiMethylSulfOxyde; ER, Endoplasmic Reticulum; ERGIC, ER-Golgi Intermediate Compartment; NBD, Nucleotide Binding Domain; NB-DNJ, N-ButylDeoxyNoJirimycin; TGN, Trans-Golgi Network; TMAO, TriMethylAmine-N-Oxide; TJ, Tight Junction.

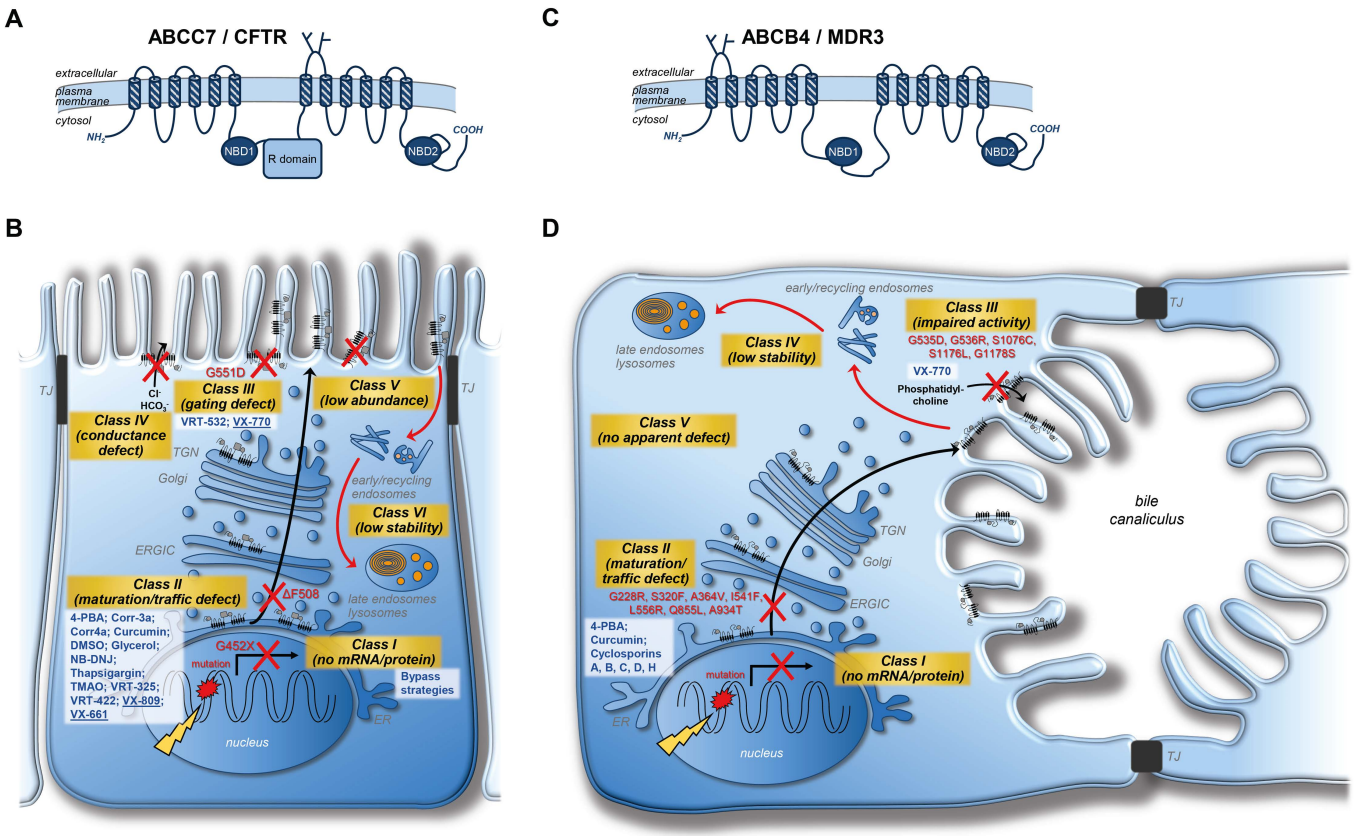

Table 1. Pharmacotherapies of ABC transporters

Sub-family	Name [1]	Alternative names	Known substrates	Associated diseases	Missense variations[2]	Cell models	Compounds[3]	References
A	ABCA1	CERP	Cholesterol; Phospholipids	Tangier disease; HDL deficiency; Atherosclerosis; Alzheimer's disease	Q579R, A594T, I659V, R1068H, T1512M, Y1767D, N1800H, R2004K, A2028V	HEK293; primary fibroblasts	4-PBA; Dithiothreitol; Thapsigargin	[62 , 98]
	ABCA3	LBM180	Phospholipids; Sphingomyelin; Cholesterol	Neonatal surfactant deficiency; Congenital cataract	L101P, G1221S	HEK293	4-PBA	[101]
	ABCA4	ABCR	N-retinyl-phosphatidyl-ethanolamine	Stargardt disease	R1108C, R1129C	HEK293	CF-106951; Corr-4a; VRT-325; VX-809	[114]
B	ABCB1	MDR1; P-gp	Xenobiotic compounds (drugs)	Multidrug resistance	More than 30 independent variations[4]	BHK; HEK293; HeLa; MDCK	4-PBA; Capsaicin; Corr-3a; Corr-4a; Corr-4b; CsA; Curcumin; FK506; Glycerol; Nilotinib; Nonylphenolethoxylates; Paclitaxel; Tariquidar; Thapsigargin; Valinomycin; Verapamil; Vinblastin; VRT-325	[26, 54, 63, 66-68, 70, 72, 73, 113]
	ABCB4	MDR3	Phosphatidyl-choline	PFIC3; LPAC; ICP	G228R, S320F, A364V, G535D, G536R, I541F, L556R, Q855L, A934T, S1076C, S1176L, G1178S	293T; HEK293; HepG2; MDCK	4-PBA; CsA; Curcumin; Cyclosporins B, C, D, H, VX-770	[54, 64, 74, 140-142]
	ABCB11	BSEP	Bile salts	PFIC2; ICP; BRIC2	G238V, A257V, E297G, E297K, D404G, V444A, D482G, A570T, G982R, R1050C, R1128C, R1128H, R1153H, T1210P, R1231Q, R1268Q	Can10; CHO; HEK293; Liver biopsies (human and rats); MDCK	4-PBA ± UDCA; Bile acids; Glycerol	[55, 56, 110-112, 122, 125, 126, 128-130]
C	ABCC1	MRP1	Organic anions; Drugs	Multidrug resistance	K513A, E521A, E535A	293T	4-PBA; DMSO; Glycerol; Polyethylene glycol; Trimethylamine-N-oxide	[57, 58]
	ABCC4	MRP4; MOAT-B	Nucleoside monophosphate derivatives; Drugs	Multidrug resistance	T796M	NIH3T3	4-PBA; Glycerol	[59]
	ABCC6	MRP6; MOAT-E	ATP	Pseudoxanthoma elasticum	R1114P, S1121W, R1138Q, T1301I, R1314W, Q1347H	MDCK; Mouse liver biopsies; Zebrafish embryos	4-PBA	[103 , 104]

	ABCC7	CFTR	Chloride and bicarbonate anions	Cystic fibrosis	More than 2000 independent variations[4]	---------- *See Fig. 1 and text for details*[4] ----------		
	ABCC8	SUR1	Potassium (with Kir6.1)	Congenital hyperinsulinism; Type 2 diabetes	C6G, G7R, V21D, N24K, F27S, D29G, A30T, L31P, L40R, G70E, R74W, M80R, G92D, G111R, A113V, A116P, E128K, R168C, G173R, V187D, R495Q, E501K, R1394H	COS-1; COS-m6; HEK293; HeLa; INS-1; Rat and human β-islets	ABT-888; Carbamazepine; Diazoxide; DMSO; Glafenine; Glibenclamide; Glycerol; KM11057; KM11060; Latonduine; Ouabain; RDR1; Repaglinide; Tolbutamide	[60, 61, 75-80, 82]
D	ABCD2	ALDP	Very long chain fatty acids	X-linked adrenoleukodys-trophy (ABCD1)	None	Rat and mouse primary hepatocytes	4-PBA; Trichostatin A (increased expression)	[102]
G	ABCG2	BCRP	Drugs; Urate	Gout; Multidrug resistance	Q141K	HEK293	4-PBA; Corr-4a; Panobinostat; Romidepsin; Vorinostat; VRT-325	[86, 99, 100]

[1] For clarity, only ABC transporters for which efficient pharmacotherapies have been proposed are indicated in this table; [2] Only variations for which a rescue of traffic and/or activity are listed here; [3] Only compounds with significant rescue of traffic and/or activity of the studied ABC transporter variants are shown; [4] For more information, the reader is invited to refer to the articles cited in this table or in the text. Abbreviations are: 4-PBA, 4-phenylbutyrate; ABC, ATP-Binding Cassette; ABCR, Retina-specific ABC transporter; ALDP, AdrenoLeukoDystrophy Protein; BCRP, Breast Cancer Related Protein; BHK, Baby Hamster Kidney; BRIC2, Benign Recurrent Intrahepatic Cholestasis type 2; BSEP, Bile Salt Export Pump; CERP, Cholesterol Efflux Regulatory Protein; CFTR, Cystic Fibrosis Transmembrane conductance Regulator; CHO, Chinese Hamster Ovary; COS, CV-1 (simian) in Origin and carrying SV40 genes; CsA, Cyclosporin A; DMSO, DiMethylSulfOxyde; HDL, High-density Lipoprotein; HEK, Human Embryonic Kidney; ICP, Intrahepatic Cholestasis of Pregnancy; LBM180, Lamellar Body Membrane of 180 kDa; LPAC, Low-Phospholipid Associated Cholelithiasis; MDCK, Madin-Darby Canine Kidney; MDR1/3, MultiDrug Resistance Proteins 1/3; MOAT, Multi-specific Organic Anion Transporter; MRP, Multidrug Related Proteins; P-gp, P-glycoprotein; PFIC, Progressive Familial Intrahepatic Cholestasis; SUR1, SulfonylUrea Receptor 1; UDCA, UrsoDeoxyCholic Acid.