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► To cite this version:

Atanu Banerjee, Jorgaq Pata, Vincent Chaptal, Ahcène Boumendjel, Pierre Falson, et al.. Structure, function, and inhibition of catalytically asymmetric ABC transporters: Lessons from the PDR subfamily. *Drug Resistance Updates*, 2023, 71, pp.100992. 10.1016/j.drug.2023.100992 . hal-04271811

HAL Id: hal-04271811

<https://hal.science/hal-04271811>

Submitted on 6 Nov 2023

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Structure, function, and inhibition of catalytically asymmetric ABC transporters: lessons from the PDR subfamily

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ABSTRACT

ATP-binding cassette (ABC) superfamily comprises a large group of ubiquitous transmembrane proteins that play a crucial role in transporting a diverse spectrum of substrates across cellular membranes. They participate in a wide array of physiological and pathological processes including nutrient uptake, antigen presentation, toxin elimination, and drug resistance in cancer and microbial cells. ABC transporters couple ATP binding and hydrolysis to undergo conformational changes allowing substrate translocation. Within this superfamily, a set of ABC transporters has lost the capacity to hydrolyze ATP at one of their nucleotide-binding sites (NBS), called the non-catalytic NBS, whose importance became evident with extensive biochemistry carried out on yeast pleiotropic drug resistance (PDR) transporters. Recent single-particle cryogenic electron microscopy (cryo-EM) advances have further catapulted our understanding of the architecture of these pumps. We provide here a comprehensive overview of the structural and functional aspects of catalytically asymmetric ABC pumps with an emphasis on the PDR subfamily. Furthermore, given the increasing evidence of efflux-mediated antifungal resistance in clinical settings, we also discuss potential grounds to explore PDR transporters as therapeutic targets.

KEYWORDS

ABC superfamily, PDR transporters, ScPdr5, CaCdr1, non-catalytic NBS

1. ABC superfamily and its clinical relevance in yeasts: PDR, the standout members

ATP-binding cassette (ABC) superfamily is a large, highly topologically conserved, and fascinating protein family with most of its members involved in transport across membranes (Srikant, 2020). The hallmark feature of all ABC proteins is the presence of a nucleotide-binding domain (NBD) involved in binding and hydrolysis of nucleotide, preferably ATP. In the case of ABC proteins that function as transporters across membranes, an additional component, i.e., a transmembrane domain (TMD) is also present (Locher, 2009). While the non-transporter ABC proteins are equally relevant due to their crucial roles in mechanisms like chromosome segregation and DNA repair, ribosome biogenesis and recycling, etc. (Navarro-Quiles et al., 2018), transport functions take the center stage due their pathophysiological and clinical implications (Leonard, 2003; Moore et al., 2023). Transporter proteins are involved in the translocation of a diverse range of substrates across membranes, including ions, amino acids, lipids, and drugs (Vasiliou et al., 2009). The bacterial ABC transporters include proteins with both import as well as export functions (ter Beek et al., 2014). On the contrary, eukaryotic ABC transporters were believed to be solely functioning as exporters, until recently, whereby new lines of evidence suggest import characteristics, especially in plants (Choi and Ford, 2021; Quazi et al., 2012; Voith von Voithenberg et al., 2019).

All functional ABC transporters share a similar structural blueprint, comprising of two NBDs containing a set of highly conserved motifs like the Walker A, Walker B, ABC signature sequence (also called the C-loop/C-motif), A-loop, Q-loop, D-loop and H-loop, and two variable TMDs that form the translocation channel (Szöllősi et al., 2018). The

function of the conserved motifs is extensively studied, and reviewed in a very recent article (Thomas and Tampé, 2020). In prokaryotes, the four domains are usually separate subunits or are combined to form homo- or heterodimerizing half-transporters, consisting of one NBD and one TMD (Thomas and Tampé, 2020). However, the eukaryotic ABC transporters are commonly constructed from two half-transporters, or they exist as full transporters, where all four domains are integrated into a single polypeptide chain (Thomas and Tampé, 2020).

As per conventional classification system, ABC superfamily comprised of 7 subfamilies, viz., ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. Two new subfamilies, viz., ABCH and ABCI were further added to make it a total of 9 subfamilies (Choi and Ford, 2021). Out of these 9 subfamilies, except ABCE and ABCF, all are vested with transport functions. It is pertinent to mention that representatives of ABCH and ABCI subfamilies are not present in humans. While ABCH subfamily of exporters is found in fishes and insects, ABCI is an elusive subfamily of plant transporters resembling the bacterial type III or energy-coupling factor (ECF) transporters and probably act as importers (Choi and Ford, 2021; Moreno et al., 2019). However, given the remarkable structural diversity, mainly concerning the TMD fold as evident from the latest structures, Thomas and colleagues proposed a new classification system for the ABC transporters (Thomas et al., 2020). As per this classification system, the transporters are grouped into seven types, I-VII, depending upon the TMD fold. While the prokaryotes contain representatives encompassing all the seven types, eukaryotic ABC transporters are representative of type IV and V only. Notably, the authors also proposed to maintain the A-G subfamilies as

subcategories under the type IV (subfamilies B–D) and type V (subfamilies A and G) systems (Thomas et al., 2020).

In yeasts and more generally fungi, ABC transporters belong to 4 subfamilies, namely, multidrug resistance (MDR), multidrug resistance associated protein (MRP), adrenoleukodystrophy protein (ALDP) and pleiotropic drug resistance (PDR), corresponding to the mammalian designations ABCB, ABCC, ABCD and ABCG, respectively. While all the transporter families play crucial functions in cellular detoxification or cellular physiology, reviewed in (Moreno et al., 2019; Prasad et al., 2015), the PDR subfamily members (type V ABC exporters) hold major implications owing to their predominant roles in antifungal resistance and MDR phenomenon exhibited by pathogenic fungi. In *Candida* species, azole resistance shows strong correlation with increased expression of PDR transporter encoding genes like *CDR1*, *CDR2*, and *SNQ2* (Moreno et al., 2019; Prasad et al., 2017).

2. Basic architecture and functioning of the PDR transporters

PDR transporters display the typical reverse topology in which each NBD precedes a TMD, also characteristic of the mammalian ABCG subfamily (**Figure 1A**). Each TMD is composed of six alpha helices interconnected by four intracellular loops (ICLs) and six extracellular loops (ECLs). Uniquely, all PDR transporters have two large, partially homologous, extracellular loops, ECL3 and ECL6 and a long cytosolic N-terminal extension (NTE) composed of phosphorylated residues (Lamping et al., 2010; Tsao et al., 2016).

Besides these topological differences, there are also a few peculiar features seen in the PDR pumps. The most remarkable characteristic of PDR transporters is their asymmetric nature, like some other clinically relevant transporters like cystic fibrosis transmembrane conductance regulator (CFTR)/ABCC1, MRP1/ABCC7 and the transporter associated with antigen processing TAP1/TAP2 proteins (Gao et al., 2000; Procko et al., 2006; Sorum et al., 2017). In these pumps, despite the presence of two NBSs (each NBS is composite with contributions from the two NBDs in the "sandwich" dimer), only NBS1 is made up of canonical (conserved) motifs and hydrolyzes ATP. On the contrary NBS2, usually referred to as the "non-catalytic" NBS contains peculiar substitutions within the conserved motifs, including the Walker A, Walker B, ABC signature sequence, Q-loop and the H-loop (**Figure 1B**) (Banerjee et al., 2021; Golin and Ambudkar, 2015; Gupta et al., 2014). Relevance of this non-catalytic NBS (alternatively referred to as the degenerate NBS or non-canonical NBS), remained elusive for long before growing body of evidence started, revealing their role in the transport mechanism (Banerjee et al., 2021). Although not a direct consequence of the sequence degeneracy, the PDR pumps also exhibit a constitutively active ATP hydrolysis machinery uncoupled to substrate transport (Golin and Ambudkar, 2015). This feature is believed to be important for keeping the transporter in an "always on" state, crucial for a rapid efflux-mediated cellular detoxification (Ernst et al., 2008).

Recently, a study from the Lutz Schmitt's lab highlighted yet another intriguing facet of Pdr5, that it functions as a proton and drug co-transporter, strictly dependent on the substrate (Wagner et al., 2022). Whether the same holds true for other PDR subfamily members is not yet known.

3. Structure-function investigations of the PDR transporters: understanding the asymmetry

The investigation into how PDR pumps operate and the impact of non-catalytic NBS initially began with groundbreaking research on ScPdr5, which was further substantiated by studies on Cdr1 from *Candida albicans* (CaCdr1). As baker's yeast provides a highly convenient platform for genetic manipulation (Decottignies et al., 1998), it was a suitable model to explore the mechanisms of not only ScPdr5 but also the *Candida* transporters (Lamping et al., 2007).

Given the asymmetric nature of the NBSs in PDR transporters, several attempts have been made to understand the consequence of atypical residues within one of the NBS. Since initial attempts to restore symmetry at the level of NBS were not successful in both ScPdr5 as well as CaCdr1 (Gupta et al., 2014; Saini et al., 2006), it was imperative to understand the functional implications of such asymmetry. A few studies from Golin's group on the S558Y mutant of Pdr5 served as initial hints towards importance of the non-canonical motifs (Ananthaswamy et al., 2010; Downes et al., 2013; Sauna et al., 2008). Since S558Y displayed strong uncoupling of ATPase and substrate transport behavior, being unable to export cycloheximide but ATPase active, it was a strong proponent for suppressor studies. Interestingly, a several suppressors emerged and were mapped to or near the otherwise conserved regions within the non-catalytic NBS (Ananthaswamy et al., 2010; Downes et al., 2013; Sauna et al., 2008). The nature of the primary mutant and those of the corresponding suppressors strongly suggested a role of the non-catalytic NBS in crosstalk across domains. Mutational studies on the D-loop also corroborated

such role, wherein D1042N in ScPdr5 and D1033A in CaCdr1 were found to harbor similar ATPase profiles to that of wild-type (WT), despite conferring complete or selective hypersensitivity towards transporter substrates, respectively (Banerjee et al., 2018; Furman et al., 2013). Two transport-defect TMH mutants of CaCdr1, i.e., L529A and V532D also yielded suppressors Q1005H and W1038S, respectively, both located either within or in close vicinity of the signature sequence and D-loop forming the non-catalytic NBS, further reinforcing the active involvement of this NBS in the transport mechanism (Banerjee et al., 2020, 2018). While both the studies pointed out a direct role of this NBS in transport modulation, the mechanistic understanding of the transport process in PDR pumps remains obscure. The recent structural evidence with ScPdr5 led to a better understanding of the process, whereby it was very clear that throughout the cycle, the non-catalytic NBS retains a bound ATP molecule (Harris et al., 2021). Notably, the ScPdr5 structure also revealed a plausible stabilizing effect of nucleotide binding at this site on the highly conserved linker domain (LD). Interestingly, LD is a critical structural determinant that influences the substrate selection as well ATP hydrolysis at the catalytic NBS. On the contrary, ATP binding and hydrolysis at the catalytic NBS leads to major structural changes responsible for substrate efflux and resetting of transporter, respectively. Due to this asymmetric nature of nucleotide hydrolysis, one half of ScPdr5 undergoes a greater conformational change than the other.

3.1 Cryo-EM and AlphaFold, a structural breakthrough

ABC pumps undergo large conformational changes for translocating substrates across membranes. In a general scheme, the protein adopts first an inward-facing (IF) state which favors the substrate binding to the drug-binding site in the TMD, accessible from

the cytoplasm in this conformation. The binding of the substrate brings together the NBDs forming 2 NBS within which an ATP-Mg²⁺ complex binds. This leads to an occluded state of the protein which prevents the substrate from moving back into the cytoplasm and favors the access of the drug-binding site from the outside, in an outward-facing (OF) state. Then ATP is hydrolyzed, which allows the protein to adopt an IF state (Chaptal et al., 2022; Thomas and Tampé, 2020).

The structural knowledge of these different states and their sequence is largely dependent on the ability to isolate the proteins in solution in a functional form i.e., capable of hydrolyzing ATP. Unfortunately, despite extensive assays, only few examples of ATPase-active purified PDR proteins have been published (Laub et al., 2017; Pierman et al., 2017; Wagner et al., 2019). Consequently, the first structures of a single member, ScPdr5, have been released only quite recently (Harris et al., 2021). However, due to the resolution revolution of cryo-EM (Kühlbrandt, 2014) and high-end microscopes becoming more accessible, a faster and easier structure determination can be expected soon (Fréchin et al., 2023). Structural data from human transporters sharing the same topology are also available, initially from the ABCG5/ABCG8 heterodimer by X-ray (Lee et al., 2016) and more recently by cryo-EM (Sun et al., 2021), then from the ABCG2 homodimer (Jackson et al., 2018; Manolaridis et al., 2018; Orlando and Liao, 2020; Rasouli et al., 2023), and more recently from the ABCG1 homodimer (Xu et al., 2022). All this information allows limited but useful mechanistic analogies to be made (Banerjee et al., 2021).

3.1.1 ScPdr5 structure

Four structures of ScPdr5 were resolved, a first one in apo state, a second with ATP and ADP bound in the non-catalytic and catalytic NBS, respectively, a third one with rhodamine 6G (R6G) bound in the drug-binding site, and a fourth one with the ADP-Mg²⁺-VO₄ complex which traps the catalytic NBS in a post-hydrolytic state. The first three structures are overall similar, with the protein in the IF conformation. Contrarily to transporters belonging to the type IV group (Thomas and Tampé, 2020), substrate binding (at least considering here R6G) does not induce large structural changes, with an overall root mean square deviation calculated on 17,713 atoms between structures with and without R6G that remains as low as 1 Å (**Figure 2A**). Structures of ScPdr5 in complex with ATP and ADP adopt an IF conformation indicating that ATP binding to the non-catalytic NBS does not induce a major conformational change. The LD remains, however, only visible when nucleotides are bound to the protein, suggesting that ATP binding to the non-catalytic NBS stabilizes the region. Of note, mutations carried on residues 801-805 in the LD display a decreased V_m and a similar K_m for ATP compared to the WT, with an added impact on drug efflux depending on the drugs (Harris et al., 2021). This highlights the coupling role of the non-catalytic NBS between the catalytic NBS and the drug-translocation region.

The nucleotide-bound structures of ScPdr5 also suggest, first, that the binding of the ATP to the catalytic site drives the IF to OF transition, and second, that the ADP produced by ATP hydrolysis is still bound after drug translocation. Accounting for the ATP concentration in yeast that ranges from sub-millimolar to millimolar levels, it is possible that this IF state of ScPdr5 with ATP and ADP bound corresponds to the resting state of the transporter.

The structures reveal a remarkable structural asymmetry. Firstly, at the level of substrate binding, R6G binds in a pocket near the membrane inner leaflet, in which it mainly establishes contacts with membrane helices of the N-terminal bundle 1, 2 and 5, mainly through van der Waals and alkyl or Pi-alkyl interactions (**Figure 2B**). Secondly, the structural asymmetry is also visible in the NBSs, the distance between conserved P-loop and signature motifs residues (considering their C-alpha) being 8 - 10 Å shorter for the non-catalytic NBS compared to the catalytic NBS, or to those of ABCG2 (**Figure 2C**). A difference of 5 Å is also observed with the same residues of ABCG5/ABCG8 (PDB 5DO7) between catalytic (K92-E217, 26 Å) and non-catalytic (C109-E197, 21 Å) NBSs (not shown), but 2-times reduced suggesting higher level of constraints in ScPdr5 which should have mechanical consequences.

Structures of ScPdr5 also show that in the external leaflet of the membrane region, residues F683 and M1373 are in contact, closing the top of the drug-translocation pathway. Those residues have been proposed to act as a gate, initially for ABCG2 (Khunweeraphong et al., 2019; Taylor et al., 2017) and recently with ScPdr5 (Alhumaidi et al., 2022). Indeed, the mutation M1373A led to a protein unable to reach the membrane, as also observed for the mutant of the equivalent residue L555A in ABCG2 (Khunweeraphong et al., 2019). In addition, the mutation F683L altered R6G transport, selectively when measured against its concentration gradient suggesting that those residues prevent a substrate reflux (Khunweeraphong et al., 2019).

The structural information obtained experimentally is both valuable and irreplaceable, especially in the case of ABC transporters whose conformational changes condition their functionality. However, obtaining these structures remains an obstacle and requires a lot

of time, even if, as introduced above, the cryo-EM revolution has permanently reduced the level of difficulty and time needed. In this context, the development of AlphaFold (AF), an artificial intelligence system recently released, achieves an outstanding accuracy in 3D structure prediction (Jumper et al., 2021) representing a “prediction revolution”. The cryo-EM structures of ScPdr5 give us the opportunity to compare and evaluate its AF-model. As shown in **Figure 2A**, the AF-Pdr5 model looks remarkably similar to the cryo-EM structures. A closer comparison shows that the AF model displays local differences which positions it as an intermediate between the IF and occluded conformations of the protein. NBDs look closer compared to the IF structure while NBSs are not formed. In addition, the C-terminal region of the LD (residues 800-806) is already positioned close to the non-catalytic NBS despite the absence of nucleotide. The drug-binding pocket is less open, due to membrane helices coming closer and moving as a rigid body, leading to a space too small for allowing R6G to bind. The ECL is also correctly predicted, close to the experimental structures. The superposition of the experimental and AF models thus shows that the latter is sufficiently accurate for use and is an asset to compensate (at least temporarily) due to the lack of experimental structures.

3.1.2 CaCdr1 model

CaCdr1 and its homologs from other *Candida* species are of critical interest due to their involvement in drug efflux. As the 3D structure of the protein is yet to be solved, all structure-function studies have been carried on using predicted models. Given that the first ABCG protein structures are recent, the first models were based on class IV transporters (PMID: 21034832), whose topology is finally different from that of ABCG and PDR transporters. The release of ABCG5/G8 (PMID: 27144356) and ABCG2 structures

(28554189, 30405239) provided much better structural templates to be used in homology modelling. In light of this, several models were built (27569110, 31734312, 30348662) to offer a better explanation of the observed phenotypes after mutagenesis studies. Their quality was greatly improved and offered a structural basis to understand the way mutations impacted transport or inhibition. However, some shortcomings were unavoidable, given the differences between templates and the actual structure of PDR transporters, featuring a remarkable asymmetry, a linker domain and a large ECD.

The recent information provided with the cryo-EM structures of ScPdr5 is a significant step in structure modelling of CaCdr1, as both proteins share 55% identity. Finally, the AF model of CaCdr1 is also helpful and, not surprisingly, looks like that of ScPdr5, standing between IF and OF conformations (**Figure 3**).

An extensive alanine-scanning mutagenesis study of the 252 residues forming most the TMD of CaCdr1 has been previously carried out and analyzed by our groups, each mutant having been functionally characterized for their capacity to export dyes, steroids and antifungals (Baghel et al., 2017; Nim et al., 2016; Rawal et al., 2013). Herein, we focus on R6G for which the Pdr5 cryo-EM structure with the dye provides an opportunity to position the critical residues for its efflux in the AF-CaCdr1 model (**Figure 3**). Interestingly, most of the residues cluster into 3 main groups. The first group corresponds to residues either in contact (red) or close to (orange) R6G, confirming the localization of the R6G-binding pocket in CaCdr1 as in the TMD of ScPdr5; of note, the pocket includes more polar residues than observed for ScPdr5 (**Figure 3B**). A second pool of residues (green) clusters well around the gating area, again confirming the key role played by the region. However, the AF model and these residues display here a distinct pattern to that of

ScPdr5. While in the former the gate is formed by F683 and M1373, the corresponding residues of CaCdr1, F673 and L1364 are indeed in contact, but their mutation into alanine does not affect R6G efflux. Alternatively, the residue F1230 seems to play a role in gating since its phenyl ring projects into the substrate pathway, contacting F673 and L1364 in the AF model. Of note, those residues were also found critical for FK506 resistance, an inhibitor of Cdr1 (30348662), strengthening their gating function.

The last pool of critical residues (in blue) is clustered in the C-terminal region of the CaCdr1 TMD, far from the binding pocket and the gating area. This group is composed majorly of charged and polar residues, pointing towards TMH9. The mutations abolish R6G efflux indicating a critical role of this region, both distant to the drug-binding site and yet in the TMD. Due to the polar nature of these residues, one can hypothesize if they could be involved in the proton transport as recently observed with ScPdr5 when transporting R6G (Wagner et al., 2022) and in a context where CaCdr1 overexpression was previously observed to modulate the intracellular pH in the yeast host strain (Wakiec et al., 2008). However, future investigations are sought to support or invalidate this hypothesis.

4 Inhibitors/modulators of PDR pumps

The investigation of modulators of ABC proteins has been and still is the subject of intensive research aiming to overcome the multidrug resistance phenotype. The clinical use of inhibitors would be in combination with antifungals, to reverse the resistance. This strategy was strongly investigated in the management of cancer, but, despite the number of highly potent inhibitors of several ABC proteins, clinical studies did not meet the

expected results. This is mainly due to the fact the cancer is a complex pathology and resistance cannot be attributed to one single mechanism, but to multiple factors (Le Borgne et al., 2023).

In fungal infections, the search for potential modulators of the efflux pumps that can reverse the resistance against drugs has risen notably during the last 15 years. In clinics, such inhibitors (also called modulators or reversal agents) can be administered in antifungal therapy if they reverse the drug resistance phenomenon mediated by the pump. The discovered modulators are of different origins: they are either already approved drugs used for unrelated pathologies or newly discovered molecules with an either natural or synthetic origin. Therefore, it is necessary to have an overview of the structures of such modulators and whenever possible, describe efflux inhibition mechanisms. A literature survey shows that the strategy of reversing resistance in the context of yeast infections is dominated by the search of modulators targeting PDR subfamily, in which CaCdr1, CaCdr2 and ScPdr5 transporters, including their homologs from other species have attracted the highest attention. Therefore, in this section, we will focus on inhibitors for these pumps.

Independent of the origin of modulators, these compounds primarily act through five possible mechanisms by which a molecule inhibits the efflux carried out by the transporter (Tran-Nguyen et al., 2017): a) inhibition of the ATPase activity of the transporter through a competition with ATP; b) inhibition of the binding of substrates into the substrate-binding site(s); c) prevention of helix-helix interactions in the transmembrane domain; e) down-regulation of the expression of the gene encoding the transporter; f) alteration of ergosterol biosynthesis.

As highlighted above, PDR inhibitors originate from three different sources, natural products, drug repurposing and synthetic compounds.

4.1 Natural products as inhibitors of PDR transporters

Naturally occurring compounds have been frequently studied as potent reversal agents of MDR in cancer. Moreover, they served as models for the design of new generations of inhibitors (Joshi et al., 2017). Obviously, these molecules have aroused high interest in the context of antifungal therapy. The first natural product was cyclosporin A (CsA). This immunosuppressant drug is a member of the first generation of inhibitors of ABCB1 (P-gp). The chemical modification of CsA led to a second generation of inhibitors (Joshi et al., 2017). CsA was also evaluated as an inhibitor of CaCdr1 (Jia et al., 2016). Combination of CsA and fluconazole showed synergistic antifungal effect on the growth and biofilm formation of clinical isolates of *C. albicans*, as evidenced by fluorescent microscopy and XTT assays. Moreover, the synergy was also associated with a down-regulated expression of the ABC transporter gene *CDR1* and the MFS transporter gene *MDR1*.

A non-exhaustive list of natural products studied as inhibitors of PDR transporters is presented in **Figure 4**.

4.1.1 Milbemycins

Milbemycins are a class of macrocyclic lactones produced by several *Streptomyces* species with potent acaricidal, insecticidal and anthelmintic activities (Yan and Xia, 2021). Experimental treatments of *C. glabrata* infections with a combination of milbemycin and fluconazole inhibited efflux in *C. glabrata* with the highest activity for A3 oxime (Silva et

al., 2013). Some derivatives also displayed an intrinsic fungicidal activity above 3.2 µg/ml. Similar values were obtained with *C. albicans* as well. In addition to a potential inhibition of CgCdr1, authors showed that the fungicidal activity could be related to reactive oxygen species formation.

4.1.2 Kaempferol

Naturally occurring flavonoids and their synthetic derivatives were extensively studied as modulators of reversal agents of MDR against anticancer drugs through the inhibition of membrane efflux pumps. The interest in flavonoids has been motivated by their intrinsic activity for human health. In the antifungal therapy, kaempferol, a naturally occurring flavonoid, was assessed for its reversal activity on azole-resistant *C. albicans* strain (Shao et al., 2016). Expression of *CDR1* and *CDR2* genes as well as that of *MDR1*, the gene encoding the MFS CaMdr1, were decreased when fluconazole and kaempferol were used concomitantly. This effect was accompanied by an increase of the accumulation in the yeast of R6G when assayed.

4.1.3 Natural products from marine sponges

Batzelladine D and norbatzelladine L are two natural alkaloids isolated from the marine sponge *Monanchora arbuscula* that were evaluated for the reversal effect of fluconazole resistance mediated by Pdr5 in *Saccharomyces cerevisiae* (Domingos et al., 2020). Both alkaloids sensitized the ScPdr5p-overexpressing strain by synergistic interaction with fluconazole. Moreover, they increased intracellular accumulation of R6G with inhibitory effect on the catalytic activity and without significant toxicity on mammalian cells.

4.1.4 Tetrandrine

Tetrandrine is a naturally occurring alkaloid isolated from *Stephania tetrandra* and other Chinese medicinal plants (Jiang et al., 2022). Tetrandrine is known for its anti-inflammatory, immunologic and antiallergenic effects. Tetrandrine was evaluated alone and in combination with fluconazole or voriconazole against *C. parapsilosis*. The susceptibility test showed that tetrandrine presented strong synergy with fluconazole and voriconazole (Zhao et al., 2019) and increased intracellular R6G accumulation, suggesting a plausible inhibitory effect on the efflux machinery.

4.1.5 Artemisinin derivatives

In the context of drug repurposing, the drug artemether derived from artemisinin, was reported to display potent antifungal activity with fluconazole and can recover the susceptibility of fluconazole-resistant clinical isolates of *C. albicans*, *C. auris*, *C. tropicalis* and *C. parapsilosis*. It was revealed that artemether enhances fluconazole efficacy by disrupting the function of ScPdr5 (Zhou et al., 2021).

4.1.6 Isocoumarins

Isocoumarins are isomers of the well-known coumarins which are frequent naturally occurring compounds. Coumarins are well studied as inhibitors of the human efflux pumps P-gp, BCRP and MRP1 (Sjöstedt et al., 2017). Isocoumarins were also studied as inhibitors of ScPdr5, assaying the ATPase and substrate transport functions of the transporter. Inhibitors were identified preventing substrate transport and/or ATPase activity with IC₅₀ values in the low micromolar range and without toxicity to human cells (Sachs et al., 2019). Thus, isocoumarins provide novel scaffolds for further pharmacomodulation and optimization as reversal agents in the context of antifungal therapy.

4.2 Synthetic compounds as inhibitors of PDR transporters

Besides natural compounds, synthetic derivatives were investigated as PDR-mediated reversal agents. Inhibitors were either conceived based on known inhibitors of other transporters, by screening of bioactive libraries of compound or through drug repurposing strategy. **Figure 5** displays the major modulators reported in the last decade.

4.2.1 Zosuquidar

Zosuquidar is a known inhibitor of P-gp, one of the human MDR efflux pumps being involved in resistance of tumor cells against cytotoxins. Since its discovery, it was used as a scaffold for the design of inhibitors of different ABC proteins including fungal transporters. Several close analogs of zosuquidar and other simpler compounds (**Figure 5**) were evaluated as inhibitors of ScPdr5 and other transporters (Infed et al., 2013). For ScPdr5, some compounds were found to inhibit R6G transport more efficiently than zosuquidar with IC₅₀ below the micromolar range.

4.2.2 Oxindoles (Azoffluxin)

The oxindole Azoffluxin was identified as a compound that synergized with fluconazole by increasing intracellular fluconazole accumulation through inhibition of the major multidrug efflux transporter Cdr1 in *C. auris* (Iyer et al., 2020). In mice infected with drug-resistant *C. auris*, azoffluxin not only enhanced fluconazole activity but also reduced fungal burden by ~1000-fold as a single agent. The conclusions of this study suggest that pharmacologically targeting *CauCdr1* in combination with azoles may be an effective strategy to control infection caused by azole-resistant isolates of *C. auris*.

4.2.3 Carbolines

A series of carbolines acting as fungal histone deacetylase (HDAC) inhibitors were studied as reversal agents against resistant *C. albicans* infection (Li et al., 2021). They showed potent synergistic effects with the antifungal drug fluconazole. One of the compounds (**Figure 5**), showed promising *in vitro* and *in vivo* synergistic antifungal activity. It reduced the virulence of *C. albicans* by inhibiting the biofilm formation. Mechanism studies revealed that the reversion of drug resistance was due to downregulation of the expression of the azole target gene *ERG11* and the efflux gene *CDR1*.

4.2.4 Piperazinyl-Quinoxaline derivatives

Quinoxaline bearing piperazinyl moieties were identified as dual inhibitors of CaCdr1 and CaMdr1 overexpressed in a *Saccharomyces cerevisiae* strain (Guillon et al., 2020). Further pharmacomodulation investigations allowed the identification of derivatives with exclusive inhibition of CaCdr1 or CaMdr1. The strongest inhibition activity of CaCdr1 was achieved with derivatives bearing either a benzyl or a diphenylmethyl substituents at the piperazine ring (**Figure 5**).

4.2.5 Clorgyline

Clorgyline is a known irreversible and selective inhibitor of monoamine oxidase A (MAO-A) and has been identified as an efflux inhibitor of CaCdr1 and CaCdr2 (Holmes et al., 2012). In another study it was also shown to enhance fluconazole susceptibility in other *Candida* species, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Nagayoshi et al., 2017). In addition, in response to the treatment with clorgyline, the expression levels of *CDR1*, *CDR2* and *PDR1* mRNAs in the *C. glabrata* wild-type strain were also elevated.

4.2.6 Alcohols

Aliphatic alcohols (from ethanol to hexanol) were evaluated for their effect on various membrane proteins including ScPdr5. The study suggested a plausible inhibitory mechanism involving the interaction of the alcohols with the pump proteins (Gášková et al., 2013).

5. Concluding remarks and future perspectives

In this review, we have explored the significant advancements made in unveiling the intricate relationship between the molecular architecture and functional properties of PDR transporters, with an emphasis on ScPdr5 and CaCdr1. While the structure-function studies highlighted in the review have provided a solid foundation, there lies many pertinent questions. Substrate selection and the remarkable promiscuity manifested by these proteins is far from understood. Secondly, while we have made inroads into the mechanistic details of the gating function, the dynamic conformational changes involved in propelling the substrate through the translocation channel, culminating into efflux remains to be addressed in more detail. With the recent advancements in cryo-EM and single-particle analysis techniques, we are poised to further unlock our understanding of these elegant molecular machines. Herein, we also provided a succinct account of inhibitors/modulators identified against PDR pumps. Of note, majority of the inhibitor screening studies with PDR pumps exploit cell-based assays, often using fluorescent substrates as surrogate for drugs. Thus, it is pertinent to mention that more direct approaches exploiting purified proteins within reconstituted systems for drug/inhibitor binding are needed, as routinely done for mammalian counterparts like ABCG2 (Jackson

et al., 2018). The advent of advanced cryo-EM data for these pumps should lead to a stronger push to this avenue, allowing a structural perspective of events. Overall, a holistic understanding of PDR pump structure and transport mechanism will pave the way for the development of novel and targeted therapeutic strategies, which holds promise for overcoming multidrug resistance, thereby improving treatment outcomes.

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FIGURE LEGENDS

Figure 1. A. Topology diagram of a typical PDR transporter. **B.** Conserved sequence motifs that are present in the NBDs of ABC proteins and the unique substitutions present in the non-canonical NBS of PDR transporters. Residues are identified by their 1-letter code, h = hydrophobic residue, x = any amino acid. Information compiled from (Banerjee et al., 2021; Eggensperger and Tampé, 2015).

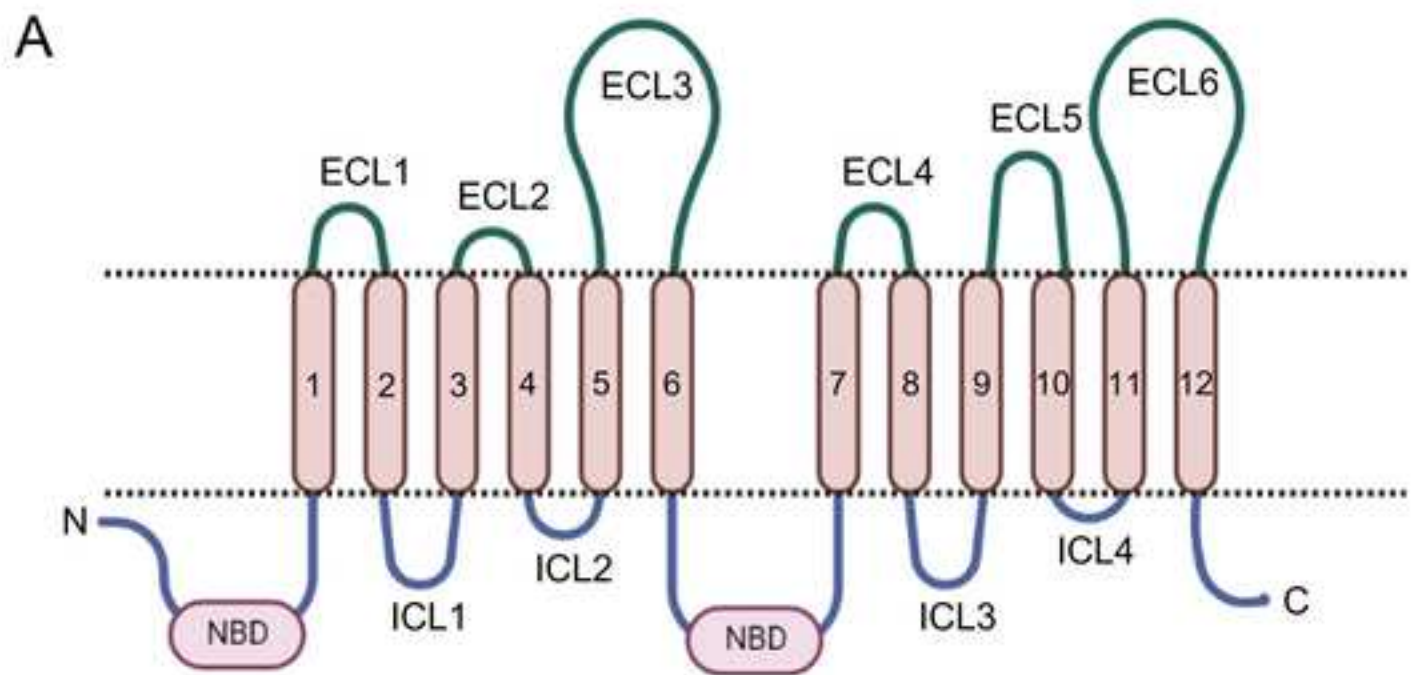
Figure 2. ScPdr5' structural information. A. ScPdr5 cryo-EM structures with and without substrate and AF model superposition. AF-ScPdr5 is in salmon, the apo-ScPdr5 (PDB 7p03) is in green and ScPdr5 in complex with ADP-Mg²⁺, ATP and R6G, bound

to the catalytic and non-catalytic NBSs and to the drug-binding site, respectively (PDB 7p05) is in light blue. Ligands are displayed in van der Waals. **B.** 2D-interaction diagram of R6G with Pdr5 based on the PDB structure 7p05, with C – H bonds (set at 3.4 Å max) shown in red, van der Waals interactions in green and alkyl and Pi-alkyl interactions (set at 5.5 Å max) in blue. **C.** Structural asymmetry of ScPdr5's NBSs. Distances are shown between C-alpha of residues of C and Walker A motifs forming each part of the NBSs. C-alpha are colored in pink (catalytic NBS) and blue (non-catalytic NBS) for ScPdr5 (salmon, 7p05), compared to those of ABCG2 (light green, 6VXI) colored in green.

Figure 3. AF model of CaCdr1 with TMD residues whose alanine substitution (glycine when alanine) abrogates R6G efflux in yeast. **A-D.** General and detailed views displaying residues, in red and orange when in contact or close to R6G (shown in black stick), in green around the gating region, and in blue when far from the binding pocket and the translocation pathway. R6G location corresponds to that found in ScPdr5 cryo-EM structure 7p05 (no docking).

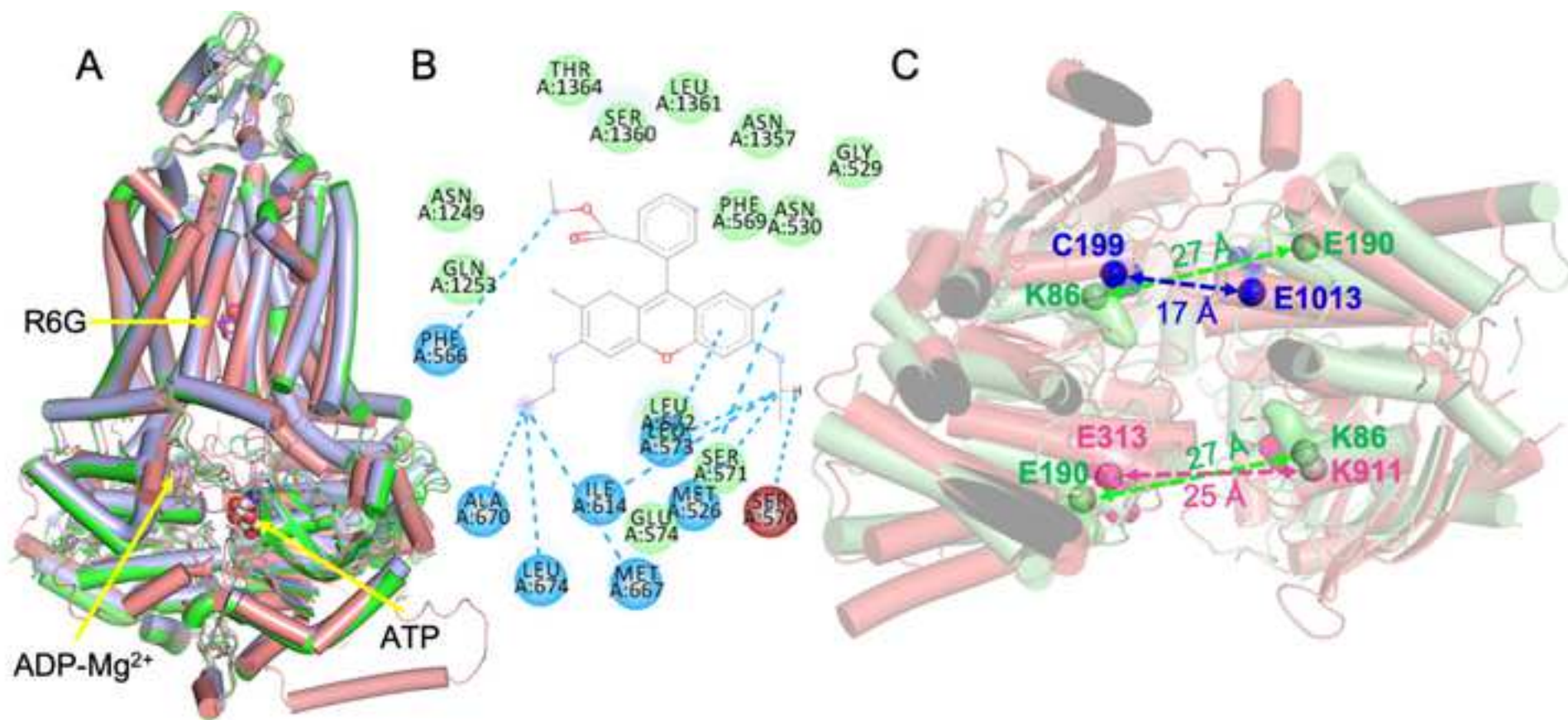
Figure 4. Structures of natural products and derivatives identified as inhibitors of PDR transporters.

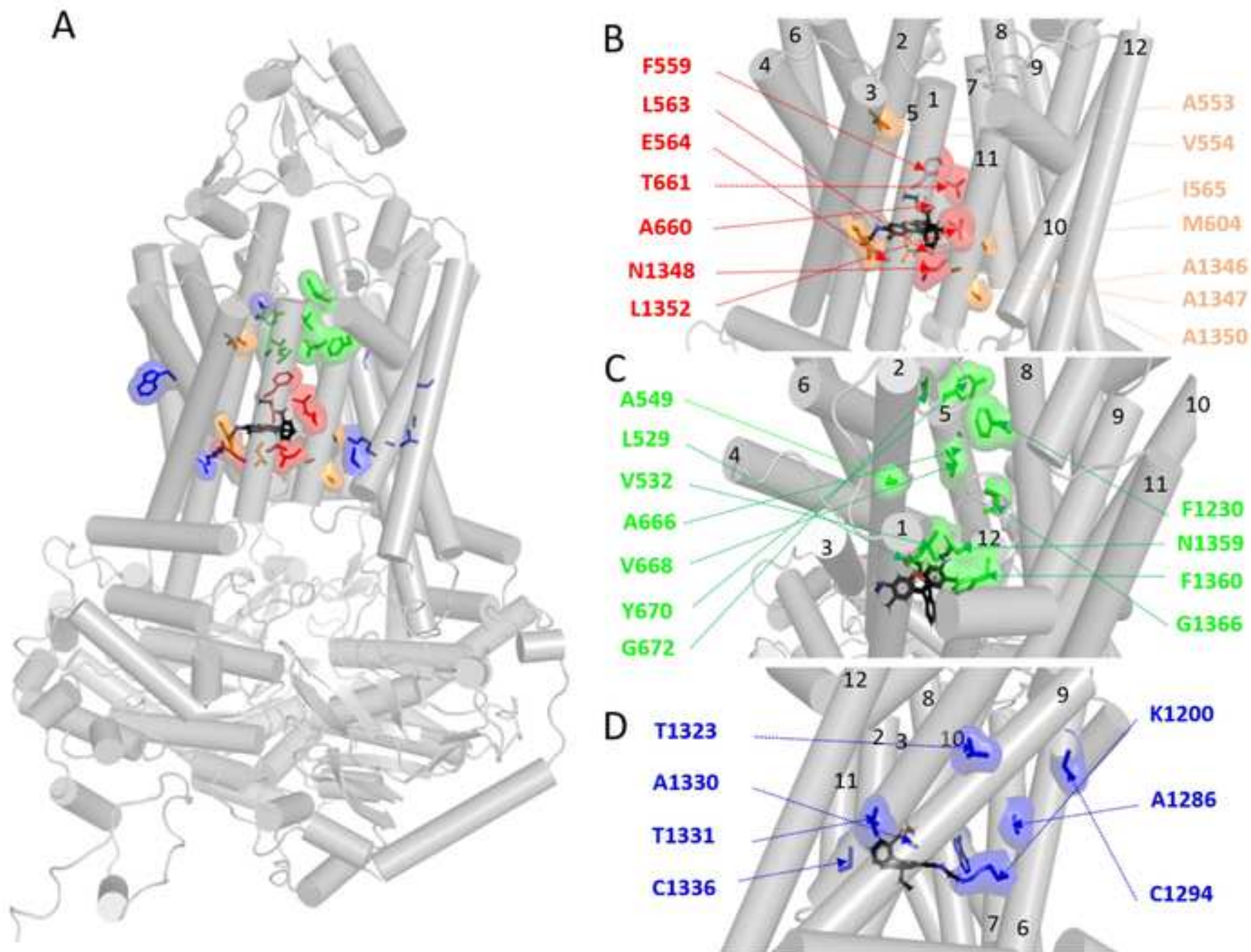
Figure 5. Structures of synthetic compounds identified as inhibitors of PDR transporters.

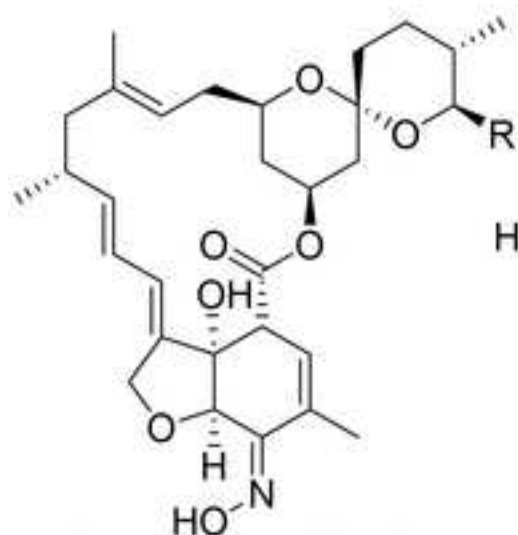


B

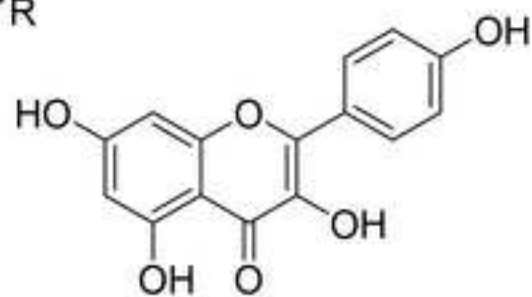
Motif	ABC consensus sequence	PDR catalytic sequence	PDR non-catalytic sequence
Walker A or P-loop	GxxGxGK(S/T)	GxxGxGK(S/T)	GxxGxG <u>C</u> (S/T)
Walker B	hhhhDE	hhhhDE	hxx <u>W</u> DN
ABC signature sequence or C-loop	LSGGQ(K/R)Q	VSGGERKR	<u>LNVE</u> QRKR
Q-loop	Q	Q	<u>E</u>
D-loop	hLD	hLD	hLD
H-loop or switch region	H(Q/R)	H(Q/R)	<u>Y</u> (Q/R)



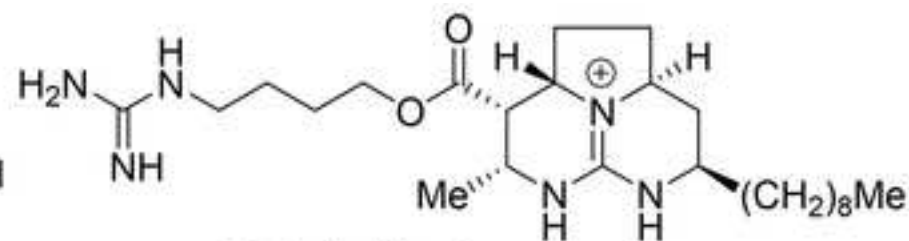




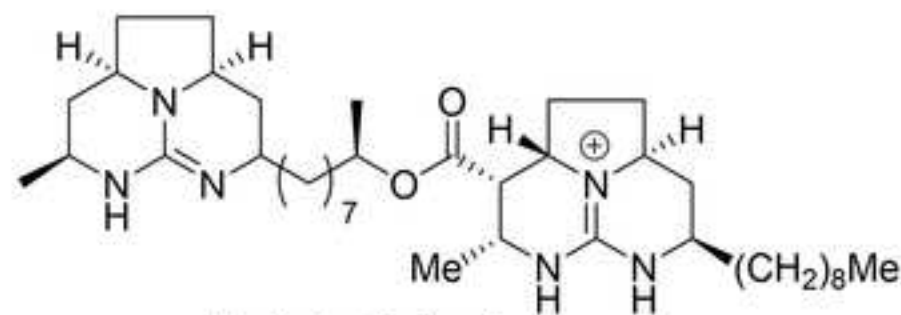
Milbemycin A3: R = CH₃
 Milbemycin A4: R = C₂H₅



Kaempferol



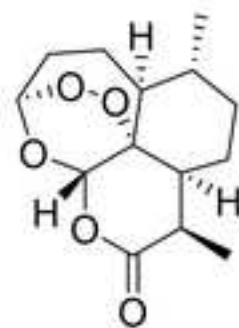
Batzelladine D



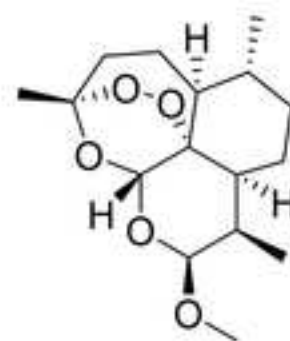
Norbatzelladine L



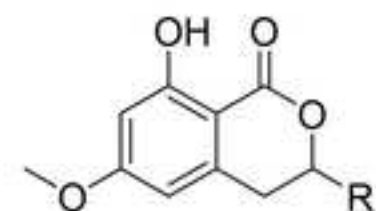
Tetrandrine



Artemisinin



Artemether



R = (CH₂)_n-R'; n = 1 - 5
 R' = H, OH, OTBS

Isocoumarins