A new twist in ABC transporter mediated multidrug resistance – Pdr5 is a drug/proton co-transporter

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Abstract

The two major efflux pump systems that are involved in multidrug resistance (MDR) are (i) ATP binding cassette (ABC) transporters and (ii) secondary transporters. While the former use binding and hydrolysis of ATP to facilitate export of cytotoxic compounds, the latter utilize electrochemical gradients to expel their substrates. Pdr5 from *Saccharomyces cerevisiae* is a prominent member of eukaryotic ATP binding cassette (ABC) transporters that are involved in multidrug resistance (MDR) and used as a frequently studied model system. Although investigated for decades, the underlying molecular mechanisms of drug transport and substrate specificity remain elusive. Here, we provide electrophysiological data on the reconstituted Pdr5 demonstrating that this MDR efflux pump does not only actively translocate its substrates across the lipid bilayer, but at the same time generates a proton motive force in the presence of Mg^{2+} -ATP and substrates by acting as a proton/drug co-transporter. Similar observations have not yet been reported for any other MDR efflux pump. We conclude from these results that the mechanism of MDR conferred by Pdr5 and likely other transporters is more complex than the sole extrusion of cytotoxic compounds and involves secondary coupled processes suitable to increase the effectiveness.

Keywords:

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Introduction

The phenomenon of multidrug resistance (MDR) exists in all cells ranging from prokaryotes to higher eukaryotes and describes the ability to prevent the efficacy of a wide range of functionally and structurally different cytotoxic compounds. One of the major mechanisms that confer MDR is the overexpression of certain efflux pumps expelling these toxic compounds into the extracellular space (1). Two classes of transporters are involved: (1) ATP binding cassette (ABC) transporters that utilize the binding and hydrolysis of ATP to transport their substrates and (2) secondary transporters of the major facilitator superfamily (MFS) as well as the RND and MATE family that use an existing electrochemical gradient across the membrane in order to export these compounds (2-5). Although studied for decades, the molecular mechanisms underlying the resistance conferred by these membrane proteins remain elusive in most cases (6).

In general, membrane transporters and channels are characterized by a high level of substrate specificity. Therefore, the large variety of substrates of individual MDR transporters raises the question whether these MDR transporters directly transport such a broad variety of structurally unrelated compounds or whether their physiological role within the cellular membrane indirectly protects the cell against cytotoxic compounds (2, 7). For the well-characterized human MDR ABC transporter P-gp (MDR1 or ABCB1), it was demonstrated that cells expressing this protein have an altered cytosolic pH and membrane potential compared to cells lacking MDR1. Earlier, *in vivo* studies revealed that Cdr1, a major MDR ABC transporter from pathogenic *Candida albicans* and a close homologue of *S. cerevisiae* Pdr5, alters the extracellular pH (8). Similar to MDR1, studies reported alterations of the membrane environment through altered ion gradients by MDR efflux pumps. In a study of the isolated transmembrane domain of the bacterial multidrug transporter LmrA from *Lactococcus lactis*, it was demonstrated that the transporter still facilitates proton motive force (pmf) driven uptake of drugs in absence of the nucleotide binding domains (NBDs) (9). This finding was later confirmed for full-length LmrA, which, however, requires that the physiological pmf results in the uptake of drugs through this membrane protein (10). The complexity of ion translocation by ABC transporters becomes more evident, if two other studies regarding this protein are taken into consideration. The first one demonstrated that LmrA is an ethidium bromide - H⁺ - Na⁺ - Cl⁻ symporter, while more recently it was shown that it can act as a Hepes⁺ - H⁺ - Cl⁻/2Na⁺ antiporter (11, 12). Another study involving the ABC transporter MsbA reported the requirement of the pmf for

this membrane protein, as ATP hydrolysis alone was insufficient to drive substrate translocation (13).

In our study, we used purified Pdr5, a long and intensively-studied member of the MDR ABC transporter family and the major player of the pleiotropic drug resistance (PDR) network of *S. cerevisiae* (14-18). Like other MDR ABC efflux pumps, *in vivo* and *in vitro* studies with plasma membrane vesicles clearly established that Pdr5 confers resistance towards a wide variety of hydrophobic compounds that are structurally unrelated, as well as metabolites (15, 17, 19). Only recently it was possible to isolate this transporter and purify it in an active state (20). Based on this possibility, we reconstituted Pdr5 into planar lipid bilayers and provide *in vitro* data demonstrating that Pdr5 actually co-transporters drug/protons in a strictly ATP- and substrate-dependent manner. Importantly, we show that contrary to LmrA or MsbA, Pdr5 does not rely on the presence of a pmf as an energy source, but rather it creates a pmf across the membrane in the presence of ATP and substrate. We conclude that the substrate/ATP induced pmf by Pdr5 serves as a self-activation and a novel mechanism for MDR.

Results

In vitro transport assay for Pdr5

In our efforts to reconstitute purified wild-type Pdr5 (Pdr5_{WT}) into synthetic liposomes and to establish an *in vitro* transport assay, we noticed robust ATPase activity comparable to the activity observed in detergent micelles (20). In plasma membrane preparations containing Pdr5_{WT}, a robust, real-time rhodamine 6G (R6G) transport assay is often used to study Pdr5_{WT} (17, 18). This assay is schematically summarized in Figure 1A. Here, plasma membranes enriched in Pdr5_{WT} likely present as inside-out vesicles, although the precise nature (sheets or vesicles) of the plasma membrane is unknown, are used. Incubation of plasma membranes vesicles enriched in Pdr5_{WT} with R6G results in a nearly quantitative insertion of R6G into the bilayer, followed by a slower equilibration between inner and outer leaflets (indicated as “1” in Figure 1A). Addition of ATP (“2”) to the Mg²⁺ containing buffer results in energization of Pdr5_{WT} and pumping of R6G from the outer to the inner leaflet, which decreases the R6G fluorescence intensity (ΔF_i). Normally, a decrease on the order of 50- 80% is achieved, which is due to oligomer formation that are non-fluorescent (17, 21). As soon as the active, Pdr5_{WT} mediated transport and the spontaneous back flip of R6G reach equilibrium, no macroscopic change in the fluorescence intensity is observed (“3”). Addition of, for example, EDTA (“4”), which complexes the cofactor Mg²⁺, restores the R6G fluorescence to its initial value prior to the addition of ATP (“5” in Figure 1A).

In contrast, in Pdr5_{WT}-proteoliposomes such a decrease was rarely observed. Only in less than 10% of the attempts, ΔF_i as read-out for substrate transport was detected with $\Delta F_i < 10\%$, not comparable to the more than 50- 80% decrease in fluorescence intensity observed when employing plasma membrane preparations (17, 18). In light of the data available for LmrA and MsbA (11-13), we wondered whether ion gradients might also have an impact on Pdr5_{WT}-mediated R6G transport. As a first step, we prepared plasma membrane preparations enriched with Pdr5_{WT} (18) and created an artificial potassium and/or proton gradient by either adding 100 mM KCl and valinomycin to the buffer (K⁺ gradient). In the case of the K⁺ gradient, R6G transport was started by addition of 5 mM ATP/Mg²⁺ (Figure 1B). After the transport induced ΔF_i reached a nearly steady state, EDTA was added to stop transport leading to nearly complete reversal of ΔF_i . Next, we tested whether a complete short-circuit of the membrane potential across the plasma membrane during the ATP/Mg²⁺ induced transport might produce a similar effect on ΔF_i .

as EDTA before. For this, after the transport induced R6G decrease of the fluorescence intensity reached a nearly steady state, nigericin, valinomycin and gramicidin were added in low μM concentrations to suppress the membrane potential completely (Figure 1B) (22, 23). Remarkably, the short circuit of the membrane potential almost completely reversed ΔF_i similar to EDTA (22). These results demonstrated that the fluorescence of R6G is sensitive to electrochemical gradients (pmf) across the membrane. This confers with the known sensitivity of R6G fluorescence towards pH (24) and the membrane potential as derived from *in vivo* and *in vitro* studies (25-27). This also suggests that the decrease in R6G fluorescence in plasma membrane preparation enriched in Pdr5_{WT} might not solely be due to formation of non-fluorescent dimers and trimers (21), but also to changes in the membrane potential.

Pdr5_{WT} shows voltage dependent ion channel activity

In order to verify whether Pdr5_{WT} requires an existing ion gradient or may facilitate voltage activated ionic currents across a membrane, purified Pdr5 was reconstituted into planar lipid bilayer (DOPC/DOPE, 7:3) and voltage dependent currents across the bilayer were monitored. The high purity of the protein used in the electrophysiological experiments was confirmed by MS analysis (see Table S1). In addition to the control experiments with MOK-samples, the same data set as for Pdr5_{WT} was also collected for the ATPase deficient E1036Q mutant (Pdr5EQ) (18).

The slope conductance of the control (“empty”) bilayer was $G_{sl} = 3.2 \text{ pS}$ (Figure 2A) when a voltage ramp ($V_h = -100 \text{ mV}$ to $+100 \text{ mV}$, see Figure 2F top) was applied under symmetrical cis/trans conditions (250 mM KCl, 10 mM HEPES pH 7.0). However, after addition of 2 μl detergent solubilized Pdr5_{WT} (0.6 mg/ml) to the cis-compartment with brief (2min) gentle stirring the voltage induced current across the bilayer increased significantly and a slightly rectifying current voltage relation was observed (Figure 2B). Addition of MOK samples did not show any effect on the *i/v*-curve. Moreover, repeated aliquot addition (10 times) of the Pdr5 protein to the cis compartment resulted in a large increase ($\sim 10^3$ -times) in the membrane currents as compared to the control (Figure 2C). Application of voltage gates on the same bilayer as in Figure 2B also showed a significant increase of the membrane currents as compared to the control (Figure 2D) and the resulting plot of the average current ($\bar{i}$) versus voltage (Figure 2E) resembled closely the *i/v*- ramp in Figure 2B. These results give clear evidence that reconstituted Pdr5 facilitates transport of K^+ and Cl^- ions at comparable high rates, which are also dependent on the amount of

reconstituted Pdr5_{WT}. Similar results have been obtained for the ATPase deficient E1036Q mutant (Pdr5_{EQ}) (see below for details). In order to check whether the high transport rates are caused either by a high number of copies of active Pdr5_{WT} in the membrane or by an "ion channel" mode, we have tested various voltage protocols, which potentially could lead to ion-channel gating of Pdr5_W.

We could indeed find such a voltage-protocol (see Figure 2F, top, 3G, H). Starting from a holding potential of $V_h = +150$ mV for 2-10 min. and using either voltage ramps (Figure 3G) or voltage gates (Figure 3H) we observed a significant increase of the Pdr5_{WT}-mediated membrane currents accompanied by brief current flickering (Figure 3B) ($G_{sl} = 420$ pS, at $V_{cmd} > +50$ mV, Figure 2B). Application of voltage gates allowed for a more detailed analysis of the Pdr5_{WT} mediated current gating (Figure 3C, D, E, F) and Figure S1). The graphs in Figure 3 first clearly show that the reconstituted Pdr5_{WT} mediates fast, flickering current gating with different open channel amplitudes.

Additionally, as indicated by the reproducible rectifying current-voltage relation, Pdr5 incorporation into the membrane occurred unidirectional with the nucleotide binding site at the cis compartment (for more details see below).

Pdr5_{WT} possesses three main conductance states

The analysis of the representative current recordings shown in Figure 3, with voltage gates from $V_h = \pm 60$ mV to $V_h = \pm 140$ mV show that the observed current gating originates from one active channel with up to three open channel amplitudes (see mean variance analysis in Figure 3E, F and Figure S1). This conclusion is proven by looking closer onto the combined current histogram and mean variance analysis of the current recordings in Figure 3D at $V_{cmd} = -140$ mV indicating that channel gating occurred with at least 3 different main open channel amplitudes. From the analysis of current recordings at different membrane potentials ($V_h = \pm 60$ mV to $V_h = \pm 140$ mV, see Figure 2C) we collected from 5 different bilayer in a total of 40 recordings all point amplitude histograms (Figure S1). The obtained currents at the given voltages were transformed into conductance values binned in a histogram and the appearing peaks plotted as a box-chart plot (Figure S1C). The following main mean conductance states of Pdr5_{WT} were obtained: $G_1 \cong 55 \pm 24$ pS, $G_2 \cong 304 \pm 106$ pS, and $G_3 \cong 665 \pm 127$ pS. Closer inspection of the time-course of gating events (see Figure 2C and Figure 2D) clearly indicate that one single Pdr5_{WT} unit displayed at least three distinct main subconductance states (for more details see Methods in SI).

The selectivity of the Pdr5_{WT} mediated ion currents was tested using asymmetric cis/trans buffer conditions (see Figure S2) gave a value of $P_{K^+}:P_{Cl^-} = 2.6:1$.

Remarkably, the mean open state G_2 was mostly occupied at membrane potentials above $V_{cmd} > \pm 100$ mV. At an ionic strength of 250 mM and using the simplified model of a cylindrical restriction channel region (28, 29) with a length of 1 nm, we calculate from this conductance state the pore diameter of Pdr5 to be $d_{restriction} \cong 6.4$ Å.

ATPase deficient E1036Q mutant (Pdr5_{EQ}) reveals similar properties as Pdr5_{WT}

After addition of 1 µl detergent solubilized Pdr5_{EQ} (2.0mg/ml) to the cis-compartment with brief (2min) gentle stirring again voltage (voltage ramp starting at $V_h = 0$ mV, see Figure 2F top) induced currents across the bilayer were observed. The amplitude of the open channel currents appeared somewhat smaller as compared to the Pdr5_{WT} with slope conductance of $G_{sl} = 150$ pS ($-V_{cmd}$) and $G_{sl} = 280$ pS ($+V_{cmd}$) were observed (Figure 4A). Again, addition of MOK-samples did not show any effect on the control i/v-curve. Repeated addition (3-times) of the Pdr5 protein to the cis compartment resulted in a large increase (~5-times) in the membrane currents as compared to the control (Figure 4D). Application of voltage gates on the same bilayer as in Figure 4D also showed a significant increase of the membrane currents and the resulting plot of the average current ($\bar{i}$) versus voltage (Figure 4E) resembled closely the i/v- ramp in Figure 4D. These results show that that reconstituted Pdr5_{EQ} facilitates transport of K^+ and Cl^- ions at high rates which are also dependent on the amount of reconstituted Pdr5_{EQ}.

However, when the pre-activation command voltage protocol shown in Figure 3H was applied to the bilayer containing the reconstituted Pdr5_{EQ} rare current gating events were only observed at negative V_{cmd} (Figure 4B). From a brief analysis of the current gating events at $V_{cmd} = -70$ mV to -140 mV, the following main mean conductance states of Pdr5_{EQ} were obtained: $G_1 \cong 35 \pm 12$ pS, $G_2 \cong 120 \pm 29$ pS. Thus, the current gating properties of the Pdr5_{WT} and the Pdr5_{EQ} mutant are different while voltage activation of Pdr5_{WT} leads to a strong current flickering mainly from the open to the closed state the Pdr5_{EQ} mutant remains mainly in the open state with current gating.

The ion selectivity of the Pdr5_{EQ} mutant as determined in asymmetric cis/trans buffer of $P_{K^+}:P_{Cl^-} = 3.5:1$ was close to the one of the Pdr5_{WT} (see Figure S2).

Modulation of Pdr5-mediated ion currents by its substrates

We further tested the effect of known substrates namely R6G, ketoconazole (KA), clotrimazole (CLO) and cycloheximide (CHX) on the voltage-induced ion channel activity of Pdr5_{WT}.

Figure 5B shows a current-voltage ramp using the command-voltage protocol shown in the inset of Figure 5A (control, empty bilayer) from a bilayer containing the reconstituted Pdr5_{WT} in symmetrical (cis/trans) buffer. As obvious, ion-conducting activity with frequent channel gating was observed (see enlarged lower panel Figure 5B). The linear slope conductance indicated by the red line was $G_{sl} = 525 \pm 2.4$ pS. This value is very close to the Pdr5_{WT} conductance state G_3 deduced from channel gating (see above). Therefore, the current-voltage ramp can likely be attributed to a single activated Pdr5_{WT} unit. The brief flickering events resemble the respective channel conductance G_1 and G_2 in their amplitudes. After addition of 2 mM Mg²⁺-ATP (cis/trans) and 330 nM R6G (1.2 μ l 330 μ M into 1.2 ml buffer) to the cis compartment of the same bilayer no further voltage dependent channel gating could be observed (Figure 5C). The current was drastically reduced while the form of the current-voltage ramp changed to a more rectifying shape (Figure 5C). In addition, the zero-current crossing (V_{rev}), changed completely and unexpectedly from $V_{rev} = 0$ mV to $V_{rev} = -46$ mV. This effect was reproducibly observed with an average $V_{rev} = -43.8 \pm 4.8$ mV ($n=7$) no matter whether the bilayer contained single activated or multiple activated copies of Pdr5_{WT}. In this context, it is important to mention that this R6G-induced shift of V_{rev} was strictly dependent on the presence of Mg²⁺-ATP in the cis compartment. Addition of Mg²⁺-AMP (trans) ($n = 3$) did not show this effect. Moreover, in this setup of Pdr5_{WT} reconstituted from the cis compartment, addition of the effectors to the trans compartment ($n=3$) did also not show any effect. One may argue that the observed shift might be due to electrode effects due to the addition of 1.2 μ l 330 μ M R6G into 1.2ml buffer of the cis compartment only. However, this can be ruled out from both theoretical and experimental considerations. We are using agar protected Ag+/AgCl electrodes ($R_{typ} \cong 10k\Omega$), which are carefully checked for electrode offset ($V_{typ}^{off} \leq 500\mu V$), which then is corrected automatically by the amplifier, at the beginning of each bilayer recording (30). In none of the five control experiments with control bilayers, the addition of 1.2 μ l 330 μ M R6G in EtOH had any effect on the electrode potentials. Moreover, as outlined in more detail below no shift in V_{rev} was observed in Pdr5_{EQ} containing bilayer at identical

experimental conditions. The change in V_{rev} can therefore only be due to an R6G induced trans-membrane ion flux as source of the additional membrane potential.

In order to explain the observed shift of V_{rev} we can use the general applied macroscopic GHK approach, based on the ion composition in the cis and trans compartments of the bilayer. Depending on the membrane permeability of the involved ions, the expected reversal potentials for the flux of the given ions in the cis/trans compartment separated by a selective permeable membrane can be calculated using the GHK current equation ((31), the details can be found in SI).

The ion concentrations in the cis/trans compartments were kept symmetrical (250 mM KCl, 10 mM HEPES pH 7.0, 2 mM Mg^{2+} -ATP) before the addition of 330 nM R6G to the cis compartment and we observed $V_{rev} = 0 \pm 1.5$ mV ($n=7$). The calculation of the expected V_{rev} by the GHK current equations (see SI for details) before and after addition of charged R6G ($n=+1$) clearly showed that the ion concentration at both sides of the Pdr5_{WT} containing membrane can by no means produce a current voltage-relation with a zero-current crossing at $V_{rev} = -43.8 \pm 4.8$ mV (see Figure S3). Therefore, we must conclude that R6G induced a Pdr5_{WT}-mediated active ion transport of one of the anions or cations present in solution. With symmetrical 250 mM KCl cis/trans, either Cl^- ions would have to be transported from trans to cis to final concentrations of $c_{trans}^{Cl^-} = 75$ mM and $c_{cis}^{Cl^-} = 425$ mM, or in the case of K^+ the ions would be transported from cis to trans to final concentrations of $c_{cis}^{K^+} = 75$ mM and $c_{trans}^{K^+} = 425$ mM (for details see SI). This massive ion pumping of one of the two ion species would in both cases reduce (Cl^-) or increase (K^+) the bulk conductance in either bilayer compartments, which in fact was not observed. Consequently, transient flux of H^+ ions is the remaining candidate to build up a membrane potential across the Pdr5_{WT} containing bilayer yielding a zero-current potential of $V_{rev} = -43.8 \pm 4.8$ mV. Introducing H^+ as an additional ion in the GHK current equations, we can calculate the amount of H^+ ions that would have to be transported from cis to trans to obey the GHK model, starting at pH 7 ($c_{cis/trans}^{H^+} = 100$ nM) symmetrically. These calculations result in final concentrations of $c_{cis}^{H^+} = 7.94$ nM and $c_{trans}^{H^+} = 0.9$ μ M (see Figure 5J and Figure S3 for calculation details). However, since we detect ion flux induced membrane potentials generated by a small number ($n \ll 100$) of channels across a distance of ~ 20 nm (see SI for details), which requires only tiny amounts of transported charges to establish the membrane potential (see example calculation in SI for details),

macroscopic changes of the H^+ concentrations would not be directly resolvable in our experimental setup.

As is obvious from the calculated current voltage relation with 2mM Mg^{2+} -ATP in cis/trans (Figure 5J), the observed shift of V_{rev} by R6G addition to the cis compartment in Pdr5_{WT} containing bilayer could be explained by active (H^+ permeability $P_{H^+} \rightarrow \infty$) H^+ transport from the cis to the trans compartment. Calculation details for Figure 5J are given in SI.

In similar experiments, we tested whether the non-charged KA, CHX and CLO, other well-known substrates of Pdr5_{WT}, also modulate the activity of reconstituted Pdr5_{WT} in planar bilayer. In a series of four different experiments, we observed a Mg^{2+} -ATP/KA induced shift of the zero-current potential of $V_{rev} = -64 \pm 8$ mV ($n = 4$) (see Figure 6).

Based on these results, we conclude analogously that also KA in the presence of Mg^{2+} -ATP induces active Pdr5_{WT}-mediated H^+ transport from the cis to the trans compartment. Interestingly, with CHX as a potential substrate, we did not observe any significant modulation of Pdr5_{WT}-mediated currents, in contrast to R6G and KA. However, CLO (1 μ M), when added to the cis compartment of a bilayer containing Pdr5_{WT}, induced a drastic shift of the reversal potential of $V_{rev} = -57 \pm 6.7$ mV ($n=4$) in the presence of Mg^{2+} -ATP (cis) (see Figure 6).

The proton gradient is ATPase dependent

In order to verify the significance of the observed modulation of Pdr5_{WT} activity by its substrates, we performed the same set of experiments as describe above except that we used the ATPase deficient Pdr5_{EQ} mutant instead of Pdr5_{WT}. This therefore is a proof whether the catalytic ATPase cycle is necessary for the observed shift of V_{rev} and, most importantly, whether the observed ion currents are Pdr5-mediated. As shown before in Figure 4 Pdr5_{EQ} also incorporates into the planar bilayer membrane and mediates voltage dependent ion currents across the membrane. However, Pdr5_{EQ}-mediated currents were observed already at membrane potentials ≥ 20 mV. In more than 90% of the attempts during voltage ramps rather large currents were observed (Figure 4A,D) and correspondingly also in voltage gates (Figure 4E, F). These results indicate that a high number of ion conducting Pdr5_{EQ} have been incorporated into the bilayer.

In addition, Pdr5_{EQ} channel gating was rarely observed and the channels remained almost exclusively in the open states as the determined conductance values of the Pdr5_{EQ} were in a similar range as for Pdr5_{WT}. This also holds true for the ion selectivity of the

Pdr5_{EQ} mutant determined to be $P_{K^+}:P_{Cl^-} = 3.5:1$ in comparison to 2.5:1 for Pdr5_{WT} (see Figure S2 and SI for details). We also tested whether channel currents of Pdr5_{EQ} could be modulated by the substrates R6G and KA in a similar set of experiments as described above for Pdr5_{WT}. We observed that in none out of the 15 attempts with R6G (see Figure 5G-I) and KA any modulation of Pdr5_{EQ}-mediated currents showing that ATPase activity and transport of the substrate is an absolute requirement for substrate induced change in V_{rev} .

Beauvericin locks Pdr5 in an open state

Beauvericin (BEA) is known as a potent inhibitor of Pdr5's ATPase and transport activity (32) and additionally can mediate ion transport across the membrane most likely in a carrier like mode (33). We therefore first investigated membrane currents after addition of 0 μ M, 3.3 μ M and 9.9 μ M BEA on the cis side (Figure 7A- C). The conductance values of these measurements are listed in Table S3. Next, we addressed the question whether BEA might suppress the substrate induced H⁺ co-transport of Pdr5_{WT}. Figure 7 D-G shows a representative series of experiments with an identical bilayer. The graphs in Figure 7E demonstrates that Pdr5_{WT} was incorporated in the membrane and after addition of R6G to the cis compartment a shift of $V_{rev} = -51$ mV was observed. Remarkably, after addition of 1.65 μ M BEA the overall current increased significantly and the shift of the reversal potential disappeared completely (Figure 7G). The conductance values of the different measurements in Figure 7 D-G are listed in Table S4. These recordings show clearly that BEA can act as a carrier, but most importantly acts as a Pdr5 inhibitor that presumably locks Pdr5 in an open channel conformation.

Molecular docking suggests Beauvericin binding to the Pdr5 substrate binding site

Only recently, structural information of Pdr5 determined by single particle cryo EM became available (34). Thus, we used molecular docking to identify possible binding sites and states of BEA. The above described results suggest that BEA could bind to the R6G binding site to lock Pdr5 in an open state. However, as the starting conformation and placement of the ligand can have an influence on the results of the docking (35), although a small one, we used two different starting placements for BEA. First, we placed BEA in the binding site of R6G (Figure 8A) as it is the most likely on based on our experiments. Second, we placed BEA close to the transmembrane domains (TMDs) in the space between the TMDs and the nucleotide-binding domains (Figure 8B). The rationale here was

that due to its size BEA might bind to both TMDs while allowing ion fluxes through its macrocycle as an alternative binding mode hypothesis. To include receptor plasticity, we employed four different cryo-EM structures of Pdr5 (34), which show different binding site conformations, after the removal of ligands present in the respective structures. Scoring grids were calculated for at least 40 Å around each initial BEA placements for each of the four Pdr5 conformations and subsequently both initial BEA conformations were docked into each grid. This resulted in 16 valid suggested binding poses. Out of these 16 binding poses nine bind to the R6G binding site in nearly identical conformations (Figure 8C) regardless of their initial placement and center of docking grid. Here, BEA utilizes one of its benzyl moieties such that it binds to a similar location as one of the benzene rings of R6Gs xanthene-like moiety. The five binding poses not binding to the R6G binding site of Pdr5 were either generated in the ADP-orthovanadate bound structure of Pdr5 (four poses) or R6G bound structure (one pose). The ADP-orthovanadate bound structure of Pdr5 shows a shift of one of the transmembrane helices at the R6G binding site. This shift renders the R6G binding site too narrow to accommodate BEA (Figure 8D). As a result, 90 % of all docked BEA binding poses in Pdr5 structures, which could accommodate BEA in the R6G binding site, bind to exactly this binding site in nearly identical conformations. This suggests that BEA binds to the R6G binding site in Pdr5.

Discussion

MDR ABC transporters have a broad range of substrates that are highly diverse in terms of structure and physico-chemical properties. There has been a long debate on how these membrane proteins confer resistance, i.e. how transporters reduce the drug concentration within a cell. Currently two models are favored: (i) the ‘drug pump model’, in which ABC transporters actively expel the compounds out of the cell is supported by numerous studies (36, 37) and (ii) the ‘altered partitioning model’, in which MDR ABC transporters confer resistance by alteration of the membrane environment through ion transport that ultimately results in lower drug uptake by the cell (7, 8, 12, 38). Essentially, the question arises whether all of the known substrates of MDR transporters are actually real substrates in terms of being actively transported out of the cell against the concentration gradient, or if these proteins mediate a decreased influx of the drugs by altering membrane properties, or whether both mechanisms are actually used.

With regard to these open questions, we tried to establish an *in vitro* transport assay for the well-studied yeast MDR ABC transporter Pdr5 (for a recent review see (16)). This transporter has been extensively studied in the last three decades and a robust *in vivo* transport assay based on the Pdr5- and ATP/Mg²⁺-dependent R6G quenching in plasma membrane preparation is present (17, 18). Despite all our efforts, we could not transfer the plasma membrane-based transport assay to proteoliposomes. Nevertheless, we also detected that the R6G fluorescence was strongly dependent on artificial ion gradients (Figure 1), which is supported by other studies (25-27). Therefore, we turned to an electrophysiological *in vitro* approach, which enabled us to measure directly Pdr5-mediated ion conductivities under varying conditions. Previously, studies performed on MsbA and LmrA demonstrated that ion gradients benefit or are even indispensable for the activity of the respective ABC transporter, while ATP, the energy source of this protein family, seems to play only a regulatory role (9-13, 39). Moreover, in the case of LmrA, it remains elusive whether this pump works as an importer or exporter. Besides these complex, slightly contradicting studies, there are to our knowledge so far, no detailed electrophysiological analysis of MDR ABC transporters *in vitro*. On basis of the functionally purified, reconstituted Pdr5, and a well-studied ATPase deficient mutant as a control as well as known substrates of Pdr5 (R6G, KA, CLO and CHX (15, 17, 18, 20, 40)) and the potent Pdr5-specific inhibitor BEA, we were able to perform an extensive electrophysiological *in vitro* characterization of this MDR efflux pump.

In particular, we were able to demonstrate that Pdr5_{WT}, but not the ATPase-deficient Pdr5_{EQ} mutant, generated a membrane potential that can only be the result of a substrate and Mg²⁺-ATP driven proton co-transport across the membrane. We therefore conclude that Pdr5 is an ATP-dependent drug/H⁺ co-transporter that actively translocates substrates and generates an additional pmf through proton efflux. Our electrophysiological measurements allowed not only to prove that Pdr5 conducts ions and thereby follows the 'altered partitioning model', but also to demonstrate that it actively transports its substrates across the bilayer. Interestingly, the H⁺ co-transport occurs independently of the charge of the substrate and both, the cationic R6G as well as the neutral KA and CLO are transported in a drug/H⁺ co-transport fashion. The zero-current potentials for KA, CLO and R6G ($V_{rev} = -64 \pm 8$ mV, -57.3 ± 6.7 mV and -47.2 ± 5.3 mV, respectively) clearly show that R6G is transported from cis to trans since the putative V_{rev} of the R6G⁺-ions contributes to the overall V_{rev} , reducing it in comparison to the neutral KA and CLO. Additionally, using cationic and neutral substrates, we demonstrate that the measured H⁺ co-transport cannot be an artifact of the efflux of a protonated or cationic substrate and instead is a real H⁺ co-transport by Pdr5 in a Mg²⁺-ATP- and substrate-dependent manner.

The results presented in this study are also in line with the widely used R6G transport assay that was established for Pdr5 located in inside-out oriented plasma membrane vesicles (17, 18, 41, 42). R6G is a mitochondrial marker and a fluorescent probe sensitive to membrane potential (27). The observed fluorescence intensity decrease in plasma membrane vesicles reflects the R6G and proton co-transport and thereby the membrane potential change during transport.

Another tested substrate, CHX, however, did not induce any proton gradient or change in conductance. In contrast to the majority of known Pdr5 substrates, CHX does not affect the ATPase activity of Pdr5 and is hydrophilic (18, 20). One can speculate that either a different transport route for this hydrophilic substrate exists, or that CHX is not a substrate at all. The observed resistance conferred by Pdr5 could be due to intracellular alkalization caused by Pdr5-mediated proton pumping as CHX undergoes degradation at basic pH (43).

An interesting feature of Pdr5 is that it can be voltage activated thus allowing high current fluxes across the membrane even in the absence of Mg²⁺-ATP or substrates. This activation occurred if Pdr5 was exposed to voltages of $V_h > +100$ mV. In a physiological context with a yeast plasma membrane potential of about -82 mV (44) and an additional Pdr5 generated $\Delta\varphi$ of about $\Delta\varphi = -50$ to -65 mV ($V_{rev, R6G} = -47.2 \pm 5.3$ mV, $V_{rev, CLO} = -$

57.3 ± 6.7 mV, and $V_{rev, KA} = -64 \pm 8$ mV), Pdr5 possibly activates itself into this high conductance state.

Next to the self-activation, the proton pumping properties of Pdr5 with multiple active copies would lead to the acidification in the trans compartment of our setup which reflects an acidic external pH in an *in vivo* setting. Moreover, drug/proton co-transport would temporarily significantly increase the membrane potential of the plasma membrane thus effecting the ion and solute homeostasis and the membrane morphology of yeast cells (45-47). This compares well with results that were obtained previously for the Pdr5 homologue Cdr1 and MDR1 *in vivo* as it has been speculated that MDR1 as well as Cdr1 may transport protons from the intra- to the extracellular space and thus alkalize the cytoplasm (8, 38). However, the complexity of the cell environment of these *in vivo* experiments did not allow to unambiguously pinpoint observations to a single protein level nor for the experimenter to control all relevant critical cellular parameters. For Cdr1 it was hypothesized that the ABC transporter creates an additional proton motive force when it was expressed in *S. cerevisiae*. In this context it is important to note that in our experiments, the proton gradient does only occur in a strictly Mg^{2+} -ATP and substrate dependent manner. This means that in the absence of a substrate, Pdr5 does not participate in pH homeostasis of the membrane even if ATPase activity is present. Therefore, the active proton pumping appears to be a second mode of action of this MDR ABC transporter.

Furthermore, we demonstrate that Pdr5 conducts K^+ and Cl^- ions with a slight cation selectivity. This is not surprising as all known charged substrates of Pdr5, like for most other MDR ABC transporters, are cationic (15, 17, 37). We were also able to show that the membrane part of the nucleotide-free Pdr5 switches between at least three major confirmations as apparent from the three distinct subconductance states of which the main state is compatible with a pore diameter of 6.4 Å (48), which is within the range reported for other ABC transporters (49). Moreover, the broad distribution of open channel states indicates that Pdr5 has a rather flexible membrane part responsible for the translocation of its substrates. Additional experiments with the Pdr5 inhibitor BEA demonstrated that inhibition of the ATPase activity abolishes H^+ -co-transport and locks Pdr5 in an open conformation as indicated by the measured high conductance. Molecular docking suggests that this is mediated via BEA binding to the R6G binding site of Pdr5. However, the structures of Pdr5 (34) did not indicate a proteon translocation pathway.

In conclusion, the presented data demonstrates that Pdr5 is an ATP-driven drug/proton co-transporter that, unlike LmrA or MsbA, does not use the pmf but creates a proton gradient across the membrane that results in self-activation and presents a novel mechanism of drug resistance.

Materials and Methods

Pdr5 Expression and Purification

Pdr5 was expressed and purified as described (20). Briefly, *S. cerevisiae* YRE1001 (18) cells were grown at 30 °C to a final OD₆₀₀ of 3.5. Cells of 4 L YPD media were harvested and disrupted using glass beads. Total membranes were collected adjusted to 10 mg/ml total protein concentration with buffer A (50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 10% (w/v) glycerol and solubilized for 1 h with 1% (w/v) trans-PCC- α -M under gentle stirring at 4 °C. Immobilized metal ion affinity chromatography of the His-tagged Pdr5 was performed with a Zn²⁺ loaded 1 ml HiTrap Chelating column (GE Healthcare) and elution was carried out with a step gradient using low and high histidine buffers (50 mM Tris-HCl pH 7.8, 500 mM NaCl, 10 % (w/v) glycerol, 0.003% (w/v) trans-PCC- α -M and 2.5 mM or 100 mM L-histidine. The elution fractions were collected, pooled and concentrated in a ultrafiltration unit (100 kDa MWCO). Size exclusion chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) with buffer A containing 0.003 % (w/v) trans-PCC- α -M. All chromatography steps were performed on Äkta protein purification systems (GE Healthcare).

Preparation of Pdr5 containing plasma membranes

Plasma membranes highly enriched in Pdr5_{WT} were prepared as described in (18).

Transport assays using Pdr5 containing plasma membranes

Transport assays were performed according to the protocol developed by Kolaczowski and co-workers (17), using a FluoroLog III fluorescence spectrometer (Horiba). Isolated plasma membranes (30 μ g of total protein content) were resuspended in 1 ml of transport buffer (50mM Hepes (pH 7.0), 10mM MgCl₂, 150 nM R6G, and 10mM NaN₃) and incubated at 35 °C. R6G Transport was initiated by addition of 5 mM ATP unless otherwise stated.

Single Channel Recordings from Planar Lipid Bilayers and Data Analysis

Planar lipid bilayer measurements using the Compact bilayer platform (Ionovation GmbH) were performed as described in detail previously (50). In brief: if not explicitly stated otherwise, symmetric conditions (250 mM KCl, 10 mM HEPES, pH 7.0) were used in cis and trans compartments. The denomination cis and trans corresponds to the half-

chambers of the bilayer unit. Reported membrane potentials are always referred to the trans compartment. Bilayer fabrication was performed on PFTE film at a 100 μm preprinted (1 % hexadecane in n-hexane) aperture with a Phosphatidylcholine (18:1) (PC) / Phosphatidylethanolamine (18:1) (PE) (7:3 ratio) lipid mixture (both lipids were purchased from Avanti Polar Lipids) in n-pentane using the “thinning method” (50). Stock solutions of purified Pdr5 with typically 0.5-2.5 mg/ml contained in a buffer of 50 mM Tris-HCl pH 7.8, 50 mM NaCl, and 0.003% (w/v) trans-PCC- α -M were added to the cis compartment under slight stirring to a final protein concentration of $\sim 1\mu\text{g/ml}$ to $2\mu\text{g/ml}$.

Ion channel currents were recorded using an EPC 10 USB amplifier (HEKA Elektronik GmbH) in combination with the Patchmaster data acquisition software (HEKA Elektronik GmbH). For data acquisition a sampling rate of 5kHz (voltage ramps) and 10 kHz (continuous recording) was used and the data were further analyzed using the Origin package (Origin Lab) and the MATLAB (MathWorks) based Ion-channel-Master software developed in our laboratory (50).

Molecular Docking

For the molecular docking BEA was drawn and converted into 3D using the ChemDraw19 suite. BEA was subsequently docked into four different Pdr5 cryo-EM structures utilizing a combination of AutoDock as a docking engine and the DrugScore2018 distance-dependent pair-potentials as an objective function. For this, the co-resolved ligands were removed and the structures aligned on the R6G bound Pdr5 structure using Pymol 2.4 (51). The DrugScore scoring grids were calculated for each Pdr5 structure twice: It was centered on BEA, which was manually docked into either the R6G binding site or the space between the TMDs and NBDs of Pdr5. Both these initial BEA structures were docked into either grid to minimized starting structure bias. During docking, default parameters were used, except for the clustering RMSD cutoff, which was set to 2.0 Å(52-54). Binding modes were considered valid if they were contained in the largest cluster with the most favorable docking energies, which comprised at least 20 % of all docking poses.

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Figures

Figure 1. R6G transport assay. (A): Real-time R6G transport in Pdr5_{WT} enriched plasma membranes. At time point 0, R6G is added to a solution containing yeast plasma membranes (“1”). After energization of Pdr5_{WT} by addition of ATP (“2”), R6G is transported from the outer to the inner leaflet until a new equilibrium value of the fluorescence intensity is reached (“3”). The Pdr5_{WT}-mediated transport results in the formation of non-fluorescent R6G oligomers in the inner leaflet as indicated by the intensity drop of approximately 60%. At equilibrium, active transport of R6G by Pdr5_{WT} and spontaneous flip-back reach steady state. At “4” the inhibitor EDTA, which complexes the essential cofactor Mg²⁺, is added, which results in restoration of the initial fluorescence intensity (“5”) which is highlight by the gray, dashed line. **(B):** Dependence of R6G fluorescence intensity in plasma membrane preparations highly enriched in Pdr5_{WT} on ATP induced R6G transport in the absence (control) and the presence of an artificial K⁺ gradient. (Control): R6G transport was initiated by addition of 5 mM ATP-Mg²⁺, at the indicated time, EDTA (1 mM) was added. (K⁺ gradient): After addition of 100 mM KCl and 5 mM ATP-Mg²⁺, nigericin (5 μM), valinomycin (0.18 μM) and gramicidin (2 μg/ml) were added at the indicated time.

Figure 2. Current recordings from a planar lipid bilayer containing reconstituted Pdr5_{WT}. (A) Current-voltage ramp measurement with a DOPE/DOPC (7:3) bilayer in symmetrical cis/trans buffer conditions (250 mM KCl, 10 mM HEPES, pH 7.0) (control). (B) Current recording of the same bilayer as in (A), but after insertion of detergent purified Pdr5_{WT} from the cis compartment using the voltage ramp protocol shown in (F) (C) Current recording from a bilayer after multiple insertion of detergent purified Pdr5_{WT}. (D) Current-voltage gates of the same bilayer as in (B) using the voltage gate protocol shown in F (lower part). (E) Current-voltage relation obtained from the mean currents in (D). (F) Applied voltage protocol for voltage-ramps (upper) and voltage gates(lower).

Figure 3. Current recordings with extension plots from a bilayer containing the reconstituted of Pdr5_{WT}

(A) Current-voltage ramp measurement with a DOPE/DOPC (7:3) bilayer in symmetrical cis/trans buffer conditions (control). (B) Current recording of the same bilayer as in (A), but after insertion of detergent purified Pdr5_{WT} from the cis compartment using the voltage ramp protocol shown in (G). (C), (D) Extension plots of current-voltage gates at the indicated V_h from the same bilayer as in (B) using the voltage gate protocol given in (H) [XXX: Please add a label for each plot and mention the voltages in this figure caption, e.g., 60 mV (C), -120 mV (D), 80 mV (E)]. (E), (F) Mean variance plots for the respective channel recordings shown in (C) and (D) depicting multiple open channel amplitudes.

Figure 4. Current recordings from a bilayer containing the reconstituted of Pdr5_{EQ}. (A) Current-voltage ramp recording with a DOPE/DOPC (7:3) bilayer in symmetrical cis/trans buffer conditions after reconstitution of Pdr5_{EQ} using the voltage ramp protocol shown in Figure 2F. (B) Current-voltage gates of the same bilayer as in (B) using the voltage gate protocol shown in F (lower part). (C) All point amplitude histograms for the recordings shown in (B). (D) Current-voltage ramp recording with a DOPE/DOPC (7:3) bilayer in symmetrical cis/trans buffer conditions after multiple additions of Pdr5_{EQ} to the cis compartment using the voltage ramp protocol shown in Figure 2F. (E) Current-voltage gates of the same bilayer as in (D) using the voltage gate protocol shown in Figure 2F (lower part). (F) Current-voltage relation obtained from the mean currents in (E).

Figure 5. Effects of R6G on Pdr5_{WT} and Pdr5_{EQ} -mediated ion conductance

Figure 5. Effects of R6G on Pdr5_{WT} and Pdr5_{EQ} -mediated ion conductance. (A)

Current-voltage ramp from an empty control bilayer under symmetrical buffer (cis/trans 250 mM KCl, 10 mM HEPES, pH 7.0) **(B)** Current-voltage ramp after reconstitution of Pdr5_{WT} in symmetrical buffer using the command voltage protocol shown in the inset of **(A)**. **(C)** Same bilayer as in **(B)** but after addition (cis) of 1 mM Mg²⁺-ATP and 5 μM R6G. **(D)** Current-voltage ramp in symmetrical buffer after reconstitution of Pdr5_{WT} using the

command voltage protocol shown in the inset below **(A)**. Current-voltage ramp from the same bilayer as in **(D)** but after addition of 1mM Mg^{2+} -ATP. **(F)** Same bilayer as in **(E)** but after addition of 5 μ M R6G. **(G)** Current-voltage ramp in symmetrical buffer (250 mM KCl, 10 mM HEPES, pH 7.0) after reconstitution of Pdr5_{EQ} using the command voltage protocol shown in the inset below **(A)**. **(H)** Current-voltage ramp from the same bilayer as in **(G)** but after addition of 1mM Mg^{2+} -ATP (cis/trans). **(I)** Same bilayer as in **(H)** but after addition of 5 μ M R6G (cis).

(J) Current-voltage relations calculated by the GHK current equations using applied experimental ionic conditions and the respective permeability (P_x , ($P_{K^+}/P_{Cl^-}/P_{R6G^+}$)) as variable (see supplemental Information for details) for 3 cases. **Red:** KCl (only permeation of K^+ and Cl^- ions), **Black:** KCl-R6G (permeation of K^+ and Cl^- ions and active transport of $R6G^+$ ions), **Blue:** KCl-R6G- H^+ (permeation of K^+ and Cl^- ions and active co-transport of $R6G^+$ and H^+ ions). Currents were normalized to i_{max} . For more details see Methods section and SI.

Figure 6. Effects of CLO and KA on Pdr5-mediated ion conductance. (A)

Control bilayer after reconstitution of Pdr5. **(B)** Same bilayer as in **(A)** but after addition of 1 μM CLO to the cis compartment. **(C)** Current-voltage recordings after addition of 330 nM KA to the cis compartment of a Pdr5_{WT} containing bilayer.

Figure 7. Current-Voltage recording in the presence of BEA in the cis compartment. (A-C) Current-voltage ramps with an empty bilayer (A) in the presence of 3.3 μM BEA, (B), 9.9 μM BEA and (C) 0 μM BEA, respectively. **(D-G)** series of current voltage ramps from a single bilayer: (D): empty bilayer, (E) Pdr5_{WT} incorporated in the presence of Mg²⁺-ATP, (F) in the presence of Mg²⁺-ATP/R6G and (D) is (F) after addition of 3.3 μM BEA.

Figure 8. Suggested binding mode of beauvericin (BEA) in Pdr5 identified by molecular docking. **(A, B)** Initial placements and centers for the docking grid generation of BEA in the Rhodamin 6G (R6G) binding site **(A)** and between the TMDs and NBDs **(B)**. BEA is shown in salmon sticks and the ectracellular domains (ECDs, magenta shades), transmembrane domains (TMDs, blue shades), and nucleotide binding domains (NBDs, yellow shades) of Pdr5 are shown as cartoon. The R6G binding site is indicated by an orange rectangle. Here the R6G bound structure of Pdr5 was chosen although R6G had been removed for docking and is thus not shown here. **(C)** Suggested binding mode of BEA in Pdr5, overlaid with the binding mode of R6G (white sticks) from the cryo-EM structure. Here, BEA places one of its benzyl moieties at a similar position as one of the benzene rings of R6Gs xanthene-like moiety. **(D)** Difference in the R6G binding site between the R6G and ADP-orthovanadate (AOV) bound Pdr5 structure (blue and green, respectively) viewed from above. The L572 and L573 containing helix in the AOV-bound structure moved towards the center of the R6G binding site and, thus, clashes with the suggested BEA binding mode. As a result, no binding mode of BEA was identified in the R6G binding pocket of the AOV-bound structure during the docking.