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Mini Review

The yeast Pdr5p multidrug transporter: How does it recognize so many substrates?

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Abstract

Multidrug transporters are of considerable importance because they present problems in the treatment of infectious disease and cancer. A central issue is the ability of efflux pumps to recognize an astounding array of structurally diverse compounds. The yeast Pdr5p efflux pump, which is a member of the ATP-binding cassette superfamily, has at least 3 substrate-binding sites, each of which appears to use different chemical properties to transport compounds. All Pdr5p substrates, however, have a size requirement that is independent of hydrophobicity.

Keywords: Yeast multidrug resistance; Pdr5; ABC transporter; Substrate specificity

The Pdr5p multidrug transporter

Although *Saccharomyces cerevisiae* has several ATP-binding cassette (ABC) multidrug-transport proteins ([1–4] for review), Pdr5p is the most extensively characterized. This transporter was initially cloned as a sequence that caused drug hyperresistance when overexpressed, but extreme hypersensitivity when inactivated by mutation [5]. Sequencing revealed a 160-kDa member of the ABC superfamily [6,7]. Although Pdr5p is a full-length ABC transporter, it exhibits unique topology because the ABC domain is present at the N-terminus followed by a transmembrane domain with six putative α helices followed by another ABC and transmembrane domain at the C-terminal end [1–3].

Pdr5p was first shown to be an efflux pump because null mutants failed to transport [^{3}H]-chloramphenicol (chl) [8]. Pdr5p transports a huge array of chemically and mechanistically distinct substrates, many of which overlap with two other ABC transporters: Yor1p and Snq2p [2]. Further studies demonstrated that its transcription is positively and negatively regulated [9–13]. The ATPase activity of Pdr5p and Yor1p has also been studied [14,15]. In many respects, their enzymology resembles that of other ABC transporters. Of note, however, is the high, basal ATPase activity of Pdr5p, which suggests that there may be an *in vivo* substrate, such as a lipid or an amphipathic peptide/protein.

The possibility of multiple transport sites

Kolaczkowski et al. [16] presented evidence suggesting that Pdr5p might use more than one substrate–transport site. This was demonstrated with an assay that measures rhodamine 6G (R6g) quenching in purified plasma membrane vesicles. The fluorescence quenching appears attributable to the sequestering of the substrate by

Abbreviations: ABC, ATP-binding cassette; chl, chloramphenicol; R6g, rhodamine 6G; clo, clotrimazole; MIC, minimum inhibitory concentration; TPCl, tripentyltin chloride; Pgp, P-glycoprotein; trit, tritylimidazole; IAAP, ^{125}iodoarylazidoprazosin.

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Pdr5p-mediated transport (because at least 50% of the membrane vesicles have inside–out orientation). The behavior of trifluoperazine is a case in point. Mutants deficient in Pdr5p were very slightly hypersensitive to this agent when isogenic strains were compared in a zone-of-inhibition assay [14]. When this compound was used as an inhibitor of R6g quenching in membrane vesicles, however, the transport kinetics were noncompetitive. These data could be interpreted as evidence that Pdr5p has at least two substrate–transport sites. Alternatively, because none of the compounds behaving noncompetitively were actually shown to be transported out of cells or into vesicles, and the difference in sensitivity between the isogenic *PDR5*$^{+}$ and *pdr5*$^{-}$ strains appeared extremely small (and was restricted to analysis of trifluoperazine), it is possible that these compounds are modulators and bind to sites such as the ABCs or transmembrane regions of the protein that are critical for Pdr5p function.

This important study raised several questions. Are there multiple substrate–transport sites? If so, how many Pdr5p transport sites are there and how are they arranged? What is the chemical basis of substrate-Pdr5p interaction at each site? These questions are central for understanding the function not only of Pdr5p but also of several other ABC drug transporters that play an important role in development of multidrug resistance in cancer [17,18].

A novel approach to the study of Pdr5p-substrate interaction

We developed a novel approach to the problem of multidrug-transporter substrate specificity. We use several series of structurally related compounds that are systematically varied to determine a minimum set of requirements for Pdr5p-substrate interaction [19]. The first series (Fig. 1A) included a set of five trialkyltin chlorides. The length of the alkyl chains was varied from methyl to pentyl. This in turn affected the overall size and hydrophobicity of these compounds. These simple compounds were shown to be Pdr5p substrates by comparing their minimum inhibitory concentrations (MICs) in several isogenic *PDR5* and *pdr5*-null mutant strains. The MICs of ethyl, propyl, butyl, and pentyl derivatives were significantly lower in the Pdr5p-deficient stocks than in strains producing the transporter. The *in vivo* efficacy of a particular substrate is expressed as the ratio of the MIC in the Pdr5p strain (numerator) to that in the Pdr5p-deficient mutant (denominator), with a ratio of 1.0 indicating no difference in the MIC between the two strains.

The importance of determining the MIC ratio for a putative substrate cannot be overemphasized because it is an *in vivo* measure of efficacy and—short of directly measuring the transport of a given compound—the best proof that it is a substrate and not merely a modulator of the transporter. This is because increased hypersensitivity to a compound in strains lacking Pdr5p, compared with an isogenic, wild-type control, is most easily explained by the absence of Pdr5p-mediated transport. It is also important to show restoration of resistance when *PDR5* (and thus Pdr5p) is returned by plasmid transformation to a pdr5-deficient stock, as was done in all cases described below.

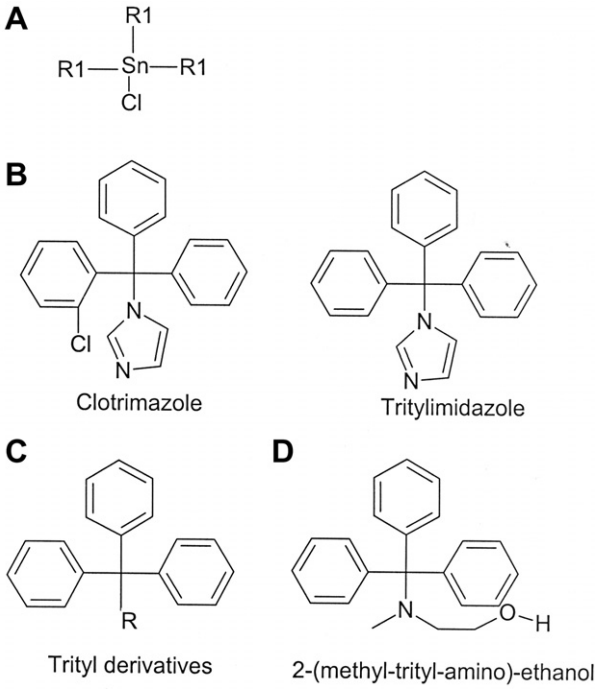

Fig. 1. The first series of compounds used to study Pdr5p specificity. (A) Trialkyltin chlorides, where R is an alkyl chain. The number of carbons varies from one (methyl) to five (pentyl). The tetraalkyltin compounds discussed in this review have an additional R group in place of the chlorine atom. (B) The behavior of xenobiotic imidazoles strongly suggests the existence of multiple sites. Although clotrimazole (left) and tritylimidazole are similar in structure and MIC ratio, their transport behavior is different. (C) Other trityl derivatives, where R = Br, OH, OCH_3, or NH_2. (D) The trityl derivative, 2-(methyl-trityl-amino)-ethanol is a site 1 substrate, as is its analog 2-(chloro-ethyl)-methyl-tritylamine, which has a Cl in place of the OH.

Pdr5p has multiple substrate–transport sites

The multisite nature of Pdr5p was demonstrated initially with the [^{3}H]-chl transport assay carried out in a pair of isogenic strains that were constructed by Dr. A. Decottignies [2]. One member of the pair carries deletions of major ABC transporters residing in plasma membrane of yeast; the other overexpresses Pdr5p. As a result, the latter shows an energy-dependent reduction in accumulated [^{3}H]-chl. When clotrimazole (clo), a potent Pdr5p substrate as determined by MIC ratio, is added to the assay, chl transport is inhibited in a concentration-dependent fashion. In contrast, tripentyltin chloride (TPCl), which has the same MIC ratio as clo, has no effect on chl efflux [20].

From these data alone, it would appear that there are two transport sites, a feature reminiscent of mammalian P-glycoprotein (Pgp, ABCB1) [21,22]. The results from four additional assays add complexity to this picture. We

Table 1
Pdr5p transport and binding assays: summary of major results

Labeled substrate	Challenged with					
	Chl	Clo	Trit	R6g	TPCl (Σ)	TBT (Ψ)
Chl (Ψ)[a]	X	++[b]	+	++	—	—
Trit (Σ)	ND	++	X	ND	++	—
Clo (Σ)	—	X	—	++	ND	ND
IAAP	ND	++	I[c]	++	—	ND
R6g (Ψ)	—	++	—	X	ND	ND

[a] The (Ψ) and (Σ) symbols are used to identify Pdr5p substrates of similar MIC ratio and therefore similar substrate strength. The other abbreviations are; chl, chloramphenicol; trit, tritylimidazole; R6g, rhodamine 6G; clo, clotrimazole; IAAP, iodoarylazidoprazosin; TBT, tetrabutyltin.

[b] ND, not determined; ++, the IC_{50} of the challenge compound is within 5-fold of the labeled substrate; +, the IC_{50} of the challenge compound is greater than 10-fold that of the labeled substrate.

[c] Trit shows weak, saturating, incomplete inhibition (30%) of IAAP binding [23].

carried out efflux assays for [^{3}H]-tritylimidazole (trit), R6g, and [^{3}H]-clo, as well as an assay that used the photoactivatable crosslinking agent ^{125}iodoarylazidoprazosin (IAAP) to measure binding in purified vesicles [23]. Table 1 shows some of our results. These lead to several important conclusions.

R6g, IAAP, and clo appear to define a single transport site, referred to here as site 1. Thus, R6g and clo inhibit each other's transport, and both of these substrates exhibit complete, concentration-dependent inhibition of IAAP-binding. Furthermore, the transport sites for chl and trit (site 2) may overlap (see [24] and [25] for examples of overlapping bacterial drug-binding sites). Thus, although TPCl inhibits trit (and not chl) efflux, trit shows weak, albeit complete inhibition of chl efflux. More specifically, it takes 10 times more trit than clo to stop chl transport. Nonreciprocity, another indication of possible site overlap, is observed in the clo–chl interaction. In this instance, clo inhibits chl efflux [20], but the converse is not true (J. Golin, unpublished data). An alternative explanation is that clo is transported from more sites than chl. Tetrabutyltin appears to define yet another site (site 3), because it has no effect on either chl or trit efflux. The nonreciprocal transport kinetics can be interpreted to suggest at least three or possibly four substrate–transport sites.

Size is a critical criterion for substrate–transporter interaction

Our recent work on Pdr5p substrate specificity [19,20,23] offered several significant observations about the requirements of Pdr5p sites. The first was that regardless of which site was used, there was a correlation between the molecular volume of the substrate and its efficacy (Fig. 2A). An optimal substrate has a surface volume of ~200 Å^3. Compounds of <90 Å^3 give MIC ratios of 1.0. It could appear that the size requirement is simply a reflection of increased hydrophobicity. This is not the case, as shown by a comparison of tripropyltin chloride (log $P = 2.66$) with dibutyltin dichloride (log $P = 1.56$). These substrates have surface volumes within 3 Å^3 of each other, but the latter is considerably less hydrophobic because of the extra, highly polar chlorine-tin bond and one less methyl group. Nevertheless, these substrates give experimentally indistinguishable MIC ratios [20]. This indicates that the striking size requirement for Pdr5-substrate interaction is largely independent of any requirement for hydrophobicity.

This observation is supported by our experimental data demonstrating that optimal substrates from three different series of compounds are all in the range of 200–225 Å^3, although their log P values vary by two orders of magnitude. Lack of a strong correlation between log P and substrate efficacy can also be seen in the plot of log P versus MIC ratio that is found in Fig. 2B. These observations were somewhat surprising because significant hydrophobicity is currently considered a prerequisite for substrate–multidrug-transporter interaction [26]. It is certainly reasonable to assume that a compound would be somewhat lipohilic to reach Pdr5p in the membrane because at least some substrates, including the relatively hydrophilic chl

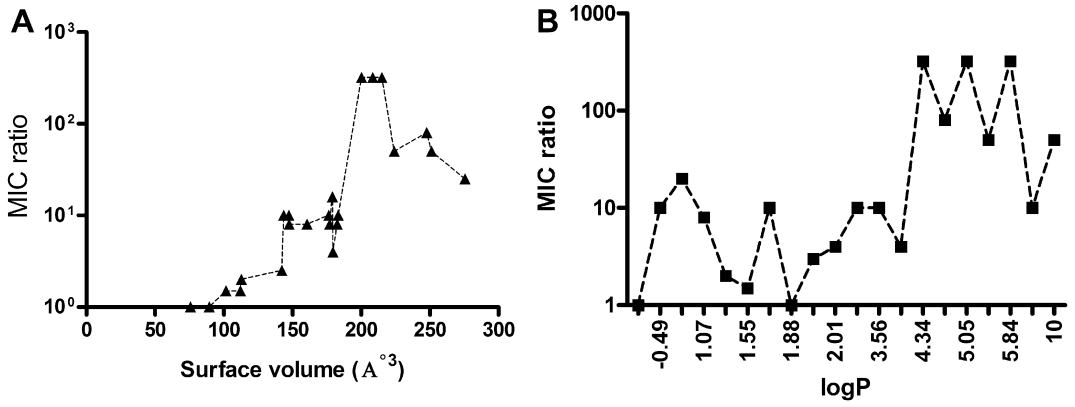

Fig. 2. Effect of size and hydrophobicity on substrate efficacy, as measured by the MIC ratio. (A) There is a strong correlation between size and efficacy. (B) The correlation between hydrophobicity, as measured by log P and the MIC ratio, is poor for Pdr5p substrates.

and the more hydrophobic clo molecules, enter by diffusion [20,27]. Furthermore, as discussed below, at least one of the transport sites uses hydrophobic interactions to recognize its substrates. Nevertheless, interaction at the other transport sites appears to depend on other chemical features.

At present, we do not know what the upper limit is for substrate size. R6g is a strong substrate (MIC ratio of ~50), even though it is larger than the compounds making up our test series. Kolaczkowski et al. [16] identified several structurally diverse compounds that are competitive inhibitors of R6g quenching and presumably act at the transport site. All of these are well above the minimum size requirement; many are in the R6g size range.

What other chemical features might be required for substrate–transporter interaction?

An important observation is that although tripropyltin chloride and TPCl show complete, concentration-dependent inhibition of [^{3}H]-trit efflux, tetrabutyltin does not [20]. Because metallic chlorides are fairly good electron-pair donors (hydrogen-bond acceptors), this suggested that site 2 required one such group. The site for tetrabutyltin (site 3), a strong Pdr5p substrate as determined by MIC ratio, is likely to depend on hydrophobic interactions because there are no ionizable groups, aromatic substituents, or atoms available for significant hydrogen bonding.

To test the idea that a single hydrogen-bond acceptor group is a prerequisite for interaction at site 2, we turned to the series of trityl derivatives found in Fig. 1C and used these as inhibitors in the [^{3}H]-trit efflux assay. When R is either an alcohol or NH_2 group, complete, concentration-dependent inhibition is observed. In contrast, when the group is an ether or bromide, weak, concentration-independent inhibition is observed. Because ethers are notoriously poor hydrogen-bond acceptors and the bromide group also has little or no ability to act as an electron-pair donor, this result strongly suggests that the initial inference is correct. In contrast to site 2, which has relatively simple substrate requirements and is therefore highly promiscuous, the chemical basis of substrate specificity at site 1 is more complex. This site is characterized by an ability of a substrate to show complete, concentration-dependent inhibition of R6g. Surprisingly, although clo does this, the closely related structure trit does not (Fig. 1B).

The differential behavior of trit and clo was originally thought to be attributable to a requirement for three electronegative atoms, one of which resided on a phenyl ring and therefore changed its electrostatic features. Indeed, all of the substrates that caused complete inhibition of R6g in our initial survey had this feature [23]. Recently, however, we have tested two analogs, 2-(methyl-trityl-amino)-ethanol (Fig. 1D) and (2-chloro-ethyl)-methyl-trityl-amine, in the R6g assay. Both are potent, competitive inhibitors of R6g efflux, even though none of the electronegative atoms residue on the aromatic rings (J. Golin and J. Janecka, unpublished data). In fact, like trit, these analogs have fewer than three electron-pair donors. This observation, along with our other data, offers no obvious unifying chemical feature specific to this binding site.

Substrate-specific binding mutants of Pdr5p are necessary

Substrate-specific mutants of Pdr5p were reported by two groups [28,29], but none of these alterations are known to be in actual binding sites or to affect binding of xenobiotic compounds. In fact, several are in the nucleotide-binding domains [28]. Although these mutants are of potentially great interest, determination of which of these, if any, directly affect substrate binding is critical for further progress in understanding the transporter–drug interaction.

Summary and comparison

Initially, Pdr5p was viewed as analogous to the mammalian transporter Pgp [16]. Like Pgp, Pdr5p uses multiple transport sites, a feature also shared with the highly homologous Cdr1p transporter of *Candida albicans* [30]. It is clear, however, that the rules of substrate specificity differ between Pdr5p and Pgp. For instance, the relatively simple Pdr5p organotin substrates are not transported by Pgp [20].

Substrates that are shared by Pdr5p and Pgp are chemically complex [16], and it is quite possible that each transporter recognizes different chemical features. Although hydrogen-bond acceptor groups, aromatic rings, and hydrophobicity are all implicated as features in Pgp/substrate interaction [31,32], the requirements of each site are unknown. Similarly, although there are numerous shared substrates between Pdr5p, Snq2p, and Yor1p, it is not clear whether the latter two also use compound size as a criterion for substrate–transporter interaction. Snq2 transports clinically important compounds such as camptothecins [33], but the transporter also mediates resistance to several substrates that appear too small to be transported by Pdr5p, including 4-nitroquinolin oxide, resazurin, helminthosporal, and numerous others [2,8,34].

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