

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE		3. REPORT TYPE AND DATES COVERED
4. TITLE AND SUBTITLE The sensitivities of yeast strains deficient in PDR ABC transporters, to quinoline-ring antimalarial drugs.				5. FUNDING NUMBERS
6. AUTHOR(S) Emerson, Lyndal R.; Skillman, Bradley C.; Wolfger, H.; Kuchler, K.; Wirth, D.F.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Walter Reed Army Institute of Research; Silver Spring, MD 20910 Institute of Medical Biochemistry, Dept of Molecular Genetics, Univ & BioCenter of Vienna, Vienna, Austria Dept of Immunology & Infectious Diseases, Harvard School of Public Health, Boston, MA 02115				8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Reprints: L. R. Emerson: lyndal.emerson@us.army.mil DISTRIBUTION STATEMENT A Approved for Public Release Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 words) Annals of Tropical Medicine & Parasitology, Vol. 98, No. 6, 643-649 (2004) Data indicate a potential role for the Pdr5p P-glycoprotein ATP-binding-cassette (ABC) transporter in mediating chloroquine sensitivity in <i>S. cerevisiae</i> . This is consistent with the hypothesis that such a transporter plays a role in mediating quinoline-drug resistance in <i>P. falciparum</i> , and complements the results of previous studies on the parasite. Additional gene products also play a major role in mediating chloroquine sensitivity and resistance, apparently through the modulation of vacuolar pH in the parasite.				
14. SUBJECT TERMS Malaria, resistance, chloroquine, quinolines				15. NUMBER OF PAGES 7
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

SHORT COMMUNICATION

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The sensitivities of yeast strains deficient in PDR ABC transporters, to quinoline-ring antimalarial drugs

Chemotherapeutic treatment of *Plasmodium falciparum* malaria has relied heavily on the quinoline-containing drugs quinine, chloroquine, mefloquine and halofantrine (Foote and Cowman, 1994; Foley and Tilley, 1998). These drugs have similar structures and are thought to act by inhibiting the polymerization of the toxic haem that is generated as the parasite breaks down haemoglobin derived from the host's erythrocytes (Slater and Cerami, 1992; Foley, 1995; Dorn *et al.*, 1998).

In some cases, resistance to these compounds may be a result of a multi-drug-resistance (MDR) mechanism involving the P-glycoprotein ATP-binding-cassette (ABC) transporters. Some strains of parasite exhibit cross-resistance to drugs such as quinine, mefloquine and halofantrine (Cowman *et al.*, 1994; Peel *et al.*, 1994), and some resistant strains exhibit amplification of the *P. falciparum* *mdr* homologue *PfMDR1* (Foote *et al.*, 1989; Wilson *et al.*, 1993). Other lines of evidence indicate, however, that a genetic locus other than *PfMDR1* is important for mediating chloroquine resistance. Data from the Wellems laboratory strongly indicate an association between chloroquine resistance and the gene *PfCRT* from a region of chromosome 7; this region was originally identified in a cross between sensitive and resistant clones of *P. falciparum* (Wellems *et al.*, 1991; Fidock *et al.*, 2000). On the other hand, a link between mefloquine resistance and *PfMDR1* is supported by the fact that mefloquine-resistant field isolates and resistant laboratory strains contain amplified *PfMDR1* (Volkman *et al.*,

1993; Wilson *et al.*, 1993). Finally, gene-replacement analysis of *PfMDR1* has demonstrated a central role for this gene product in mefloquine, quinine and halofantrine resistance, and a contributory role in chloroquine resistance (Reed *et al.*, 2000).

The contribution of ABC transporters to chloroquine and mefloquine resistance in *P. falciparum* is further supported by the reversal phenomenon observed when these drugs are combined with agents such as verapamil or penfluridol, resulting in a sensitive phenotype. Hundreds of compounds have been identified that can reverse mammalian MDR, and the results of several studies show that these compounds have similar activity in *Plasmodium* (Oduola *et al.*, 1998; Sowunmi *et al.*, 1998).

The difficulty of manipulating *P. falciparum* experimentally makes the use of heterologous systems, for the investigation of parasite genes and their homologues, attractive. Systems based on the yeast *Saccharomyces cerevisiae* have the advantages of mutant strains that are readily available and ease of handling. In *S. cerevisiae*, MDR is referred to as pleiotropic drug resistance (PDR) and is associated with a number of P-glycoprotein homologues. The project to sequence the entire *S. cerevisiae* genome revealed 30 ABC genes that have been classified into six distinct subfamilies based on the results of phylogenetic analysis (Decottignies and Goffeau, 1997; Kuchler and Egner, 1997; Taglicht and Michaelis, 1998; Bauer *et al.*, 1999; Schuller and Ruis, 2002). With 10 members, the PDR family is the largest of these subgroups and, to date, there have been a total of seven ABC genes

directly associated with modulation of resistance to xenobiotics in *S. cerevisiae*. The focus of the present study was the role of five of the PDR genes (*PDR5*, *PDR10*, *PDR12*, *PDR15* and *SNQ2*). *PDR5* has been linked to resistance to cycloheximide, mycotoxins and cerulenin, and has been found to transport steroids (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994; Hirata *et al.*, 1994; Kralli *et al.*, 1995; Mahe *et al.*, 1996; Bauer *et al.*, 1999; Schuller and Ruis, 2002). A second member of the PDR group, *SNQ2*, has been linked to resistance to 4-nitroquinoline-*N*-oxide (4-NQO), methyl-nitro-nitrosoguanidine (MNNG) and metal ions such as Na⁺, Li⁺ and Mn⁺ (Servos *et al.*, 1993; Miyahara *et al.*, 1996; Bauer *et al.*, 1999). Yeast cells with deletions in *SNQ2* and *PDR5* exhibit a more pronounced sensitivity to metal ions and other drug substrates (Bauer *et al.*, 1999). In terms of their sequences, *PDR10* and *PDR15* appear closely related to *PDR5* (65% sequence identity), although the functional relatedness, if any, of these genes remains to be determined. Interestingly, both *PDR10* and *PDR15*, like *PDR5* and *SNQ2*, have been reported to localize on the cell surface (Bissinger and Kuchler, 1994; Parle-McDermott *et al.*, 1996; Egner *et al.*, 1998; Bauer *et al.*, 1999). Like *PDR5*, *SNQ2* has a closely related homologue (60% identity), *PDR12*. Expression of Pdr12p mediates resistance to weak organic acids, including sorbate, benzoate, propionate, acetate, and fluorescein-like compounds (Parle-McDermott *et al.*, 1996; Holyoak *et al.*, 1999). *PDR12* is induced by exogenous weak-acid stress, and to a lesser extent, by high osmolarity or ethanol, and is essential for adaptive growth during metabolic stress (Ruis and Schuller, 1995; Piper *et al.*, 1998).

MATERIALS AND METHODS

Strains and Growth Conditions

The strains of *S. cerevisiae* used are listed in Table 1. The cells were grown in YPAD

(yeast-peptone-adenine-dextrose) liquid medium or on YPAD agar. Deletion of the ABC transporter genes *PDR5*, *SNQ2*, *PDR12*, *PDR10* or *PDR15*, either as single or multiple gene deletions, did not alter the normal growth of the yeasts under the conditions used in these experiments (see Table 2).

Gradient Drug Plates

Gradient plates, which contained a continuously increasing concentration gradient of drug across the width of a square Petri dish (see Table 2), were made by adding YPAD medium containing 2% agar in two steps (Cunningham *et al.*, 1986; Memisoglu and Samson, 2000). The plates were inoculated with a yeast strain and incubated at 30°C for 48 h before the extent of yeast growth was accessed and recorded as a percentage of that seen with the wild-type strain (YPH499).

Chloroquine-uptake Assay

The assay used to measure uptake of quinoline-3-[¹⁴C]chloroquine ([¹⁴C]chloroquine) was based on that employed by Krogstad *et al.* (1987), to measure chloroquine uptake in *P. falciparum*, and the method, for measuring Fe⁺⁺ uptake in *S. cerevisiae*, described by Eide *et al.* (1992). Exponentially-growing cells were pelleted at 1000 × *g* for 5 min at 4°C, resuspended in approximately 1% of the original culture volume of YPAD medium, and kept on ice until use. The [¹⁴C]chloroquine was mixed with chilled YPAD medium to give a concentration of 3 nM. To measure uptake, 450 µl of this assay solution were mixed with 50 µl of cell suspension and incubated at 30°C for 6–12 h. Each assay culture was then chilled on ice, vortexed, vacuum-precipitated through a glass-fibre filter with a pore size of 1.2 µm (GF/C; Whatman) and washed with 10 ml of ice-cold, double-distilled water. The cell-associated [¹⁴C]chloroquine was then measured on an LS5000TD liquid-scintillation system (Beckman). The

TABLE 1. *The strains of Saccharomyces cerevisiae that were investigated*

Strain	Genotype
WILD-TYPE	
YPH499	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52</i>
SINGLE-KNOCKOUT MUTANTS	
YKKA-7	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1</i>
YHW10N	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr10::hisG-URA3-hisG</i>
YRE106	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr12::hisG-URA3-hisG</i>
YHW15K	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr15::loxP-KanMx-loxP</i>
YYM 5	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δsnq2::hisG</i>
DOUBLE-KNOCKOUT MUTANTS	
YHW105	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr10::hisG, Δpdr5::TRP1</i>
YRE107	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1, Δpdr12::hisG-URA3-hisG</i>
YHW515	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1, Δpdr15::loxP-KanMx-loxP</i>
YYM4	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1, Δsnq2::hisG</i>
YHW1015KN	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr10::TRP1, Δpdr15::loxP-KanMx-loxP</i>
YRE108	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δsnq2::hisG, Δpdr12::hisG-URA3-hisG</i>
TRIPLE-KNOCKOUT MUTANTS	
YHW1052	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1, Δsnq2::hisG, Δpdr10::hisG</i>
YHW10515K	<i>MATa, ade2-101oc, his3Δ200, leu2-Δ1, lys2-801am, trp1-Δ1, ura3-52, Δpdr5::TRP1, Δpdr10::hisG, Δpdr15::loxP-KanMx-loxP</i>

levels of non-specific uptake, resulting from cell-surface adsorption, were determined by preparing parallel assay cultures that were incubated on ice, not at 30°C, for 6–12 h.

RESULTS AND DISCUSSION

Compared with the wild-type, strains of *S. cerevisiae* deficient in *PDR5* are known to have higher sensitivity to cycloheximide (Egner *et al.*, 1998). Therefore, in order to validate the experimental system used in the present study, all the yeast strains employed were tested for sensitivity to cycloheximide, using the gradient agar-plate assay. All the strains deficient in *PDR5* were confirmed to have relatively high sensitivities to cycloheximide (Table 2).

The sensitivities of the strains to chloroquine, mefloquine, quinidine, quinacrine and quinine were then explored in the gradient assay (Table 2). On a linear gradient of chloroquine from 0–197 mM, yeast strains deficient in *PDR5* were found to have increased sensitivity to the drug (compared with the wild-type) unless they had the $\Delta pdr5/\Delta pdr12$ double deletion. Similarly, except for the $\Delta pdr5/\Delta pdr12$ double deletion, deletion of *PDR5* resulted in cells with increased (but <2-fold higher) sensitivity to mefloquine and quinine. Sensitivity to quinacrine and quinidine was not affected by the deletion of *PDR5* alone, although cells deficient in *PDR5*, *PDR10* and *SNQ2* were found to be more sensitive to both of these drugs in the gradient assays, indicating a potential overlap in substrate specificities for these ABC transporters.

Since ABC transporters have been implicated in drug export, yeast strains deficient in ABC transporters were investigated for increased drug accumulation. In these experiments, [¹⁴C]chloroquine accumulation in the strains YPH499 (wild-type) and YHW1052 ($\Delta pdr5/\Delta pdr10/\Delta snq2$) increased over time; there was greater accumulation, however, by the PDR deletion mutant,

YHW1052 [with mean (s.d.) values of 384.8 (57.7) fmoles/10⁶ cells for YHW1052, and 260.0 (28.2) fmoles/10⁶ cells for YPH499].

Yeast strains deficient in the ABC transporter gene *PDR5* therefore have increased sensitivity to the quinoline drug chloroquine and increased accumulation of [¹⁴C]chloroquine. Such strains also have increased sensitivity to mefloquine and quinine, but not to quinacrine and quinidine. The association between the deletion of ABC transporters and both chloroquine sensitivity and increased chloroquine accumulation indicates an efflux-mediated model of chloroquine resistance, similar to that previously demonstrated for *PDR5* and cycloheximide resistance (Egner *et al.*, 1998).

A somewhat paradoxical result was observed, in the present study, when both *PDR5* and *PDR12*, another ABC transporter responsive to weak-acid stress, were deleted. The double-knockout mutant ($\Delta pdr5/\Delta pdr12$) was less sensitive to chloroquine than the single $\Delta pdr5$ knockout. Increased sensitivity to cycloheximide was observed in this strain, similar to that observed in the $\Delta pdr5$ strains, which indicates that drug sensitivity is not generally altered in the $\Delta pdr5/\Delta pdr12$ strain. The decrease in sensitivity to chloroquine may indicate an indirect role for *PDR12* in responding to chloroquine stress or may indicate that changes in vacuolar pH are associated with chloroquine action in *S. cerevisiae*. This is consistent with previous work showing that chloroquine treatment can result in changes in vacuolar pH (Pearce *et al.*, 1999).

The transcriptional response of yeast strains YPH499 (wild-type) and YHW1052 ($\Delta pdr5$) to treatment with chloroquine has already been investigated (Nau *et al.*, 2000; Emerson *et al.*, 2002). The results of these experiments indicated no appreciable transcriptional response of ABC transporters in these yeast strains. These findings were somewhat surprising and indicated that the protection provided by ABC transporters against quinoline-ring compounds is not modulated at the transcriptional level.

TABLE 2. The growth of the PDR mutant yeasts on yeast-peptone-adenine-dextrose (YPAD) agar that contained an antimalarial drug, or no drug, as a percentage of that seen with the wild-type YPH499

Strain and (deletions)	Mean growth (s.d.) on medium (% of that seen with the wild-type)*					
	Chloroquine	Quinine	Quinacrine	Quinidine	Mefloquine	Cycloheximide
YKKA-7 (<i>pdr5</i>)	62.9 (9.4)	82.1 (4.1)	88.8 (7.3)	97.7 (3.0)	71.0 (11.6)	54.3 (7.1)
YHW10N (<i>pdr10</i>)	101.6 (3.4)	95.2 (2.2)	91.5 (5.6)	100.2 (2.2)	93.9 (5.8)	102.8 (3.0)
YRE106 (<i>pdr12</i>)	102.6 (13.2)	94.3 (4.7)	106.6 (4.9)	98.2 (10.6)	127.4 (12.6)	100.0 (13.0)
YHW15K (<i>pdr15</i>)	97.2 (5.0)	98.8 (3.2)	93.1 (3.5)	101.2 (1.2)	92.4 (6.2)	102.0 (2.5)
YYM5 (<i>snq2</i>)	99.3 (14.1)	99.1 (4.9)	100.7 (11.0)	97.6 (3.9)	114.6 (11.6)	100.5 (12.0)
YHW105 (<i>pdr5</i> , <i>pdr10</i>)	40.7 (2.7)	78.1 (5.2)	80.4 (8.8)	98.2 (1.8)	66.9 (10.7)	42.2 (4.2)
YRE107 (<i>pdr5</i> , <i>pdr12</i>)	93.5 (14.0)	118.9 (5.9)	79.0 (6.8)	109.2 (1.1)	87.3 (14.4)	43.6 (5.7)
YHW515 (<i>pdr5</i> , <i>pdr15</i>)	41.8 (10.5)	77.0 (14.3)	78.9 (4.8)	96.0 (6.0)	63.7 (12.5)	42.9 (7.3)
YYM4 (<i>pdr5</i> , <i>snq2</i>)	43.6 (6.5)	67.9 (3.4)	73.7 (7.4)	72.7 (7.8)	62.8 (11.3)	36.7 (4.8)
YHW1015KN (<i>pdr10</i> , <i>pdr15</i>)	102.8 (1.9)	97.7 (4.6)	96.9 (8.5)	99.4 (6.4)	94.6 (7.2)	102.4 (2.4)
YRE108 (<i>snq2</i> , <i>pdr12</i>)	99.3 (15.0)	91.5 (4.6)	94.7 (8.2)	64.5 (2.3)	116.4 (6.3)	94.7 (12.3)
YHW1052 (<i>pdr5</i> , <i>pdr10</i> , <i>snq2</i>)	32.0 (4.8)	66.0 (3.3)	55.2 (4.3)	55.2 (12.2)	61.9 (13.7)	44.7 (5.8)
YHW10515K (<i>pdr5</i> , <i>pdr10</i> , <i>pdr15</i>)	61.7 (9.3)	75.5 (3.8)	79.0 (5.1)	87.5 (9.8)	65.5 (15.4)	6.7 (0.9)

*The agar medium, held in a square Petri dish, contained no drug or a continuous concentration gradient of chloroquine (0-197 mM), mefloquine (0-0.6 mM), quinine (0-26.6 mM), quinacrine (0-21.1 mM), quinidine (0-15.4 mM) or cycloheximide (0-0.88 mM). Each mean shown is of the values from at least three independent experiments.

Taken together, the present data indicate a potential role for the Pdr5p ABC transporter in mediating chloroquine sensitivity in *S. cerevisiae*. This observation is consistent with the hypothesis that such a transporter plays a role in mediating quinoline-drug resistance in *P. falciparum*, and complements the results of previous biochemical and genetic studies on the parasite (Reed *et al.*, 2000). Interestingly, additional gene product(s) also play a major role in mediating chloroquine sensitivity and resistance, apparently through the modulation of vacuolar pH in the parasite (Fidock *et al.*, 2000; Ursos *et al.*, 2000, 2001).

ACKNOWLEDGEMENTS. This work was supported financially by the U.S. Department of the Army (via AASERT grants DAAG55-98-1-0186 and DAMD 17-98-1-8003) and the National Institutes of Health (via R01 grant AI27872-11). K.K. was supported by the *Fonds zur Förderung der wissenschaftlichen Forschung* (grant P12661-BIO) and, in part, by funds from the *Herzfelder'sche Familienstiftung* and by COST action B16. The authors thank R. Egner and Y. Mahé for help with strain construction, and W. Milhous and M. Novak, both of the Walter Reed Army Institute of Research (WRAIR), for the [¹⁴C]chloroquine (WR1544). They also wish to thank N. Roy (also of the WRAIR) for his technical assistance. A portion of this work comes from the doctoral work of L.R.E. performed at Harvard University. This article has been reviewed and approved for publication by the WRAIR. The opinions or assertions contained herein are, however, the private views of the authors, and are not to be construed as official, or as reflecting the true views of the U.S. Department of the Army or the U.S. Department of Defense.

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Received 14 November 2003,

Revised 10 March 2004,

Accepted 12 March 2004

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