Curcumin Modulates Efflux Mediated by Yeast ABC Multidrug Transporters and Is Synergistic with Antifungals

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Curcumin (CUR), a natural product of turmeric, from rhizomes of Curcuma longa, is a known agent of reversal of drug resistance phenotypes in cancer cells overexpressing ATP-binding cassette (ABC) transporters, viz., ABCB1, ABCG2, and ABCC1. In the present study, we evaluated whether CUR could also modulate multidrug transporters of yeasts that belong either to the ABC family or to the major facilitator superfamily (MFS). The effect of CUR on multidrug transporter proteins was demonstrated by examining rhodamine 6G (R6G) efflux in Saccharomyces cerevisiae cells overexpressing the Candida albicans ABC transporters Cdr1p and Cdr2p (CaCdr1p and CaCdr2p, respectively) and the MFS transporters CaMdr1p and ScPdr5p. CUR decreased the extracellular concentration of R6G in ABC transporter-expressing cells but had no effect on methotrexate efflux mediated through the MFS transporter CaMdr1p. CUR competitively inhibited R6G efflux and the photolabeling of CaCdr1p by [125I]iodoarylazidoprazosin, a drug analogue of the substrate prazosin (50% inhibitory concentration, 14.2 μM). Notably, the mutant variants of CaCdr1p that displayed abrogated efflux of R6G also showed reduced modulation by CUR. Drug susceptibility testing of ABC protein-expressing cells by spot assays and checkerboard tests revealed that CUR was selectively synergistic with drug substrates such as R6G, ketoconazole, itraconazole, and miconazole but not with fluconazole, voriconazole, anisomycin, cycloheximide, or FK520. Taken together, our results provide the first evidence that CUR modulates only ABC multidrug transporters and could be exploited in combination with certain conventional antifungal drugs to reverse multidrug resistance in Candida cells.

Overexpression of ATP-binding cassette (ABC) multidrug transporters, including P-glycoprotein (ABC1), multidrug resistance protein (ABCB1), and mitoxantrone resistance protein (ABCG2), plays a major role in the development of multidrug resistance (MDR) in cancer cells (19). Among the various strategies to combat MDR, blocking the functioning of MDR transporters represents an attractive approach (11). Notably, several functional inhibitors of MDR proteins have been tested, but thus far none are clinically successful, due to the dose-limiting toxic effect of the modulators.

To circumvent this problem, extensive efforts have been under way in recent years to identify natural inhibitors of MDR exporters, since natural products have the potential to yield a large number of new drugs. Curcuminoids, from the rhizomes of Curcuma longa, have been reported to reverse the drug resistance phenotype in cancer cells overexpressing ABC transporters, viz., ABCB1, ABCG2, and ABCC1 (2, 4, 5). Curcuminoids blocked the efflux of fluorescent substrates calcein AM, rhodamine 123, and bodipy-FPlviblastine in MDR cervical carcinoma cell lines overexpressing ABCB1 and the efflux of mitoxantrone and phosphorhod A, mediated by ABCG2, in HEK293 cells (3, 4).

In yeasts, including species of the pathogenic genus Candida, upregulation of multidrug transporter genes belonging either to the ABC family or to the major facilitator superfamily (MFS) is frequently observed in cells exposed to drugs and leads to the phenomenon of MDR (29, 31, 32). For clinical isolates of Candida albicans, it has been established that the ABC transporters C. albicans Cdr1p (CaCdr1p) and CaCdr2p and the MFS transporter CaMdr1p are major MDR transporters that contribute to azole resistance. There are compounds, such as FK506, enniatins, milbemycins, synthetic D-octapeptides, cyclosporine, isonitrile, disulfiram, ibuprofen, and unnarminics (12, 30), that inhibit fungal ABC transporters. Such inhibitors or chemosensitizers probably act directly by affecting substrate binding and transport mediated by MDR efflux proteins.

Notably, the effect of curcuminoids on fungal ABC transporters is not known. However, due to functional and structural similarities between ABCB1 and ABC transporters in yeasts, it is very likely that the curcuminoids could act as “reversal agents” of drug resistance in yeasts as well. In this study, we have examined the potency of curcumin (CUR) in modulating the efflux activity of CaCdr1p and have compared it with those of CaCdr2p and Saccharomyces cerevisiae Pdr5p (ScPdr5p). Our results demonstrate that CUR behaves as a specific modulator of rhodamine 6G (R6G) efflux mediated by CaCdr1p, CaCdr2p, and ScPdr5p in S. cerevisiae cells overexpressing these transporters. Notably, CUR had no impact on efflux activity mediated by the MFS transporter CaMdr1p.
Furthermore, CUR reversed drug resistance by displaying synergism with selected drugs.

**MATERIALS AND METHODS**

**Materials.** R6G, a commercial-grade mixture of curcuminoids (commonly known as CUR), protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, \(\beta\)-tosyl-L-phenylalanine chloromethyl ketone \[TPCK\]), and \(\alpha\)-tosyl-L-lysine chloromethyl ketone \[TLCK\], and \(\alpha\)-tosyl-L-phenylalanine chloromethyl ketone \[TPCK\], a biinchonic acid protein determination kit, micnazone (MCZ), ketoconazole (KTC), itraconazole (ITC), anisomycin (ANISO), cycloheximide (CYH), FK520, oligomycin, dinitorphenol, deoxyglucose, 3-[4-(5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other molecular-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). \[^{3}H\]-labeled fluconazole (FLC; specific activity, 19 Ci/mmol) and \[^{3}H\]MTX (specific activity, 8.60 Ci/mmol) was procured from Amersham Biosciences, United Kingdom. Radiolabeled \[^{125}\]Iiodoarylazo-doprazoin (IAAP) (2,200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA).

**Yeast strains and growth media.** The strains used in this study are listed in Table 1. The yeast strains were cultured in yeast extract-peptone-dextrose (YEPD) broth (Bio 101, Vista, CA). For agar plates, 2.5% (wt/vol) Bacto agar (Difco, BD Biosciences, NJ) was added to the medium. All strains were stored overnight-grown culture were transferred to YEPD medium and allowed to grow to 10^8 cells (wt/vol) in PBS without glucose. The cells were then de-energized for 45 min in deoxyglucose (5 mM) and dinitrophenol (5 mM) in PBS (without glucose), and resuspended as a 2% cell suspension, which corresponds to 10^8 cells (wt/vol) in PBS without glucose. The cell suspension was then washed and resuspended as a 2% cell suspension in PBS without glucose. For competition assays, CUR (100 \(\mu\)M) was added to the de-energized cells 5 min before the addition of R6G and allowed to equilibrate.

**Measurement of drug accumulation.** The accumulation of \[^{3}H\]FLC (specific activity, 19 Ci/mmol) and \[^{3}H\]MTX (specific activity, 8.60 Ci/mmol) was determined essentially by the methods described previously (22). Briefly, cells from mid-log phase (5 \(\times\) 10^7) were centrifuged at 3,000 \(\times\) g for 3 min and resuspended in PBS as a 2% cell suspension. For accumulation studies, 100 nM FLC and 25 \(\mu\)M MTX were routinely used (22). CUR at 100 \(\mu\)M was added 5 min before the addition of drugs and was allowed to equilibrate. A 100-\(\mu\)l volume of the cell suspension containing drugs alone or drugs plus CUR was incubated at 30°C for 40 min, filtered rapidly, and washed twice with PBS (\(pH\) 7.4) on a Millipore manifold filter assembly using a 0.45-\(\mu\)m-pore size cellulose nitrate filter (Millipore). The filter discs were dried and put in cocktail “O,” and the radioactivity was measured in a liquid scintillation counter (Beckman). Accumulation was expressed as picomoles per milligram (dry weight).

**Photoaffinity labeling with IAAP.** The crude membrane proteins (50 \(\mu\)g) prepared from AD-CDR1 cells (27) were incubated with CUR or with R6G for 10 min at 37°C in 0.1 ml of 50 mM Tris-HCl (pH 7.5). The samples were brought to room temperature, and 3 to 6 nM \[^{125}\]IAAP (2,200 Ci/mmol) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp assembly (PCC, Scientifics, Gaithersburg, MD) fitted with two black-light (self-filtering) UVA long-wavelength F15T8BLB tubes (365 nm) for 10 min at room temperature (21 to 23°C). One milliliter of radioluminoprecipitation assay buffer was added to the samples, and CaCdr1p cross-linked with \[^{125}\]IAAP was immunoprecipitated with 10 \(\mu\)g of a monoclonal antibody (BD Biosciences, Palo Alto, CA) against green fluorescent protein (GFP) (27). The samples were then separated on a 7% Tris-acetate gel at a constant voltage, and the gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at \(\sim\)80°C for 12 to 24 h.

**ATPase assay.** The ATPase activity of the plasma membrane (PM) fractions was measured as oligomycin-sensitive release of inorganic phosphate either alone, as described previously (27), or in the presence of CUR (100 \(\mu\)M) and varying concentrations of ATP (0.5 mM to 7 mM).

**Immunodetection of ABC proteins.** PMs were prepared from *S. cerevisiae* cells overexpressing ABC transporters as described previously (27) or in the presence of CUR (100 \(\mu\)M). The PM protein concentration was determined by a biocinchonic acid assay using bovine serum albumin as the standard. Western blot

**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Strain name or mutation</th>
<th>Genotype or description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AD1-8u^-</td>
<td>MAT (\alpha) pdr-3 his1 ura3 (\Delta)yor1-1:his1G (\Delta)pdr5:his1G (\Delta)pdr10:his1G</td>
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<td>CDR1-GFP cells carrying an A1346G mutation in the CDR1 ORF and integrated at the PDR5 locus</td>
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<td>CDR1-GFP cells carrying an A1347G mutation in the CDR1 ORF and integrated at the PDR5 locus</td>
<td>24</td>
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<tr>
<td>5</td>
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<td>24</td>
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<tr>
<td>6</td>
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<td>24</td>
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<tr>
<td>7</td>
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<td>CDR1-GFP cells carrying a G1362A mutation in the CDR1 ORF and integrated at the PDR5 locus</td>
<td>24</td>
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<td>Puri et al., unpublished data</td>
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<td>CAI4</td>
<td>(\Delta)ara3::imm343/(\Delta)ara3::imm343</td>
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* ORF, open reading frame.
FIG. 1. Effects of CUR on efflux of substrates in yeast cells. (A) Extracellular R6G concentrations in \textit{S. cerevisiae} control cells (AD1-8u−) (diamonds) and in cells overexpressing CaCdr1p (AD-CDR1) (squares), incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). Filled triangles represent AD-CaMDR1 cells. Energy-dependent R6G efflux was initiated by adding 2% glucose (arrow) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments. (B) \[^{3}H\text{MTX}\] accumulation in \textit{S. cerevisiae} control cells (AD1-8u−) and in cells overexpressing CaMdr1p (AD-CaMDR1). Cells were incubated either with \[^{3}H\text{MTX}\] (25 μM; specific activity, 8.60 Ci/mmol) alone (shaded bars) or with \[^{3}H\text{MTX}\] plus CUR (100 μM) (open bars). The solid black bar represents AD-CDR1 cells. The accumulated \[^{3}H\text{MTX}\] was measured, 40 min after the initiation of efflux, using a liquid scintillation counter (Beckman). Values are means ± standard deviations (error bars) for three independent experiments. (C) \[^{3}H\text{FLC}\] accumulation in \textit{S. cerevisiae} control cells and in cells overexpressing CaCdr1p. Cells were incubated with either \[^{3}H\text{FLC}\] (100 nM; specific activity, 19 Ci/mmol) alone (shaded bars) or \[^{3}H\text{FLC}\] plus CUR (100 μM) (open bars). The accumulated \[^{3}H\text{FLC}\] was measured 40 min after the addition of glucose (2%). Values are means ± standard deviations (error bars) for three independent experiments. (D) Extracellular R6G concentrations in \textit{C. albicans} strain CAI4. Cells were incubated either with R6G (10 μM) alone (filled triangles) or with R6G plus CUR (100 μM) (open triangles). Energy-dependent R6G efflux was initiated by adding 2% glucose (arrow) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments. (E) Extracellular R6G concentrations in \textit{S. cerevisiae} control cells (AD1-8u−) (diamonds) and in cells overexpressing CaCdr2p (AD-CDR2) (squares), incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). (F) Extracellular R6G concentrations in \textit{S. cerevisiae} control cells (AD1-8u−) (diamonds) and in cells overexpressing ScPdr5p (AD-PDR5) (squares), incubated either with R6G (10 μM) alone (filled symbols) or with R6G plus CUR (100 μM) (open symbols). Energy-dependent R6G efflux was initiated by the addition of 2% glucose (arrows) and was quantified by measuring the absorbance of the supernatant at 527 nm. Values are means and standard deviations (error bars) for three independent experiments.
showed energy-dependent efflux of R6G, which was inhibited by CUR (100 μM). However, the addition of CUR had no effect on the leakage of preloaded R6G from de-energized *S. cerevisiae* cells. We tested if CUR could also affect a multidrug transporter belonging to the MFS, and we examined efflux mediated by CaMdr1p expressed in a similar heterologous background. As shown in Fig. 1B, the transport of [³H]MTX, a well-known substrate of CaMdr1p, remained unaffected by a fourfold excess of CUR. In the experiments for which results are shown in Fig. 1A, strain AD-CaMDR1 was used as a negative control for R6G transport, and in the experiments for Fig. 1B, strain AD-CDR1 was used as a negative control for MTX transport. The effect of CUR was also substrate specific, as evidenced by the fact that efflux of the well-known substrate FLC remained unimpeded in CaMdr1p-expressing *S. cerevisiae* cells, even though CUR was supplied in a 1,000-fold excess (Fig. 1C). Of note, CUR could also modulate R6G efflux in *C. albicans* cells (Fig. 1D); however, for subsequent studies, we used an *S. cerevisiae* strain overexpressing MDR transporters.

**CUR selectively modulates ABC transporters.** Before investigating whether CUR affects drug transporters, we examined whether it affected the viability of cells. For this purpose, control cells and transporter-overexpressing cells were exposed to various concentrations of CUR for 48 h, and cytotoxicity was determined by an MTT assay (3, 4). The percentage of viable cells was calculated in order to determine the 50% inhibitory concentrations (IC₅₀) (Fig. 2). As shown in Table 2, the IC₅₀ for control cells (AD1-8u) and those for cells expressing various transporters (AD-CDR1, AD-CDR2, AD-PDR5, and AD-CaMDR1) were not very different, ranging from 410.6 ± 9.4 μM to 498 ± 5.5 μM. Further, our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extracellular concentration of CUR remained the same even after the initiation of efflux (data not shown). Our data suggest that CUR interacts with the yeast transporters, but these multidrug transporters may not transport it, since the IC₅₀ and relative resistance factors were similar whether cells were overexpressing a transporter or not. We investigated whether the effect of CUR is specific to ABC transporters, and we examined R6G efflux mediated by CaCdr1p homologues, such as CaCdr2p and ScPdr5p, which were expressed in similar backgrounds. It was observed that CUR could inhibit the efflux of R6G mediated by both the proteins (Fig. 1E and F). Our transport assays confirmed that CUR is not a substrate of ABC or MFS proteins, since the extracellular concentration of CUR remained the same even after the initiation of efflux (data not shown). This was further confirmed by a cytotoxicity assay (3, 4), where the IC₅₀ were similar for cells that did and did not

**RESULTS**

**CUR inhibits R6G efflux.** We used a commercial preparation of CUR in order to observe its effect on MDR efflux proteins of the pathogenic yeast *C. albicans*. For this purpose, we monitored the efflux of R6G in cells where GFP-tagged CaCdr1p (Cdr1p-GFP) was stably overexpressed from a genomic *PDR5* locus in the *S. cerevisiae* mutant strain AD1-8u⁻ (20). The host AD1-8u⁻, constructed by Goffeau's group (7), was derived from a Pdr1-3 mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the *PDR5* promoter (20). As shown in Fig. 1A, *S. cerevisiae* cells overexpressing CaCdr1p...
A) 

Curcumin I (Curcumin)

Curcumin II (Demethoxycurcumin)

Curcumin III (Bisdemethoxycurcumin)

IC$_{50}$ = 40 ± 5 μM

IC$_{50}$ = 45 ± 5 μM

IC$_{50}$ = 40 ± 5 μM

B) 

Rhodamine 6G

Miconazole

Itraconazole

Anisomycin

Cycloheximide

Fluconazole

Ascomycin

Voriconazole

Ketoconazole
overexpress transporters (Table 2). It should be mentioned that the functionality of GFP-tagged versions of ABC and MFS transporters was similar to that of untagged proteins (22, 27). To further examine the effect of CUR, the ABC transporter CaCdr1p was selected for detailed functional analyses.

**CUR competitively inhibits R6G efflux.** Commercially available CUR is a mixture of three major curcuminoinds: curcumin, or diferuloylmethane (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III). This mixture contains predominantly curcumin I (~77%), followed by curcumin II (17%) and curcumin III (3%), which display a wide range of biological and pharmacological properties (1, 16). We used purified curcuminoinds (curcumin I, II, and III) to see if these compounds showed any selectivity as modulators of R6G efflux. The efflux of R6G mediated by CaCdr1p was inhibited by all the pure forms of CUR in a concentration-dependent manner, with IC_{50} ranging from 40 ± 5 to 45 ± 5 µM (Fig. 3A). The Lineweaver-Burk plot revealed that CUR competitively inhibits R6G efflux, with an increase in apparent K_m (5.87 to 11.83 µM) but no effect on the V_{max} (Fig. 4A).

**CUR inhibits drug binding and has no effect on ATPase activity or the expression of CaCdr1p.** We had shown previously that IAAP (a photoaffinity analogue of the human P-glycoprotein substrate prazosin) and azidopine (a dihydropyridine photoaffinity analogue of its modulator, verapamil) specifically bind to CaCdr1p (27). To monitor whether CUR affects drug binding, we labeled CaCdr1p with [125I]IAAP as described in Materials and Methods. Figure 4B demonstrates that CUR effectively inhibited the photoaffinity labeling of CaCdr1p with [125I]IAAP, with an IC_{50} of 14.2 µM. We also monitored [125I]IAAP labeling in the presence of R6G as described in Materials and Methods. Interestingly, R6G could not inhibit [125I]IAAP binding. In contrast to well-known inhibitors of CaCdr1p ATPase activity, such as vanadate, oligomycin, sodium azide, and N-ethylmaleimide (15, 26, 27), CUR at 100 µM had no effect on ATPase activity in the presence of varying concentrations of ATP (Fig. 4C). CUR also had no effect on the expression of CaCdr1p (Fig. 4D). Taken together, the data strongly indicate that although CUR is not transported, it acts as a competitive inhibitor at one of the transport sites used to transport clinically significant antifungal agents.

**CUR displays synergism with selected azoles.** When the control (AD1-8u-) cells and the CaCdr1p-expressing cells were grown either in the presence of drugs alone (FLC at 6.52 µM, VORI at 5.72 µM, MCZ at 0.167 µM, KTC at 0.037 µM, ITC at 0.141 µM, ANISO at 2.97 µM, CYH at 0.28 µM, FK520 at 12.6 µM, R6G at 0.209 µM) or in the presence of both CUR (75.6 µM) and the indicated drug, it was observed that CaCdr1p-expressing cells displayed the expected drug resistance and thus were able to grow in the presence of drug alone. Similar results were obtained with CaCdr2p- and ScPdr5p-expressing S. cerevisiae (data not shown). However, the simultaneous presence of CUR with either R6G or azoles, viz., KTC, ITC, or MCZ, sensitized the cells, as evidenced by inhibition of the growth of the cells (Fig. 5ii). Interestingly, the presence of CUR along with noncompeting drugs, such as ANISO, CYH, FLC, VORI, and FK520, did not affect the level of resistance or the growth of cells expressing ABC proteins (Fig. 5iii). The observed inhibition of growth by CUR in the presence of drugs was not due to loss of viability, as determined by an MTT assay (Fig. 2). Notably, CUR (75.6 µM) alone did not inhibit the growth of control cells (AD1-8u-) or that of cells overexpressing CaCdr1p (Fig. 5i) or CaMdr1p (Fig. 5iv). The growth of CaMdr1p-overexpressing cells in the presence of MTX remained insensitive to CUR (Fig. 5v).

**DISCUSSION**

Among the 28 putative ABC transporter genes and 95 putative MFS transporter genes identified in the C. albicans genome (9, 10), there is overwhelming clinical and experimental evidence that only ABC transporters, such as CaCdr1p and CaCdr2p, and the MFS transporter CaMdr1p are major determinants of azole resistance (23, 25). The reversal of the functionality of these multidrug efflux pump proteins represents an
FIG. 4. Biochemical analysis of CaCdr1p in the presence of CUR. (A) Lineweaver-Burk plot of CaCdr1p-mediated R6G efflux in the presence of CUR 5 min after the addition of 2% glucose. Filled diamonds, open squares, and filled triangles represent 0, 50, and 100 μM CUR, respectively. The rate of each reaction was calculated as nanomoles of R6G released per minute per 5 × 10⁶ cells. (B) Effect of CUR or R6G on the photoaffinity labeling of CaCdr1p with [125I]IAAP. The autoradiogram represents the amounts of [125I]IAAP incorporated into CaCdr1p in the presence of the indicated concentrations of CUR or R6G. The graph represents the amounts of [125I]IAAP incorporated into CaCdr1p in the presence of the indicated concentrations of CUR. (C) Effect of CUR on the ATPase activity of CaCdr1p. PMs from cells overexpressing CaCdr1p were incubated with or without 100 μM CUR and varying concentrations of ATP (0.5 mM to 7 mM) in the ATPase buffer. The assay was performed essentially as described in Materials and Methods. The data are plotted using GraphPad Prism. (D) Effect of CUR (100 μM) on the expression of CaCdr1p. Western blot analyses were performed with an anti-GFP monoclonal antibody. Equal loading of protein was assessed by using a Coomassie-stained gel.
forms of CUR—curcumin I, II, and III—showed similar levels of modulation of R6G efflux in \textit{S. cerevisiae} cells expressing ABC transporters (Fig. 3A). The modulatory effect of CUR was restricted to R6G; it had no effect on the efflux of another substrate, FLC. We could observe a direct correlation between the modulatory effect of CUR and the status of R6G efflux. For instance, those mutant variants of CaCdr1p that show abrogated efflux of R6G also display decreased modulation by CUR.

Notably, R6G and FLC are both substrates of CaCdr1p, but only the former is competed with CUR (Fig. IA and C). If the structure of CUR is compared with the structures of R6G and FLC, it is apparent that electronic factors, such as the number of \(\pi\) rings and an extended \(\pi\) surface, could be important for CUR and other substrates, such as ITC, KTC, and MCZ, which compete with R6G efflux (Fig. 3B). In this context, it is noteworthy that noncovalent \(\pi-\pi\) interactions have tremendous biological implications (13, 14). On the other hand, if one considers the structures of FLC, VORl, ANISO, and CYH, which do not compete with R6G, there are no such electronic factors but a good number of tetrahedral sites. Therefore, these subtle differences in properties between the structures of competing and noncompeting substrates could explain why CUR is a selective modulator.

The probability that the modulation of ABC transporter function would result in an increase in the intracellular concentrations of the drugs to toxic levels became apparent from the growth studies. When CUR was used in combination, it was synergistic with drugs in cells overexpressing ABC transporters. This synergism was restricted to those drugs whose efflux was modulated by R6G (24). Thus, the chemosensitization of cells by CUR was specific to competing drugs, such as KTC, ITC, and MCZ, and was not observed with noncompetitive drugs, such as FLC, VORI, ANISO, CYH, and FK520. The fact that the presence of CUR along with some drugs did not inhibit the growth of cells not only points to the selectivity of CUR for certain compounds but also suggests that R6G, KTC, ITC, MCZ, and CUR may share overlapping binding sites of ABC multidrug transporter proteins. The modulatory and synergistic effects of CUR confirm our previous observation that KTC, MCZ, and ITC share CaCdr1p binding sites with R6G (24).

A natural CUR mixture contains three major curcuminoids: curcumin I, curcumin II, and curcumin III. We tested these individual curcuminoids in our earlier studies with the mammalian ABC drug transporters P-glycoprotein, MRPI,
and ABCG2 (3, 4, 5). We reported that these individual curcuminoids inhibited the function of these drug transporters with different efficiencies and that curcumin I was the most potent among them (3, 4, 5). In addition, we have also reported that tetrahydrocurcumin, a major metabolite of CUR, also inhibits these three mammalian ABC drug transporters (18). In this study, based on the initial data discussed above for the natural CUR mixture and its purified individual components with mammalian transporters, we evaluated the CUR mixture alone for its activity to synergize the activities of antifungal agents. There are several CUR derivatives that are synthetic analogues, and some of them may have better activity than the CUR mixture. Thus, these analogues merit further study.

There are reports to suggest that CUR can downregulate the expression of an MDR-linked transporter (ABCB1) and can even affect the function of several transcription factors (2, 6). For this reason, we tested the effect of CUR on the expression of an ABC transporter and observed that CUR did not affect the expression levels of CaCdr1p (Fig. 4D), implying that the modulation of R6G efflux by CUR is restricted to its direct effect on the functionality of ABC transporter proteins. The direct effect of CUR on CaCdr1p was confirmed by its ability to compete the photoaffinity labeling of CaCdr1p with [125I]IAAP (Fig. 4B) and by its competitive inhibition of R6G efflux (Fig. 4A). We excluded the possibility that CUR could be a preferred substrate of the ABC transporters studied (Fig. 2).

Our study shows that CUR, which is not a transport substrate of CaCdr1p, specifically modulates the efflux of R6G mediated by the transporter. This is not surprising, since it has been observed previously that curcuminoids can modulate drug transport without being a substrate of mammalian ABCG2 (4). Our cytotoxicity data (Fig. 2; Table 2) suggest that the presence or absence of efflux pump proteins did not affect the growth and viability of yeast cells, again pointing to the fact that CUR is not a substrate of these pumps. It is not clear, however, whether CUR modulates R6G efflux by binding to the substrate or to an allosteric site(s) of CaCdr1p. Considering the fact that the structures and substrate specificities of fungal ABC transporters such as CaCdr1p, CaCdr2p, and ScPdr5p are very different, our finding from this study that yeast transporters can be modulated by CUR is very significant.

It is reported that the poor bioavailability of CUR and its low concentrations in plasma decrease its effectiveness in modulating the function of ABC drug transporters in rodents and humans. However, recent studies indicate that the use of piperine to prevent the glucuronidation of curcumin, as well as the encapsulation of CUR in liposomes, can increase the absorption of CUR and its levels in plasma (28). It is, however, not known whether CUR is metabolized via glucuronidation in yeast cells or whether the intracellular efflux by CUR (100 μM), calculated by taking the level of R6G efflux with each mutant CaCdr1p variant in the absence of CUR as 100%. Values are means ± standard deviations (error bars) for three independent experiments.
level of CUR is lower than that in medium or plasma. These issues need to be resolved before CUR can be used as an effective in vivo or in vitro antifungal. In summary, the modulation of antifungal efflux by CUR is substrate and transporter specific. Nevertheless, curcuminoids are not toxic to the cell, nor are they transported by their target efflux pumps. Thus, their ability to sensitize cells to azoles toxic to the cell, nor are they transported by their target efflux pumps.

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