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Rhodamine 6G efflux for the detection of *CDR1*-overexpressing azole-resistant *Candida albicans* strains

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We investigated the drug efflux mechanism in azole-resistant strains of *Candida albicans* using rhodamine 6G (R6G). No significant differences in R6G uptake were observed between azole-sensitive B2630 (9.02 ± 0.02 nmol/10^8 cells) and azole-resistant B67081 (8.86 ± 0.03 nmol/10^8 cells) strains incubated in glucose-free phosphate buffered saline. A significantly higher R6G efflux (2.0 ± 0.21 nmol/10^8 cells) was noted in the azole-resistant strain (B67081) when glucose was added, compared with that in the sensitive strain B2630 (0.23 ± 0.14 nmol/10^8 cells). A fluconazole-resistant strain C40 that expressed the benomyl resistance gene (*CaMDR*) also showed a low R6G efflux (0.16 ± 0.06 nmol/10^8 cells) as did the sensitive strains. Accumulation of R6G in growing *C. albicans* cells was inversely correlated with the level of *CDR1* mRNA expression. Our data also suggest that measurement of intracellular accumulation of R6G is a useful method for identification of azole-resistant strains due to *CDR1*-expressed drug efflux pump.

Introduction

Persistent or recurrent oropharyngeal candidosis (OPC) caused by *Candida albicans* is common in patients with AIDS.[1] Fluconazole has been used widely to treat OPC, but several publications have described its failure in the treatment of patients with advanced AIDS, particularly when used over a long period of time.[2,3] The Subcommittee for Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (NCCLS) has collaborated with many other investigators to develop the *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Now, NCCLS has published its approval-level version as document M27-A.[4] Interpretive breakpoints were proposed for the antifungal testing of *Candida* spp. with fluconazole by the NCCLS M27-T broth macrodilution method, with MICs of >64 mg/L indicating resistance.[5] The trailing growth phenomenon remains a problem in antifungal testing of certain yeast isolates by the NCCLS method. The M27-A method addressed this problem by requiring an 80% reduction in growth after 48 h of incubation; MICs are several dilutions above the 24 h results.[6] The development of another test for the detection of azole-resistant *C. albicans* strains may be helpful for the clinical laboratory.

Many studies have investigated the mechanisms of azole resistance in *Candida* spp. isolates.[7–9] The results of these studies indicate that resistance to azole antifungal agents may be a consequence of one or more mechanisms. One of the major mechanisms imparting resistance to azole antifungal agents can be drug efflux, similar to the multidrug-resistant (MDR) pumps in tumour cells. Among the MDR genes, *CDR1* has been cloned in *C. albicans* and the level of mRNA of the *CDR1* gene was found to be significantly higher in fluconazole-resistant strains than in sensitive strains.[10] The product of another *C. albicans* gene, *CaMDR1*, formerly known as *BEN*^{r}, resembles proteins of the major facilitator superfamily (MFS) class of MDR proteins.[11]

In the present study, we used a fluorescent dye, rhodamine 6G (R6G), a substrate of MDR protein in *Saccharomyces cerevisiae*, to identify MDR activity in azole-resistant isolates of *C. albicans*.

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Materials and methods

Strains, medium and azole antifungal agents

Two isolates of *C. albicans* (B2630 and B67081) held at the Janssen Research Foundation (Beerse, Belgium) were used. Another 10 strains of *C. albicans* that were originally isolated from AIDS patients with OPC, were obtained from the Institute of Microbiology (Centre Hospitalier Universitaire, Vaudois, Switzerland). The species were identified using standard procedures. All strains were maintained at –80°C. Strains of *C. albicans* were grown in a CYG medium containing 0.5% casein hydrolysate (Merck, Darmstadt, Germany), 0.5% yeast extract (Difco, Detroit, MI, USA) and 0.5% glucose (Difco). Fluconazole was obtained from Pfizer (Sandwich, UK), and ketoconazole and itraconazole from the Janssen Research Foundation (Beerse, Belgium). Azole antifungal agents were dissolved in dimethyl sulphoxide (DMSO) at 1.5 g/L and used as stock solution.

MIC determination for C. albicans isolates

The stock solution was diluted 100-fold with susceptibility-testing culture medium and a series of 10 two-fold dilutions was prepared. These solutions were pipetted in 100 μL volumes into rows of wells of flat-bottomed microdilution plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ, USA). The final concentrations of fluconazole ranged from 100 to 0.13 mg/L, and those of ketoconazole and itraconazole from 8 to 0.02 mg/L, in two-fold serial dilutions.

The MIC of antifungal agents was determined by the microdilution method using the 96 flat-bottomed microdilution plate modified from the NCCLS macrodilution method. From deep frozen stock culture, the cells of *C. albicans* were inoculated into 5 mL of CYG broth and incubated at 37°C for 18–24 h with shaking. The final inoculum size was adjusted to 10^3 cfu/mL. The plates were sealed with plastic stickers and incubated for 48 h at 35°C. MICs were determined as the minimum concentration of the antifungal agents yielding at least 80% inhibition of the growth compared with the growth of control.

Measurement of R6G uptake and glucose-induced efflux

Yeast cells were grown in 100 mL of CYG broth at 37°C for 14 h. 1×10^8 yeast cells/mL were transferred to 100 mL of fresh CYG broth and incubated at 37°C for 4 h. The cells were harvested in 50 mL Falcon tubes (Becton Dickinson) and centrifuged at 5000**g** for 5 min. The pellets were washed twice with 20 mL of phosphate buffered saline (PBS; Life Technologies, Paisley, Scotland). They were then suspended in a glucose-free PBS buffer, at a concentration of 1×10^8 cells/mL and incubated at 37°C for 1 h in a reciprocating shaker. A stock solution of R6G (Sigma Chemical Co., St Louis, MO, USA) was prepared by dissolving the dye in DMSO at a concentration of 10 mM. A final concentration of 10 μM of R6G was added to the cell suspension and incubated at 37°C in a reciprocating shaker. After incubation for 5, 10, 15, 20, 25 min, 1 mL samples were withdrawn and centrifuged at 9000**g** for 2 min. The supernatants (750 μL) were collected and absorption was measured at 527 nm. To examine the effect of glucose, the cell suspension was centrifuged after 25 min incubation, at 5000**g** for 5 min and pellets were resuspended in PBS containing 1 mole of glucose and incubated at 37°C. Samples of 1 mL volume were withdrawn at 5 min intervals and centrifuged at 9000**g** for 2 min. Then 750 μL of supernatant was collected and absorption was measured at 527 nm. The concentration of R6G was calculated using a standard concentration curve of R6G.

Intracellular concentration of R6G in growing isolates of C. albicans

Yeast cells were grown in 100 mL of CYG medium at 37°C for 14 h in a reciprocating shaker. A total of 1×10^8 cells/mL was transferred to 100 mL of fresh CYG medium and the cells were harvested after 4 h incubation by centrifugation at 5000**g** for 5 min, and then washed with PBS. Cells were resuspended in CYG medium to a cell concentration of 2.5×10^8 cells/mL. One millilitre of the cell suspension was incubated in a 10 mL glass tube with R6G (final concentration 10 μM) at 37°C for 1 h, under continuous shaking at 300**g** in an orbital shaker. Cells were collected by centrifugation at 9000**g** for 2 min and were resuspended with 750 μL of PBS buffer and absorption was measured at 527 nm.

Measurement of CDR1 and CaMDR (BEN^r) mRNA expression

The measurement of *CDR1* and *CaMDR* (BEN^r) mRNA expression was reported in our previous study.[10] Yeast cells were grown to logarithmic phase in 100 mL of Yeast Nitrogen Base (YNB) medium at 30°C with shaking. The cells were harvested and were ground to a fine powder under liquid nitrogen. The powder was immediately dissolved in a denaturing solution provided by the RNAeasy kit (QIAGEN Inc., Chatsworth, CA, USA). For the Northern analysis, RNA was first denatured in a loading buffer (50% formamide, 100 mM MOPS pH 7.0, 6.4% formaldehyde, 5% glycerol, 5% of a water solution saturated with bromophenol blue) at 85°C for 5 min and then subjected to electrophoresis in 1% agarose. The agarose was melted in a buffer containing 0.1 M MOPS, 0.6 M formaldehyde. The electrophoresis buffer was 0.1 M MOPS pH 7.0.

Northern transfer was performed overnight on GeneScreen Plus (DuPont NEN, Boston, MA, USA) with 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)

as a transfer buffer. RNA was fixed on the membrane by baking at 80°C under vacuum. Membranes were pre-hybridized at 42°C with a buffer consisting of 50% formamide, 1% sodium dodecyl sulphate (SDS), 4 × SSC, 10% dextran sulphate and salmon sperm DNA 100 mg/L. ^{32}P-labelled DNA probes were generated by random priming and were added to the hybridization solution overnight. After the washing step, membranes were exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA) for documentation. For quantitative analysis, the signals were subsequently analysed by a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Results

Effect of glucose on uptake and efflux of R6G by C. albicans

To investigate the efflux mechanisms of azole antifungal agents, we measured the uptake and efflux of R6G by *C. albicans* cells. For this purpose, we compared R6G uptake and efflux in azole-resistant (B67081) and -sensitive (B2630) strains. Intracellular R6G uptake increased immediately when both types of cells were incubated in glucose-free PBS, as was evident from a sharp drop in the measured extracellular concentrations of R6G (Figure 1). However,

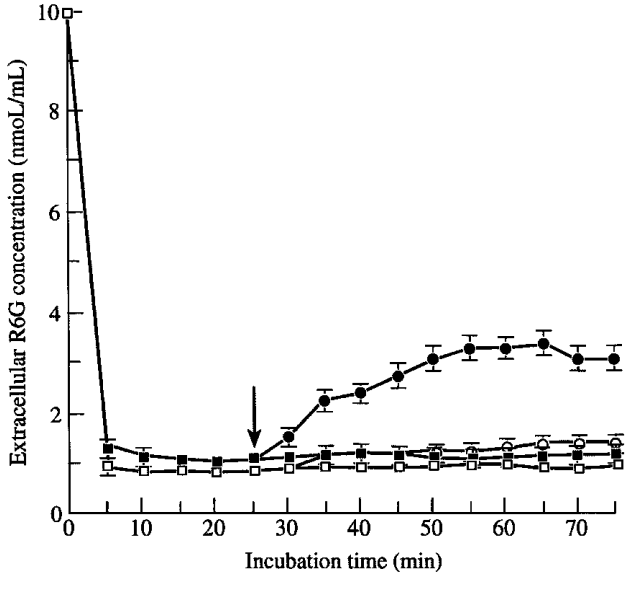

Figure 1. R6G uptake and glucose-induced R6G efflux by azole-resistant (B67081) and -sensitive (B2630) *C. albicans* strains. One mole of glucose was added after 25 min incubation (arrow) to a glucose-free PBS. Each data point represents the mean ± S.D. of 10 measurements of the extracellular concentration of R6G at the specified time interval. (●) B67081 azole-resistant strain in the presence of glucose, (○) B67081 strains without glucose, (■) B2630 azole-sensitive strain with glucose, (□) B2630 without glucose.

the uptake reached equilibrium 25 min after incubation. No significant difference in R6G uptake was observed between B67081 (8.86 ± 0.03 nmol/10^9 cells, n = 10) and B2630 (9.02 ± 0.02 nmol/10^9 cells, n = 10) 25 min after incubation. In the next step, the cells were resuspended in PBS and 1 mol of glucose was added 30 min after incubation. Azole-resistant *C. albicans* (B67081) pumped out higher concentrations of R6G (2.00 ± 0.21 nmol/10^9 cells, n = 10) into the extracellular fluid than azole-sensitive *C. albicans* (B2630) (0.23 ± 0.14 nmol/10^9 cells, n = 10). However, no R6G efflux occurred when both strains were maintained for another 35 min in the absence of glucose (Figure).

Table I summarizes R6G uptake and glucose-induced R6G efflux in different clinical isolates of azole-resistant and -sensitive *C. albicans* strains. Intracellular accumulation of R6G was observed in all strains when incubated in PBS. In addition, glucose-induced R6G efflux was observed in all azole-resistant strains. However, one azole-resistant strain (C40) showed only a small amount of glucose-induced R6G efflux.

Intracellular R6G accumulation in growing C. albicans cells

We compared the intracellular accumulation of R6G in growing *C. albicans* cells, both fluconazole-sensitive and -resistant strains (Table II). R6G accumulation measured by the photometric method in the azole-resistant strain (B67081) was markedly lower than in the sensitive strain (B2630). In contrast, intracellular R6G concentration in four of five fluconazole-sensitive strains (MIC of fluconazole was <6.3 mg/L for C27, C33, C23 and C37) was more than 4.0 nmol/mL. However, the concentration in one azole-sensitive strain, C32 (MIC of fluconazole was 3.2 mg/L) was lower than in the other four sensitive strains. Intracellular R6G concentration in three fluconazole-resistant strains (C82, C26 and C39) was <3.0 nmol/mL. The highest accumulation of R6G was noted in azole-resistant strains C34 and C40 (MIC of fluconazole was 25 and 100 mg/L, respectively). We correlated the concentration of intracellular R6G in growing *C. albicans* strains with the level of *CDR1* mRNA (Table II). Six strains with a low expression of *CDR1* mRNA showed a high accumulation of intracellular R6G whereas three other azole-resistant strains with a high level of *CDR1* expression showed very low intracellular concentration of R6G. These results indicated that accumulation of R6G in the cell was inversely related to the expression level of *CDR1* mRNA.

Discussion

In our assay, we used the fluorescent dye rhodamine which is known to be transported into or out of the cell in a number of organisms that maintain MDR,[12] from yeast to

Table I. R6G uptake and glucose-induced efflux in clinically isolated azole-sensitive and -resistant *C. albicans* strains

Patient	Strain	MICs (mg/L)			R6G concentration (nmol/10^9 cells)[a]	
		fluconazole	itraconazole	ketoconazole	uptake	glucose-induced efflux
I	C23	3.2	0.05	0.1	9.09	0.22
	C32	6.3	0.2	0.4	9.06	0.46
	C39	100	6.3	25	9.01	1.93
III	C33	0.4	<0.025	<0.025	9.09	0.19
	C34	25	0.4	0.8	9.06	0.39
	C26	>100	13	>25	9.08	2.82
	C82	50	0.8	0.4	8.99	2.17
IV	C27	0.4	<0.025	0.2	9.03	0.09
	C37	6.3	0.2	0.2	9.05	0.18
	C40	100	1.6	1.6	9.04	0.16
	B2630	0.13	0.032	0.016	9.02	0.23
	B67081	64	1.0	1.0	8.86	2.0

[a]R6G uptake was calculated from the extracellular R6G concentration incubated with 10 μmol/mL of R6G in PBS buffer after 25 min. The concentration of R6G efflux induced by glucose was calculated thus: extracelluar R6G concentration with glucose–extracellular concentration without glucose after 30 min incubation. Each experiment was carried out three times for each strain, and the mean values at each R6G concentration are given. The variation in each concentration was <10%.

Table II. The accumulation of R6G in growing fluconazole-sensitive and -resistant *C. albicans* strains

Strain	Fluconazole MIC (mg/L)	Relative expression level of mRNA		R6G accumulation (nmol/mL)[a]
		CDR1	*CaMDR*	
C27	0.4	0.2	0	4.38 ± 0.10
C33	0.4	0.2	0	4.09 ± 0.15
C23	3.2	0.3	0	4.30 ± 0.11
C32	6.3	0.6	0	3.03 ± 0.05
C37	6.3	0.2	0	4.50 ± 0.15
C34	25	0.2	0	3.99 ± 0.10
C82	50	1.7	0	2.63 ± 0.13
C26	100	1.4	0	2.58 ± 0.07
C39	100	1.3	0	2.62 ± 0.16
C40	100	0.2	1.0	4.46 ± 0.09

[a]The values are mean ± S.D. (n = 6).

mammalian cells.[13] In our previous study, the accumulation of fluconazole in strains C26 and C40 was lower than that in strain B2630, however, only C26 showed a marked reduction in the accumulation of itraconazole.[14] Our results showed that R6G moved from the extra- into the intracellular compartment in both azole-resistant and -sensitive strains when incubated in glucose-free conditions. The R6G efflux from azole-resistant strains was significantly enhanced when glucose was added to PBS. These results suggest that these strains are capable of inducing the MDR protein to reduce intracellular accumulation of azole compounds. It is noteworthy that in strains of azole-resistant *Candida glabrata* and *Candida krusei*, no significant differences in R6G efflux were observed between azole-sensitive and -resistant strains (data not shown). Considered together, we believe that the mechanisms of resistance of *C. glabrata* and *C. krusei* against azole antifungal agents may be different from those of *C. albicans* or, alternatively, R6G may not be a substrate of the MDR protein in these strains.

The resistance of C40 to fluconazole was as high as that of strain C26. The latter strain also showed cross-resistance to ketoconazole and itraconazole, but strain C40 was less resistant to ketoconazole and itraconazole.[10] In the present study, R6G uptake was not significantly different between the two resistant strains. However, we observed R6G efflux only in C26, since the level of *CDR1* mRNA expression of C40 was similar to that of the sensitive strain; R6G efflux in C40 was not induced by glucose. The differences in R6G efflux between strains C26 and C40 suggest that R6G is a substrate of the MDR protein produced by *CDR1* mRNA.

Intracellular concentrations of R6G were high in fluconazole-sensitive growing *C. albicans* cells but low in resistant strains. However, one of the sensitive strains, C32, had a low concentration of R6G in growing cells. Comparison between C32 and C37 strains showed that the susceptibility of both strains to fluconazole was similar, but the level of expression of *CDR1* mRNA in C37 was almost twice that in the C32 strain. Thus, the differences in expression of *CDR1* mRNA between these two strains may explain the difference in intracellular accumulation of R6G. Among azole-resistant strains, C34 and C40 showed a higher accumulation of R6G than other resistant strains. These two strains also showed a low level of expression of *CDR1* mRNA. The C40 strain was the only resistant strain included in this study that expressed *CaMDR* mRNA. The methods used to measure accumulation of R6G could not confirm whether the mechanism of azole resistance was due to the *CaMDR* gene. The differences in R6G accumulation between these two strains suggest that the efflux mechanism of resistance was induced by a phenotype different from that of the *CDR1* gene, or perhaps was caused by an entirely different mechanism. Future studies should compare the accumulation of cytochrome P450 or the binding affinity of azole compounds in these strains.

This method may be simple and convenient for measuring the level of *CDR1* mRNA expression in azole-resistant strains of *C. albicans* and could be useful in determining the basis of azole resistance among clinically isolated strains. The method is based on the molecular mechanism of *C. albicans* resistance to azoles and can be used for comparing azole-resistant strains of *C. albicans* defined using other procedures. We suggest that measurement of intracellular accumulation of R6G could be used clinically to identify resistant strains of *C. albicans* isolated from patients during azole antifungal therapy and to help in selecting a suitable antifungal therapy.

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References

1. Willocks, L., Leen, C. L. S., Brettle, R. P., Urquhart, D., Russell, T. B. & Milne, L. J. R. (1991). Fluconazole resistance in AIDS patients. *Journal of Antimicrobial Chemotherapy* **28**, 937–9.

2. Johnson, E. M., Warnock, D. W., Luker, J., Porter, S. R. & Scully, C. (1995). Emergence of azole drug resistance in *Candida* species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidiasis. *Journal of Antimicrobial Chemotherapy* **35**, 103–14.

3. Law, D., Moore, C.B., Wardle, H.M., Ganquli, L.A., Keaney, M. G., Denning, D.W. *et al.* (1994). High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS. *Journal of Antimicrobial Chemotherapy* **34**, 659–68.

4. National Committee for Clinical Laboratory Standards. (1997). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard M27-A*. NCCLS, Villanova, PA.

5. Rex, J. H., Pfaller, M. A., Galgiani, J. N, Bartlett, M. S., Espinel-Ingroff, A., Ghannoum, M. A. *et al.* (1996). Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro–in vivo correlation data for fluconazole, itraconazole, and Candida infections. *Clinical Infectious Diseases* **24**, 235–47.

6. Revankar, S. G., Kirkpatrick, W. R., McAtee, R. K., Fothergill, A. W., Redding, S. W., Rinaldi, M. G. *et al.* (1998). Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. *Antimicrobial Agents and Chemotherapy* **36**, 153–6.

7. Vanden Bossche, H., Marichal, P. & Odds, F. C. (1994). Molecular mechanisms of drug resistance in fungi. *Trends in Microbiology* **2**, 393–400.

8. Vanden Bossche, H., Warnock, D. W., Dupont, B., Kerridge, D., Gupta, S. S., Improvisi, L. *et al.* (1994). Mechanisms and clinical impact of antifungal drug resistance. *Journal of Medical and Veterinary Mycology* **32**, *suppl 1*, 189–202.

9. White, T. C. (1998). Antifungal drug resistance in *Candida albicans*. *ASM News* **63**, 427–33.

10. Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M. & Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrobial Agents and Chemotherapy* **39**, 2378–86.

11. Goldway, M., Teff, D., Schmidt, R., Oppenheim, A. B. & Koltin, Y. (1995). Multiple resistance in *Candida albicans*: Disruption of the *BEN*^{r} gene. *Antimicrobial Agents and Chemotherapy* **39**, 422–6.

12. Lee, J. S., Paull, K., Alvarez, M., Hose, C., Monks, A., Grever, M. *et al.* (1994). Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Molecular Pharmacology* **46**, 627–38.

13. Clark, F. S., Parkinson, T., Hitchcock, C. A. & Gow, N. A. R. (1996). Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species: Possible role for drug efflux in drug resistance. *Antimicrobial Agents and Chemotherapy* **40**, 419–25.

14. Maesaki, S., Marichal, P., Hossain, M. A., Sanglard, D., Vanden Bossche, H. & Kohno, S. (1998). Synergic effects of tacrolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. *Journal of Antimicrobial Chemotherapy* **42**, 747–53.

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