The Monoamine Oxidase A Inhibitor Clorgyline Is a Broad-Spectrum Inhibitor of Fungal ABC and MFS Transporter Efflux Pump Activities Which Reverses the Azole Resistance of *Candida albicans* and *Candida glabrata* Clinical Isolates

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Resistance to the commonly used azole antifungal fluconazole (FLC) can develop due to overexpression of ATP-binding cassette (ABC) and major facilitator superfamily (MFS) plasma membrane transporters. An approach to overcoming this resistance is to identify inhibitors of these efflux pumps. We have developed a pump assay suitable for high-throughput screening (HTS) that uses recombinant *Saccharomyces cerevisiae* strains hyperexpressing individual transporters from the opportunistic fungal pathogen *Candida albicans*. The recombinant strains possess greater resistance to azoles and other pump substrates than the parental host strain. A flow cytometry-based HTS, which measured increased intracellular retention of the fluorescent pump substrate rhodamine 6G (R6G) within yeast cells, was used to screen the Prestwick Chemical Library (PCL) of 1,200 marketed drugs. Nine compounds were identified as hits, and the monoamine oxidase A inhibitor (MAOI) clorgyline was identified as an inhibitor of two *C. albicans* ABC efflux pumps, CaCdr1p and CaCdr2p. Secondary *in vitro* assays confirmed inhibition of pump-mediated efflux by clorgyline. Clorgyline also reversed the FLC resistance of *S. cerevisiae* strains expressing other individual fungal ABC transporters (*Candida glabrata* Cdr1p or *Candida krusei* Abc1p) or the *C. albicans* MFS transporter Mdr1p. Recombinant strains were also chemosensitized by clorgyline to other azoles (itraconazole and miconazole). Importantly, clorgyline showed synergy with FLC against FLC-resistant *C. albicans* clinical isolates and a *C. glabrata* strain and inhibited R6G efflux from a FLC-resistant *C. albicans* clinical isolate. Clorgyline is a novel broad-spectrum inhibitor of two classes of fungal efflux pumps that acts synergistically with azoles against azole-resistant *C. albicans* and *C. glabrata* strains.

There are several mechanisms by which fungi can become resistant to azoles, including changes in the ergosterol biosynthesis pathway, overexpression of Erg11p (CYP51p), mutations in Erg11p, and the overexpression of drug efflux pumps (6, 18). Of these mechanisms, increased expression of drug efflux pumps in the cell plasma membrane is widely recognized to be the major cause of high-level ($\text{MIC} \geq 64 \mu\text{g ml}^{-1}$), clinically relevant resistance of *Candida albicans* to azoles (1, 13, 37, 39, 41). There are two classes of efflux pumps: ATP-binding cassette (ABC) transporters that use the hydrolysis of ATP as their energy source, and major facilitator superfamily (MFS) pumps that utilize membrane potential to drive efflux. The *C. albicans* ABC transporter Cdr1p (CaCdr1p) is reported to be a major contributor to azole resistance in clinical isolates of fluconazole (FLC [Diflucan])-resistant strains of the opportunistic pathogen *C. albicans* (13, 47). ABC efflux pumps are often responsible for azole resistance in other pathogenic fungi, such as CneMdr1p in *Cryptococcus neoformans* (23). In *Candida glabrata*, an increasingly important fungal pathogen, the main mechanisms of azole resistance include overexpression of the *CgCDR1* and *CgPDH1* ABC efflux pump genes (42, 43). In another emerging fungal opportunistic pathogen, *Candida krusei*, ABC efflux pump CkAbc1p is a crucial factor in azole resistance (19, 24). MFS pumps can also contribute to azole resistance in fungi (50, 51).

An important approach to combating antifungal resistance is to develop inhibitors of these pumps that will chemosensitize cells to existing effective antifungal drugs (5, 31, 44, 45). A specific efflux pump inhibitor will not inhibit the normal growth of cells

expressing the efflux pump but will sensitize the cells to sub-MIC levels of their antifungal substrates. Screening for chemosensitization in agar diffusion assays or liquid growth assays has been used to identify inhibitors of fungal efflux pumps. The application of these usually non-high-throughput screens, to panels of xenobiotics (23), combinatorial peptide libraries (33), and libraries of natural products (46) has identified tacrolimus (FK506), milbemycins (23), enniatins, beauvericin, unnarmicins (46), ibuprofen (40), tetrandrine (52), cerulenin analogs (9), and some octapeptides (33) as specific fungal ABC pump inhibitors. Ideally, such compounds will inhibit a range of clinically important efflux pumps but not human orthologs.

Flow cytometry-based assays of fluorescently tagged whole cells are readily automated and adaptable for high-throughput screening (HTS) (10). As there are several fluorescent substrates of efflux pumps, fluorescence-based assays of activity are applicable to measuring the activity of ABC transporters (16, 21, 44). A dif-

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TABLE 1 Yeast strains used in this study

Strain	Description	Genotype
<i>S. cerevisiae</i> strains		
AD1-8u ⁻	Host strain (abbreviated to AD)	<i>mataα</i> <i>PDR1-3</i> <i>ura3</i> <i>his1</i> Δ <i>yor1::hisG</i> Δ <i>snq2::hisG</i> Δ <i>pdr10::hisG</i> Δ <i>pdr11::hisG</i> Δ <i>ycf1::hisG</i> Δ <i>pdr3::hisG</i> Δ <i>pdr15::hisG</i> Δ <i>pdr5::hisG</i>
AD/pABC3	AD1-8u ⁻ + pABC3 empty cloning cassette (control)	AD1-8u ⁻ Δ <i>pdr5::pABC3</i>
AD/CaCDR1	<i>C. albicans</i> ATCC10261 <i>CDR1</i> ORF ^d (A allele) in cloned cassette	AD1-8u ⁻ Δ <i>pdr5::pABC3</i> -CaCDR1
AD/CaCDR2	<i>C. albicans</i> ATCC10261 <i>CDR2</i> ORF (A allele) in cloned cassette	AD1-8u ⁻ Δ <i>pdr5::pABC3</i> -CaCDR2
AD/CaMDR1	<i>C. albicans</i> ATCC10261 <i>MDR1</i> ORF (A allele) in cloned cassette	AD1-8u ⁻ Δ <i>pdr5::pABC3</i> -CaMDR1
AD/CgCDR1	<i>C. glabrata</i> CBS138 <i>CDR1</i> ORF in cloned cassette	AD1-8u ⁻ Δ <i>pdr5::pABC3</i> -CgCDR1
AD/CkABC1	<i>C. krusei</i> B2399 <i>ABC1</i> ORF in cloned cassette	AD1-8u ⁻ Δ <i>pdr5::pABC3</i> -CkABC1
<i>Candida</i> clinical isolates		
MML604	<i>C. albicans</i> 2-76 ^a	FLC-sensitive clinical isolate
MML605	<i>C. albicans</i> 12-99 ^a	FLC-resistant clinical isolate; derived from MML604
MML609	<i>C. albicans</i> FH8 ^b	FLC-resistant clinical isolate
MML610	<i>C. albicans</i> TL1 ^b	FLC-sensitive clinical isolate
MML611	<i>C. albicans</i> TL3 ^b	FLC-resistant clinical isolate; derived from MML610
MML99	<i>C. glabrata</i> CBS138 ^c	Clinical isolate

^a Reference 50.^b Reference 29.^c Strain obtained from the Centraalbureau voor Schimmelcultures (Utrecht, Netherlands).^d ORF, open reading frame.

ferential signal can be obtained between cells which are actively effluxing a previously loaded fluorescent substrate (low signal at endpoint) and cells for which efflux is inhibited by a test compound (high signal at endpoint). We have recently developed a flow cytometry-based HTS assay suitable for the identification of inhibitors of fungal efflux pumps, using a yeast-based assay of accumulation of fluorescent pump substrates rhodamine 6G (R6G) or Nile Red (16). The screening assay employed recombinant strains of the model yeast *Saccharomyces cerevisiae* in which the individual transporter genes were cloned and functionally expressed (23). The AD host strain has deletions of seven ABC transporters so that pump activity can be assayed in a background of depleted endogenous pumps. Furthermore, the gene encoding the transporter of interest is integrated at the *S. cerevisiae* *PDR5* genomic locus downstream of a promoter under the control of a mutant transcriptional regulator, *Pdr1-3p*, producing constitutive high-level expression of functional heterologous proteins in the plasma membrane of the derived recombinant strain. A panel of *S. cerevisiae* strains has been created that express a range of pumps (ABC and MFS) from important fungal pathogens including *C. albicans*, *C. glabrata*, and *C. krusei* (16).

In this study, the Prestwick Chemical Library (PCL, Illkirch, France; a collection of off-patent drugs and alkaloids) was screened for inhibitors of *C. albicans* Cdr1p and Cdr2p by flow cytometry, using the recombinant strains AD/CaCDR1 and AD/CaCDR2 (expressing *C. albicans* Cdr1p and *C. albicans* Cdr2p, respectively) with the fluorescent substrate R6G. Nine hits were obtained including the monoamine oxidase A (MAO-A)-selective inhibitor clorgyline. Secondary assays of pump function confirmed that clorgyline is a broad-spectrum inhibitor of several fungal efflux pumps, including *C. albicans* Mdr1p, that are considered to be responsible for azole resistance.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* and *Candida* sp. strains used in this study are listed in Table 1. The genotype of the parental *S. cerevisiae* strain,

AD1-8u⁻ (8), is common to all the derivative strains, with the addition of the individual efflux pump gene and *URA3* at the *PDR5* locus (23). The *C. albicans* strains used in this study were kindly provided by T.C. White (University of Missouri-Kansas City). Strains MML604 and MML605 were originally isolated by S. W. Redding (University of Texas Health Science Center at San Antonio, Texas).

All fungal strains were maintained on yeast extract, peptone, and glucose (YPD) medium which contained 1% (wt/vol) Bacto yeast extract (Difco Laboratories, Detroit, MI), 2% (wt/vol) Bacto peptone (Difco), and 2% (wt/vol) glucose. For HTS assays, yeast were grown in complete supplement medium without uracil (CSM-ura), which contained 2.67% (wt/vol) minimal synthetic defined (SD) base (Clontech Laboratories Inc., CA), 0.077% (wt/vol) –Ura Drop Out (DO) supplement (Clontech Laboratories Inc.), and 2% (wt/vol) D-glucose. For secondary assays of growth inhibition, yeast were grown in complete supplement mixture (CSM) adjusted to pH 7.0, containing 0.67% (wt/vol) yeast nitrogen base (Difco), 0.077% (wt/vol) CSM supplement (Bio 101, Vista, CA), and 2% (wt/vol) glucose. Where required for solid media, 2% (wt/vol) agar or 0.6% (wt/vol) agarose (Gibco: Invitrogen Corporation, Auckland, New Zealand) was included.

Compounds. Fluconazole (FLC; aqueous solution) was purchased from Pfizer Laboratories Ltd. (Auckland, New Zealand); itraconazole (ITC) was purchased from Janssen-Kyowa (Tokyo, Japan); and miconazole (MCZ), rhodamine 6G (R6G), nystatin (NYS), enniatin B, 2-deoxyglucose, and HEPES were purchased from Sigma-Aldrich New Zealand Ltd. (Auckland, New Zealand).

Flow cytometry-based HTS. The flow cytometry-based high-throughput screen (HTS) was formatted for 384-well microtiter plates as described previously (16). The 384-well microplates were configured with 32 control wells for negative, unblocked, transporter activity controls (low fluorescence) and positive, blocking, controls (high fluorescence), respectively. Positive controls utilized the known inhibitor of Cdr1p, enniatin B (14) (50 μ M final concentration), or wells seeded with parental strain AD1-8u⁻. Exponential-phase yeast cells of either AD/CaCDR1 or AD/CaCDR2 were incubated with medium containing R6G at a final concentration of 15 μ M before distribution into 384-well plates (5 μ l per well). Test compounds (5 μ M final concentration), stored as dimethyl sulfoxide stocks, were added to the noncontrol wells. After a 20-min incubation at room temperature, the HyperCyt system (22, 38) was used to aspirate

samples for delivery to a cyan flow cytometer (Dako Cytomation, Fort Collins, CO) for analysis. The samples were excited with a 488-nm laser, and passage through a PE filter enabled detection of R6G fluorescence. Specialized software (IDLeQuery) was used to analyze the data file. Hits were determined for a test compound if the fluorescence reading obtained showed a ≥ 5 -fold change in median fluorescence signal relative to the negative controls without added inhibitor. This represented a separation of the hit sample fluorescence from the negative control by ~ 100 standard deviations (SDs).

Direct determination of R6G efflux from whole cells. Hits detected in the HTS were confirmed using a non-HTS microtiter assay of R6G efflux (14), which measured effluxed R6G rather than the R6G remaining in cells. Exponential-phase yeast cells (100 ml, optical density at 600 nm [OD₆₀₀] of 1.3 to 1.7) were preloaded with R6G under energy-depleted conditions. Cells were washed twice with distilled water, resuspended in HEPES buffer (100 ml, OD₆₀₀ 1.0, 50 mM HEPES-NaOH, pH 7.0), incubated with shaking (200 rpm) at 30°C in the presence of 5 mM 2-deoxyglucose for 30 min, and then incubated for 30 min with R6G (15 μ M). Cells were then washed twice, resuspended in HEPES buffer at an OD₆₀₀ of 10 to 15, and incubated with hit compounds, at the concentrations indicated, with shaking (200 rpm, 30°C) for 30 min. Cell samples (50 μ l) were added to wells of a glass-fiber filter microtiter plate (Unifilter; Whatman, Maidstone, UK) and preincubated at 30°C for 5 min before the addition of 50 μ l of 0.8% glucose or HEPES only. After a 6-min incubation at 30°C (predetermined as a time at which optimum efflux had occurred), supernatants were collected by suction (-50 kPa) into the wells of 96-well microtiter plates (Greiner, transparent). The fluorescence of R6G in the samples was measured by comparison to R6G standards prepared in assay buffer containing equivalent amounts of the inhibitor, using a Synergy 2 microplate reader (BioTek, Winooski, Vermont) with excitation and emission wavelengths of 485 and 520 nm, respectively.

Drug susceptibility assays.

(i) Liquid microdilution MIC and checkerboard chemosensitization assays. The MICs of compounds for *C. albicans* strains were determined in accordance with the CLSI microdilution reference method (5a). For *S. cerevisiae* strains, the method was modified by using a CSM-based medium (32) because *S. cerevisiae* AD1-8u⁺, and its derivative strains, do not grow in the RPMI medium used in the CLSI method. Briefly, CSM (200 μ l) was inoculated with 4×10^3 yeast cells per well and incubated at 30°C for 48 h with shaking (150 rpm) in the presence of a series of 2-fold dilutions of the hit compound. Cell growth was monitored at 590 nm with the Synergy 2 microplate reader (BioTek). This assay determined the MIC of hit compounds for growth inhibition, and concentrations at or below the MIC were used in checkerboard chemosensitization assays. For azoles, MICs were defined as the minimum concentrations giving $>80\%$ growth inhibition compared with the no-drug control.

Microplate checkerboard assays of the chemosensitization of yeast strains to FLC by various inhibitor compounds were undertaken as described previously (33). In brief, doubling dilutions of an individual hit compound were prepared in rows of a sterile microplate (100 μ l per well at $2 \times$ final concentration). In a separate microplate, doubling dilutions of FLC (or other azoles) were prepared in columns (100 μ l per well; $4 \times$ final concentration) before transfer of 50 μ l from each well to the plate containing the dilutions of the hit compound. Finally, 4×10^3 cells (50 μ l) were added to each well of the plate containing the checkerboard of compound and FLC to give a final volume of 200 μ l. Microplates were incubated at 30°C with shaking (150 rpm), and cell growth was determined by optical density measurement at 24 h. Synergistic activity of hit compounds with azoles was demonstrated quantitatively in terms of FIC (fractional inhibitory concentration) and FIC index (FICI) determinations. FICs were calculated by the following formula: (MIC of drug A, tested in combination with another drug B)/(MIC of drug A alone) (28). The FICI was defined as the FIC of drug A plus the FIC of drug B. In accordance with published guidelines (35), an FICI of ≤ 0.5 was considered to indicate synergism.

(ii) Agarose diffusion chemosensitization assays. Chemosensitization of *S. cerevisiae* cells expressing fungal efflux pumps to a range of

TABLE 2 PCL hits for *S. cerevisiae* strains expressing either CaCdr1p or CaCdr2p

Compound ID ^a	Inhibition (%) ^b	
	CaCdr1p	CaCdr2p
Clorgyline hydrochloride	98.4	93.8
Ebselen	39.8	100
Flupentixol dihydrochloride	48.9	0
Prochlorperazine dimaleate	46.4	0
Econazole nitrate	0	91.1
Droperidol	0	86.7
Ethoxyquin	0	67
Sulconazole nitrate	0	57.5
Disulfiram	0	54.2
Average Z' (SD)	0.78 (0.07)	0.68 (0.06)

^a All compounds were tested in the screen at a final concentration of 5 μ M.

^b Test compound percentage inhibition of efflux pump activity at each point was calculated as $100 \times [1 - (MFI_{PC} - MFI_{Test}) / (MFI_{PC} - MFI_{NC})]$, where MFI_{Test} , MFI_{PC} , and MFI_{NC} represent the median fluorescence intensity (MFI) of cells in wells containing test compound, the average MFI of cells in positive control wells, and the average MFI of cells in negative control wells, respectively.

xenobiotics was carried out by agarose diffusion. Disks containing hit compounds were placed on a lawn of *S. cerevisiae* cells plated on agarose containing sub-MIC levels of the xenobiotic (34). CSM (20 ml) containing a sub-MIC level of FLC (or in some cases ITC or MCZ) was solidified with 0.6% agarose in an Omnitray (126 by 86 by 19 mm; Nunc, Roskilde, Denmark) or petri dish. Yeast cells (1×10^5 to 2×10^5) were suspended in 20 ml of melted top CSM-ura containing 0.4% agarose plus sub-MIC azole drug and poured onto the agarose in the Omnitray. An appropriate volume of hit compound (~ 10 μ l) was applied to paper disks (5-mm diameter; BBL, Becton, Dickinson Co., Sparks, MD) and placed on the solidified medium surface. The tray was incubated at 30°C for 48 h. FLC-free medium was also used in the disk assay to assess the effects of test hit compounds alone on the growth of strains.

(iii) Agarose dilution susceptibility assays to demonstrate chemosensitization. Serial 2-fold dilutions of exponential-phase yeast cells (initial cell concentration, 1.0×10^6) were prepared in CSM, pH 7.0. A 5- μ l portion of each dilution was spotted onto CSM (pH 7.0) agarose medium containing either no added drug, FLC alone at the indicated concentration, clorgyline alone at the indicated concentration, or both FLC and clorgyline at these concentrations. Cell growth was monitored after incubation at 30°C for 48 h.

RESULTS

HTS screen of the Prestwick Chemical Library (PCL) using *S. cerevisiae* strains expressing *C. albicans* Cdr1p or Cdr2p. Nine compounds were identified in the primary HTS screen of the 1,200-member PCL as inhibitors of CaCdr1p or CaCdr2p (Table 2). However, only two, clorgyline and ebselen, resulted in retention of R6G by both AD/CaCDR1 and AD/CaCDR2. Seven other compounds were identified as hits with just one of the recombinant strains; flupentixol and prochlorperazine were active against the CaCdr1p-expressing strain, and econazole nitrate, droperidol, ethoxyquin, sulconazole nitrate, and disulfiram were active against the CaCdr2p-expressing strain. Clorgyline inhibited CaCdr1p efflux activity by 98.2% and also inhibited CaCdr2p by 93.8%. Clorgyline has not previously been reported as a pump inhibitor or as having any antifungal activity.

The HTS hit clorgyline promoted retention of R6G by a recombinant *S. cerevisiae* strain expressing *C. albicans* Cdr1p, at concentrations that did not inhibit yeast growth. The R6G re-

FIG 1 Effect of clorgyline on R6G efflux from *S. cerevisiae* AD/CaCDR1 and control AD/pABC3 yeast cells. The efflux of R6G from either AD/CaCDR1 (filled circles) or AD/pABC3 (open circles) cells in the presence of 0.4% glucose was measured 6 min following glucose addition in the presence of clorgyline at the concentrations indicated, as described in Materials and Methods. Glucose-dependent efflux is expressed relative to the amount of R6G effluxed from controls with no added clorgyline (100% values). Each data point represents the mean of quadruplicate determinations in a representative experiment repeated once. Variation from the mean did not exceed 15%.

tention observed in the HTS in the presence of a putative pump inhibitor could have resulted from an effect of the compound on R6G uptake or on viability/metabolic activity, giving rise to a false-positive result. Four of the compounds detected as hits in the primary screen (ebfelen, econazole, sulconazole, and disulfiram) have known antifungal activity. Therefore, in order to confirm that clorgyline had an effect on efflux, rather than just on R6G retention, an assay of R6G efflux from yeast cells into the assay supernatant was applied. We measured the glucose-dependent release of R6G into the supernatant from the AD/CaCDR1 strain preloaded with R6G under glucose-deprived conditions. Released R6G was measured in supernatants harvested at a fixed time point (6 min after addition of glucose) demonstrated previously to give optimal efflux under similar conditions (13). R6G efflux from the *C. albicans* Cdr1p-expressing strain was inhibited 50% by 12.5 $\mu\text{g ml}^{-1}$ (47.2 μM) clorgyline (Fig. 1). Efflux of R6G from the strain AD/CgCDR1, expressing *C. glabrata* Cdr1p, was also inhibited by 50% by clorgyline at 50 $\mu\text{g ml}^{-1}$. Negligible R6G efflux was detected from the FLC-sensitive parental strain AD1-8u⁻ (without heterologous efflux pumps), with or without added glucose or clorgyline, at concentrations of up to 20 $\mu\text{g ml}^{-1}$. Only at clorgyline concentrations of $\geq 50 \mu\text{g ml}^{-1}$ was increased fluorescence from the control strain detected in the supernatant (Fig. 1). Thus, nonspecific efflux was detected only at concentrations at or above 50 $\mu\text{g ml}^{-1}$. The MICs of clorgyline for both the AD/CaCDR1 strain and the control AD/pABC3 strain were 20 $\mu\text{g ml}^{-1}$ (Table 3). These data indicated that the concentration of clorgyline used in the HTS (5 μM ; 1.35 $\mu\text{g ml}^{-1}$), and the 50% inhibitory concentration (IC_{50}) (47.2 μM ; 12.5 $\mu\text{g ml}^{-1}$) determined for direct inhibition of R6G efflux were not growth inhibitory. In contrast, consistent with previous reports (3), the MIC for ebselen against *S. cerevisiae* was $<10 \mu\text{M}$ (results not shown), so that for ebselen, the concentration used in the HTS (5 μM) was very close to the MIC. The clorgyline MIC data also indicated that clorgyline is not a substrate of *C. albicans* Cdr1p. If it was a substrate of the pump, strain AD/CaCDR1 would have had lower susceptibility to clorgyline than the control strain AD/pABC3.

Clorgyline reversed the resistance of efflux pump-expressing *S. cerevisiae* strains to FLC and other azoles. Although inhibition of R6G efflux from the pump-expressing strains indicated that the compound has specific activity against the fungal ABC transporter expressed, it is also important to demonstrate that an inhibitor acts to reduce or prevent the efflux of azoles as well as R6G. The effect of clorgyline on azole transport by efflux pumps was investigated by determining its ability to increase the FLC susceptibility of strains expressing individual fungal ABC transporters that are known to be involved in the FLC resistance of clinically relevant yeast species (*C. albicans* Cdr1p and Cdr2p, *C. glabrata* Cdr1p, and *C. krusei* Abc1p). The ability of clorgyline to chemosensitize the strains AD/CaCDR1, AD/CaCDR2, AD/CgCDR1, and AD/CkABC1 to FLC was demonstrated by agarose diffusion assays (Fig. 2). Similarly, clorgyline also chemosensitized a strain (AD/CaMDR1) expressing the *C. albicans* MFS transporter Mdr1p to FLC (Fig. 2). When 50 or 100 μg of clorgyline was added to disks, it had no significant effect on yeast growth in the absence of FLC but enhanced the growth inhibitory effect of FLC against all strains. Clorgyline also reversed the resistance of AD/CDR1 to ITC (a triazole) and MCZ (an imidazole) (results not shown). At high concentrations (150 or 200 μg per disk), clorgyline showed antifungal activity which was increased in the presence of sub-MIC concentrations of FLC (results not shown). Some toxicity is also evident against the AD/pABC3 strain at 100 μg added to the disk. Quantitation of the synergistic activity of clorgyline with azoles against both *S. cerevisiae* strains expressing efflux pumps, and for clinical isolates of *C. albicans*, was demonstrated using checkerboard liquid MIC chemosensitization assays (Table 4). Figure 3 shows representative checkerboard assays used to generate the data for Table 4.

Clorgyline reversed the azole resistance of *C. albicans* and *C. glabrata* clinical isolates and showed quantitative synergy with azoles. Agarose diffusion assays and agar dilution assays (Fig. 4) showed that clorgyline enhanced the growth-inhibitory effect of FLC against three FLC-resistant *C. albicans* clinical isolates (MML605, MML609, and MML611; FLC MICs of 128, 64, and 64 $\mu\text{g ml}^{-1}$, respectively) and two FLC-sensitive isolates (MML610

TABLE 3 Fluconazole and clorgyline MICs for yeast strains used in this study

Strain name	MIC ($\mu\text{g ml}^{-1}$)	
	FLC	Clorgyline
<i>S. cerevisiae</i> strains		
AD1-8u ⁻	0.5	20
AD/pABC3	0.5	20
AD/CaCDR1	200	20
AD/CaCDR2	80	20
AD/CaMDR1	15	20
AD/CgCDR1	300	20
AD/CkABC1	32	20
<i>Candida</i> clinical isolates		
<i>C. albicans</i> MML604	0.5	>40
<i>C. albicans</i> MML605	128	>40
<i>C. albicans</i> MML609	64	>20
<i>C. albicans</i> MML610	0.5	>20
<i>C. albicans</i> MML611	64	>20
<i>C. glabrata</i> MML99	128	>40

FIG 2 Effect of clorgyline on the growth of *S. cerevisiae* strains expressing individual fungal efflux pumps in the absence or presence of FLC. Growth of the control *S. cerevisiae* strain AD/pABC3 or recombinant strains expressing either *C. albicans* Cdr1p, Cdr2p, or Mdr1p, *C. glabrata* Cdr1p, or *C. krusei* Abcb1p were analyzed by agarose diffusion chemosensitization assays as described in Materials and Methods, using CSM medium (pH 7.0) containing either no added FLC (top rows) or FLC (bottom rows) at the relevant concentrations ($0.25 \times \text{MIC}$ values). Paper disks containing clorgyline at the concentrations indicated were placed on the surface of agarose plates seeded with yeast. Plates were incubated at 30°C for 24 h.

and MML604). Although FLC sensitive, both MML610 and MML604 express low levels of Cdr1p, explaining their response to clorgyline. In addition, clorgyline also reversed the FLC resistance of a *C. glabrata* isolate (MML99; FLC MIC $120 \mu\text{g ml}^{-1}$) (Fig. 4). These data were confirmed by checkerboard liquid MICs allowing FICI determinations (Table 4). Clorgyline showed synergy with FLC and MCZ against AD/CaCDR1 (FICI values of 0.13 and 0.11, respectively) and AD/CaMDR1 (FICI values of 0.25 and 0.24, respectively). Synergy between clorgyline and FLC was demonstrated for three FLC-resistant *C. albicans* strains, MML605, MML609, and MML611 (FICI values 0.22, 0.11, and 0.35, respectively). Clorgyline also directly inhibited R6G efflux from *C. albicans* MML611; at a concentration of $12 \mu\text{g ml}^{-1}$, no net R6G efflux into the supernatants was detectable.

DISCUSSION

Azoles are the most commonly prescribed class of antifungal drugs, in particular, FLC and ITC (7). FLC has good pharmacokinetic properties, is cost-effective, and very well tolerated, but

several fungi that can cause serious infections show innate or acquired resistance. Approximately 30% of clinical *C. glabrata* isolates show moderate innate azole resistance, and they can also acquire increased resistance during patient therapy with azole drugs (30). Given that there are very few other antifungal drug classes (5), there is a need to develop drugs that counter such resistance and extend the utility of azoles such as FLC. The flow cytometry-based screen for FLC-enhancing agents using *S. cerevisiae* strains expressing either CaCdr1p or CaCdr2p was developed because these ABC transporters are major contributors to the clinical resistance of *C. albicans* to azoles (13, 47). The PCL was selected as the compound library because it is compact (1,200 compounds) and also because published data which would aid interpretation of the results are available on each compound. Of the nine hits identified in the PCL screen, four (flupentixol, prochlorperazine, droperidol, and ethoxyquin) are known inhibitors or inducers of human ABC transporters (15, 25, 26, 36), and four (ebfelen, econazole, sulconazole, and disulfiram) have known antifungal activity (2, 3, 20, 49, 53). The finding that eight of nine hits

TABLE 4 Clorgyline shows synergism with FLC and MCZ as demonstrated by FICI

Inhibitor/azole	Yeast strain ^c	MICs ^a ($\mu\text{g ml}^{-1}$) and FIC						
		Azole alone	Combined ^b	FIC ^c	Clor alone	Combined ^b	FIC ^c	FICI ^d
Clorgyline/FLC	Sc AD/CaCDR1	200	<2.5 (20)	≤ 0.01	20	2.5 (80)	0.125	$\leq 0.13^*$
Clorgyline/MCZ		2	<0.1 (20)	≤ 0.05	20	<1.25 (4)	0.06	$\leq 0.11^*$
Clorgyline/FLC	Sc AD/CaMDR1	15	1.9 (10)	0.13	10	<1.25 (7.5)	<0.12	<0.25*
Clorgyline/MCZ		0.25	<0.03 (10)	0.12	10	<1.25 (0.12)	<0.12	<0.24*
Clorgyline/FLC	Ca MML605	128	12.5 (20)	0.10	>20	2.5 (100)	<0.12	<0.22*
Clorgyline/FLC	Ca MML609	64	3.15 (20)	0.05	>20	1.25 (25)	<0.06	<0.11*
Clorgyline/FLC	Ca MML611	64	16 (32)	0.25	>20	2 (64)	<0.1	<0.35*

^a Cells for MIC determinations were incubated in CSM medium (pH 7.0) at 30°C for 24 h; all determinations were obtained by checkerboard titrations (Fig. 3).

^b Combined MIC measured with the other compound (either clorgyline or azole) at the concentration ($\mu\text{g ml}^{-1}$) indicated in parentheses.

^c Fractional inhibitory concentration.

^d Fractional inhibitory concentration index. * denotes synergistic combination (FICI ≤ 0.5) (35).

^e Sc, *S. cerevisiae*; Ca, *C. albicans*.

FIG 3 Representative checkerboard susceptibility assays showing chemosensitization to FLC by clorgyline of *S. cerevisiae* strains AD/CaCDR1 and AD/CaMDR1 and *C. albicans* azole-resistant clinical isolate MML611. Yeast cells were grown in CSM medium pH 7.0 containing the indicated concentrations of FLC and clorgyline at 30°C for 24 h. Growth was measured spectrophotometrically as described in Materials and Methods, and values are given as a percentage of growth in control wells without added compounds.

detected in the screen, excepting clorgyline, had been shown previously to interact with efflux pumps and/or show antifungal activity validated our assay and this approach to screening. Compounds known to inhibit human ABC transporters were not studied further; neither were compounds active only against the CaCdr2p-expressing strain.

Two of the hit compounds, clorgyline and ebselen, were active against both the strain expressing CaCdr1p and the strain expressing Cdr2p (Table 2). Clorgyline is a monoamine oxidase A (MAO-A)-selective inhibitor, which is no longer in use as an antidepres-

sant because of dietary interactions, i.e., the “cheese effect” (4). Ebselen, a seleno-organic compound showing glutathione peroxidase-like activity, has previously reported antifungal activity (2, 3) and is believed to act via inhibition of the fungal plasma membrane ATPase (3). However, only clorgyline inhibited R6G efflux from both strains by more than 93% at a concentration of 5 μ M, yet did not show antifungal activity at concentrations that inhibited efflux (Fig. 1 and Table 3). Clorgyline has not previously been reported as a pump inhibitor, and our further analysis showed that it has potential as a lead for an azole resistance reversal agent.

FIG 4 Effect of clorgyline on the growth of *C. albicans* or *C. glabrata* clinical isolates with decreased FLC susceptibilities in the absence or presence of FLC. (A) Inhibition of *C. albicans* MML605, MML609, or MML611 or *C. glabrata* MML99 growth in the absence (top row) or presence (bottom row) of FLC at the relevant concentrations ($0.25 \times \text{MIC}$ values) as demonstrated by agarose diffusion chemosensitization assays using CSM medium (pH 7.0). Clorgyline (100 μ g) was added to each disk. FLC MIC values are reported as μ g ml⁻¹. (B) Growth of *C. albicans* isolates MML604 and MML610 in the absence or presence of FLC, without or with added clorgyline, at the indicated concentrations ($0.25 \times \text{MIC}$ values for FLC; 15 μ g ml⁻¹ for clorgyline) as demonstrated by agarose dilution assays.

In contrast, the activity of ebselen in the HTS was probably a result of antifungal activity at the concentration (5 μM) used, as the reported IC_{50} for ebselen against *S. cerevisiae* is 4.6 μM (3) and our experiments found it to have an MIC of <10 μM against AD/CaCDRI.

The activity of clorgyline in the primary HTS was confirmed in secondary assays by demonstrating inhibition of efflux of the fluorescent substrate R6G from CaCdr1p- and CaCdr2p-expressing *S. cerevisiae* strains (Table 2; Fig. 1), and reversal of the resistance of these pump-expressing strains to FLC and other azoles (Fig. 2). Clorgyline was also active against other fungal efflux pumps. It reversed the FLC resistance of *S. cerevisiae* strains expressing other individual fungal ABC transporters as well as showing activity against the *C. albicans* MFS efflux pump Mdr1p (Fig. 2).

Pump inhibitory activity was demonstrated at concentrations that had a minimal effect on yeast cell viability, in contrast to the effects of ebselen. The clorgyline concentration required for chemosensitizing activity ($\geq 5 \mu\text{M}$) was ~ 10 fold greater than its reported activity (IC_{50} 0.54 μM) against MAO-A (12). However, concentrations of up to 50 μM have been demonstrated to be nontoxic to cultured human cells (11), and clorgyline is under investigation as a lead compound for treatment of heart failure (17) and also for prostate cancer (11). Another key finding was that clorgyline is not a substrate of fungal efflux pumps; therefore, the inhibitory activity observed is not a result of competition with the substrates (R6G or azoles) used in the secondary assays. Importantly, clorgyline chemosensitized clinical isolates of both *C. albicans* and *C. glabrata* to FLC. The PCL was also screened recently in an HTS using a growth inhibition-based assay (45). Six compounds were identified that were active in potentiating FLC antifungal activity against four fungal species, including the FLC-sensitive *C. albicans* strain CAF2-1, although the screen did not use azole-resistant isolates. Interestingly, although clorgyline was found to sensitize two cryptococcal strains to FLC, it was not identified as a hit for *C. albicans*. This was probably because the screen employed a FLC-sensitive *C. albicans* strain, with growth inhibition as an endpoint, in contrast to our use of FLC-resistant pump expressing *S. cerevisiae* strains to detect specific inhibition of efflux.

Our data cannot distinguish between a direct or indirect effect of clorgyline on yeast efflux pumps, although, as shown by the HTS data, it acts rapidly on R6G efflux. As it has a broad-spectrum effect on both ABC and MDR transporters, which have different mechanisms of pumping, it is more likely to have an indirect effect, possibly by affecting the plasma membrane environment. The human target of clorgyline is monoamine oxidase A, a member of the flavin monooxidase family (48). Yeast species also possess members of this family of enzymes, and the *C. albicans* homolog is the *CBP1* gene, encoding a corticosteroid binding protein (27) which has $\sim 30\%$ similarity to human monoamine oxidase. We speculate that clorgyline may act on this sterol binding protein and affect plasma membrane proteins such as efflux pumps.

In conclusion, we have identified that clorgyline, a drug previously used as an antidepressant, enhanced the antifungal effect of FLC against azole-resistant yeast species, including strains of *C. albicans* and *C. glabrata*, via inhibition of ABC and MFS fungal efflux pumps.

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