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Amino Acid-Derived 1,2-Benzisothiazolinone Derivatives as Novel Small-Molecule Antifungal Inhibitors: Identification of Potential Genetic Targets

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We have identified four synthetic compounds (DFD-VI-15, BD-I-186, DFD-V-49, and DFD-V-66) from an amino acid-derived 1,2-benzisothiazolinone (BZT) scaffold that have reasonable MIC_{50} values against a panel of fungal pathogens. These compounds have no structural similarity to existing antifungal drugs. Three of the four compounds have fungicidal activity against *Candida* spp., *Cryptococcus neoformans*, and several dermatophytes, while one is fungicidal to *Aspergillus fumigatus*. The kill rates of our compounds are equal to those in clinical usage. The BZT compounds remain active against azole-, polyene-, and micafungin-resistant strains of *Candida* spp. A genetics-based approach, along with phenotype analysis, was used to begin mode of action (MOA) studies of one of these compounds, DFD-VI-15. The genetics-based screen utilized a homozygous deletion collection of approximately 4,700 *Saccharomyces cerevisiae* mutants. We identified mutants that are both hypersensitive and resistant. Using FunSpec, the hypersensitive mutants and a resistant *ace2* mutant clustered within a category of genes related directly or indirectly to mitochondrial functions. In *Candida albicans*, the functions of the Ace2p transcription factor include the regulation of glycolysis. Our model is that DFD-VI-15 targets a respiratory pathway that limits energy production. Supporting this hypothesis are phenotypic data indicating that DFD-VI-15 causes increased cell-reactive oxidants (ROS) and a decrease in mitochondrial membrane potential. Also, the same compound has activity when cells are grown in a medium containing glycerol (mitochondrial substrate) but is much less active when cells are grown anaerobically.

Candida species rank fourth among the causes of bacterial/fungal nosocomial infectious diseases, and *Candida* vaginitis and oropharyngeal (OPC) diseases occur at high frequencies in humans (20, 48, 53). As for other fungal infections, *Aspergillus fumigatus* is one of the most common causes of mold infections of humans (48). For these and other fungal infections, the cost-burden analysis is estimated to be ~$2.6 billion per year (21, 36, 37, 44, 60). Fungal infections are also of global importance. Oropharyngeal candidiasis (OPC) in the HIV/AIDS patient is a heavy health burden in countries of Africa as well as in India and China (7, 11, 12, 29, 43, 62). Cryptococcal meningitis in HIV/AIDS populations exceeds that of other kinds of bacterial meningitis, including tuberculosis, and is among the most reported infections in HIV/AIDS patients in developing countries (42, 43, 58). The incidence of these diseases is a compelling reason to continue efforts into developing new preventative therapies.

Antifungal drugs that are currently used in patient treatment of candidiasis include the polyene amphotericin B (AmpB), azoles, and the echinocandins (45). These compounds bind to membrane ergosterol (AmpB) and perturb membrane functions, inhibit ergosterol synthesis (azoles), or inhibit cell wall β-1,3-glucan synthesis (echinocandins). The use of azoles and especially triazoles is not without problems, as resistance to fluconazole has resulted in an increase in several non-*albicans* species of *Candida*, such as *C. parapsilosis*, C. *glabrata*, and *C. krusei*, as well as *A. fumigatus*, as pathogens (23, 32, 34, 47–49, 54, 59).

It is quite clear that progress toward the identification of new classes of antifungals has been slow. Newer trizoles are remodeled existing drugs. This is similar to the situation in antibacterial drug development, such that phrases like "bad bugs, no drugs" now reflect the current scenario where pathogens resistant to nearly all available antibacterial drugs are being encountered in both hospital and community settings (8).

We have identified a series of 4-amino-acid-derived-1,2-benzisothiazolinone (BZT) derivatives that have broad activity against diverse fungal pathogens (18) (see Fig. S1 in the supplemental material). The current data in this paper further define the activity of these compounds and suggest a mode of action (MOA) for one of the active compounds.

MATERIALS AND METHODS

Compound library. A series of four broad-spectrum antifungal agents based on the 1,2-benzisothiazol-3(2H)-one (BZT) scaffold were used in this study. We have previously reported their structures, shown in Fig. S1 in the supplemental material (18).

Strains. For MIC_{50} screening experiments with each of the four compounds, we used *Candida albicans* (CAF2 and SC5314) as well as *C. glabrata* (RC-201), *Candida lusitaniae* (RC-301), *Candida guilliermondii*

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TABLE 1 MIC_{50}s of the BZT compounds against human pathogenic fungi[a]

Strain	MIC_{50} (μg/ml)						
	DFD-VI-15	BD-I-186	DFD-V-49	DFD-V-66	Fluconazole	Micafungin	AmpB
C. albicans CAF2	**1.6**	**1.6**	**1.6**	**1.6**	0.2	**0.016**	**0.25**
C. glabrata RC-201	**1.6**	**0.8**	**3.2**	**1.6**	3.2	**0.016**	**0.25**
C. tropicalis RC-501	**1.6**	**1.6**	**3.2**	**3.2**	16	**0.016**	**0.5**
C. parapsilosis RC-601	**1.6**	**0.8**	**3.2**	**3.2**	0.4	0.5	**0.25**
C. lusitaniae RC-301	**1.6**	**0.8**	**1.6**	**3.2**	3.2	**0.03**	**0.25**
C. guilliermondii RC-401	**1.6**	**1.6**	**3.2**	**3.2**	6.4	0.5	**0.25**
C. apicola RC-701	**1.6**	**3.2**	**3.2**	**3.2**	0.2	**0.016**	**0.25**
A. fumigatus AF-294	12.5	6.4	6.4	**3.2**	16	1.0	**0.5**
C. neoformans H99	**1.6**	**1.6**	**6.4**	**3.2**	>64	**>2**	**>10**
C. neoformans JEC-21	**1.6**	**0.8**	**6.4**	**1.6**	>64	**>2**	**>10**

[a] MIC_{50}s (μg/ml) of the BZT compounds are shown against a panel of human pathogenic fungi. The antifungal activities of the compounds were also compared to those of fluconazole, micafungin, and amphotericin B (AmpB) against the same strains. Results for fungicidal compounds are indicated in bold numbers. (Fungicidal, MFC/MIC_{50} ≤4; fungistatic, MFC/MIC_{50} >4.) Data are shown as averages of results from five experiments.

(RC-401), *Candida tropicalis* (RC-501), *C. parapsilosis* (RC-601), and *Candida apicola* (RC-701), *A. fumigatus* (AF-294), *Cryptococcus neoformans* strains (H-99 and JEC-21), *Trichophyton rubrum* (22403), *Trichophyton mentagrophytes* (22402), *Epidermophyton floccosum* (1252), and *Microsporum canis* (22349). *S. cerevisiae* BY4743 was used in phenotype profiling against one of the four compounds (DFD-VI-15). The RC series of strains was obtained from the Microbiology and Immunology Laboratory of the Georgetown University-MedStar Hospital, Washington, DC. MIC susceptibility assays also included strains that were resistant to fluconazole, micafungin, or amphotericin B (see below).

MIC determinations (broth microdilution method) and growth curves. The MIC_{50} of all strains was determined by following the guidelines in CLSI standards M27-A3 (14) and M38-A2 (15). A 50% growth inhibition was used as an endpoint. A total of 1,000 cells in RPMI were added to each well of 96-well microtiter plates to a final volume of 200 μl of RPMI. All assays were read after 24 h for *Candida* spp., 48 h for *A. fumigatus*, 72 h for *C. neoformans*, and 5 to 7 days for dermatophyte cultures. The dermatophytes were initially grown on Sabouraud agar and then screened according to the method described above. Growth curves were plotted by measuring the optical density at 595 nm (OD_{595}) of the test plate wells every 30 min for 48 h in treated and untreated cultures.

MFC determinations. The *in vitro* minimum fungicidal concentrations (MFC) were determined by subculturing 10 μl of a 10^3 cell suspension with or without compounds from each microtiter plate well on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose). However, to rule out low-cell-density effects that could influence MFC determinations, we also used the entire inoculum of 1,000 cells with compounds. Concentrations in wells that showed complete inhibition in the MIC assays as well as the last well that indicated growth were determined. Cultures were incubated at 30°C for 48 h. The MFC was defined as the lowest drug concentration that resulted either in no growth or in fewer than five colonies. If the ratio of the MFC_{50} to the MIC_{50} concentration was ≤4, the compound was designated fungicidal, and if higher, fungistatic (24).

Time-kill assays. *C. albicans* CAF2 cells from log growth phase were used at a concentration of 10^4 cells/ml in 10 ml of RPMI with and without compounds. A total of 100 μl of cell samples was taken after mixing every hour for 14 h and plated on YPD agar for 24 to 48 h at 30°C. The viable cell concentration was calculated from the number of CFU after 48 h (10). The maximum kill rate was calculated at the 6- to 8-h time period point which represents the average time-kill rate.

Toxicity assays. Cell viability was measured by neutral red and MTT (dimethyl diphenyl tetrazolium) assays in tissue culture with each of the four BZT compounds at defined concentrations in the human hepatoma cell line HepG2, as described by Mosmann et al. (40) and Repetto et al. (50), respectively. The 50% cell cytotoxicity (CC_{50}) concentrations were calculated for each compound after 24, 48, and 72 h of incubation. Data are indicated as CC_{50} (μg/ml per compound). CC_{50}/MIC_{50} ratios were calculated to determine fold changes in toxicity.

Synergy experiments. *In vitro* synergy interactions between the BZT compounds and current antifungals such as micafungin, fluconazole, and amphotericin B were determined. *C. albicans* CAF2 was screened using a checkerboard assay method described by others (4, 26, 55). The fractional inhibitory concentration index (FICI) value of each pair of compounds was calculated and classified as follows: antagonism, >2; additive, 0.5 to 2; and synergy, <0.5. Interactions between the four compounds and known antifungals were determined.

***S. cerevisiae* screens for hypersensitive mutants with DFD-VI-15.** We used the YSC Homozygous Diploid library (YSC1056; Thermo Scientific) in susceptibility assays against DFD-VI-15. The library contains ~4,700 null mutants in nonessential genes in 96-well microtiter plates. Individual deletion strains (5 μl) were transferred from frozen stocks to 200 μl of YPD (10 g yeast extract, 20 g peptone, and 20 g glucose per liter) for overnight growth. Then cultures were transferred to fresh YPD in 96-well plates for dilutions to achieve a 1:40 dilution of cells. Following this, each mutant was printed (pin replicator) onto YPD agar plates (150-mm diameter) with or without compound (6 μg/ml) (see Fig. S2 in the supplemental material). We chose a concentration that yielded approximately 1 to 5% mutants per 96-well plate with a hypersensitive phenotype, according to previously published protocol and preliminary data (5). The growth of each deletion mutant was determined at 48 h and 72 h and compared to that of the same

TABLE 2 MIC_{50}s of the BZT compounds against species of dermatophytic fungi[a]

Strain	MIC_{50} (μg/ml)				
	DFD-VI-15	BD-I-186	DFD-V-66	DFD-V-49	Ketoconazole
Trichophyton rubrum (22403)	**3.2**	3.2	**3.2**	**3.2**	0.5
Trichophyton mentagrophytes (22402)	**3.2**	**3.2**	**3.2**	**3.2**	0.25
Epidermophyton floccosum (1252)	**3.2**	**0.8**	3.2	**12**	0.13
Microsporum canis (22349)	**0.8**	0.8	3.2	**3.2**	**0.5**

[a] The activities of all compounds were compared to that of ketoconazole. Results for fungicidal compounds are shown in bold numbers as in Table 1. All data are averages of results from three experiments.

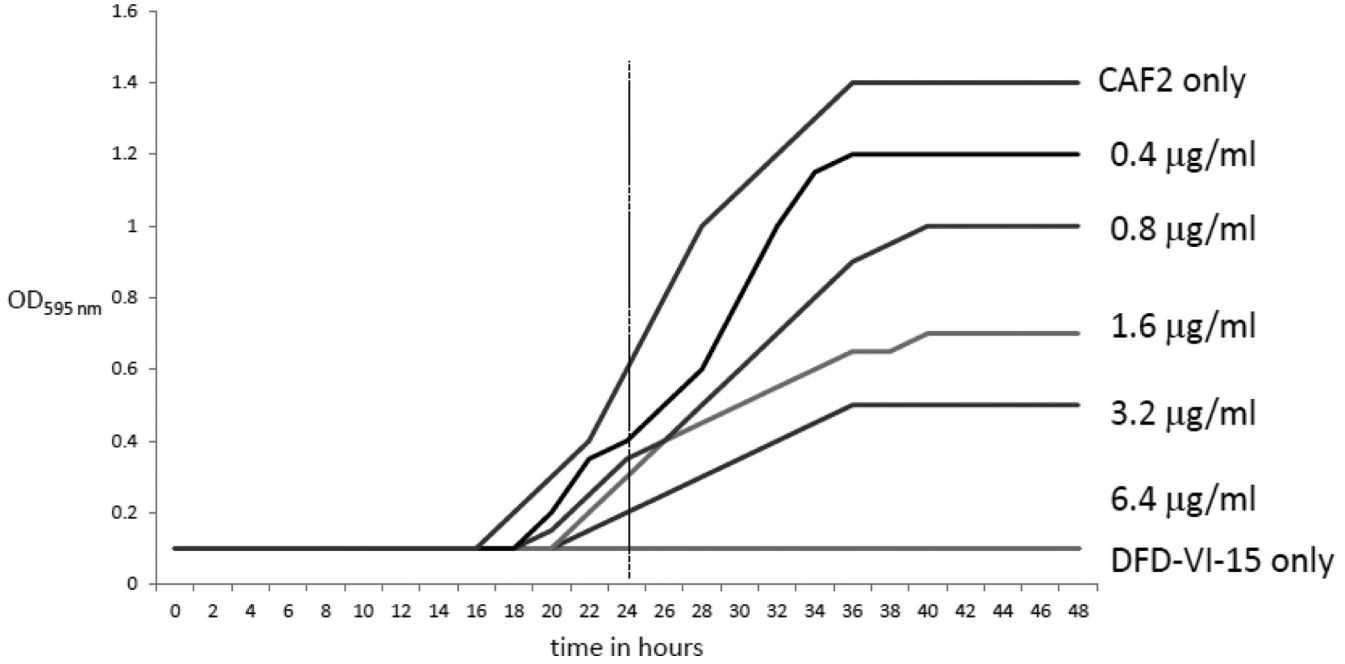

FIG 1 The growth curves of *C. albicans* CAF2 in untreated (CAF2 only) and DFD-VI-15-treated cultures are shown. A concentration-dependent inhibition is observed. The OD_{595} at 24 h for 1.6 μg/ml (MIC_{50}) of DFD-VI-15 is approximately half that of untreated cells. Complete inhibition of growth was seen at 6.4 μg/ml (~4× MIC_{50}). Similar profiles were observed for the other BZT compounds.

mutant grown in the absence of compound. Mutants that displayed hypersensitivity after 48 h and confirmed after 72 h were considered hypersensitive. These experiments were done three times with the entire yeast library. Hypersensitive mutants were identified, and a secondary screen was done with the hypersensitive mutants using the MIC microtiter dilution method but in YPD broth, since *S. cerevisiae* BY4743 grows less in RPMI broth. This screen was used to rule out false positives that may have occurred during the primary screens of all mutants. As all mutants are coded, we identified strains and their corresponding deleted genes that resulted in either hypersensitivity or resistance to DFD-VI-15 (see below). Screens included the parental strain *S. cerevisiae* BY4743.

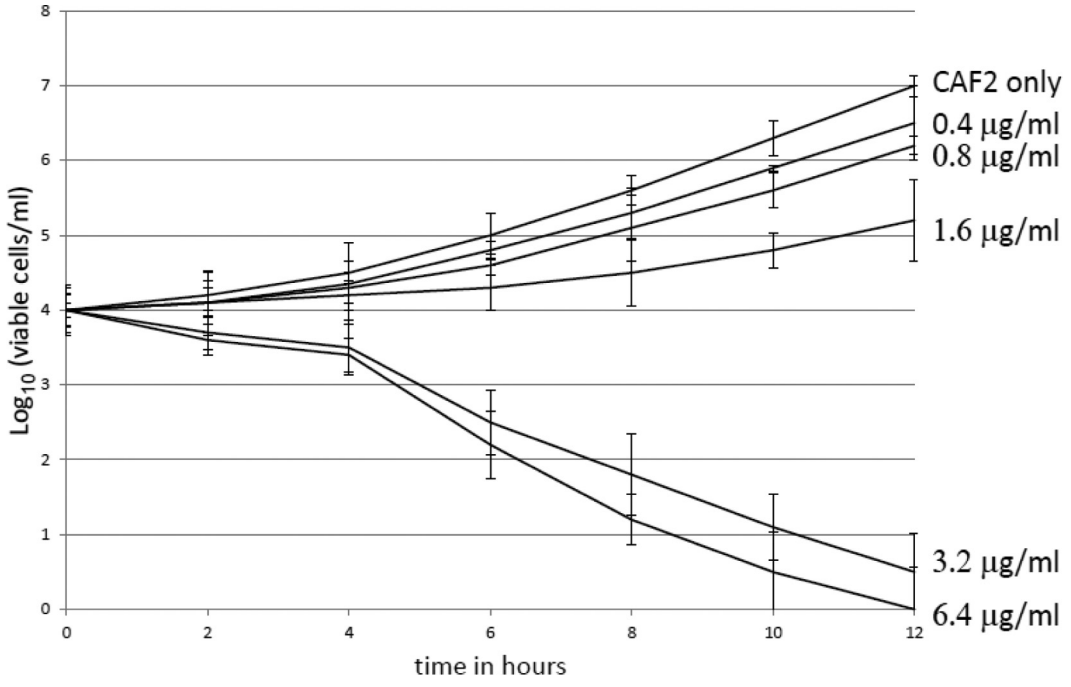

FIG 2 Time-kill experiments with DFD-VI-15. Data are shown as $\log_{10}$ viable cells/ml. Cell counts are shown for different concentrations of DFD-VI-15 against *C. albicans* CAF2. The maximum kill concentration was 3.2 to 6.4 μg/ml after 24 h of incubation with DFD-VI-15.

TABLE 3 Maximum kill rates (KR_{max}) against *C. albicans* CAF2[a]

Compound	KR_{max} (per h)[b]
DFD-VI-15	0.85 ± 0.05
BD-I-186	0.75 ± 0.07
DFD-V-66	0.80 ± 0.03
DFD-V-49	0.67 ± 0.08
Fluconazole	0.25 ± 0.10
Micafungin	0.90 ± 0.04
Amphotericin B	0.80 ± 0.08

[a] The maximum kill rates for DFD-VI-15, BD-I-186, DFD-V-66, DFD-V-49, fluconazole, micafungin, and amphotericin B against *C. albicans* CAF2 were compared. The BZT derivatives have comparable maximum kill rates to amphotericin B and micafungin but those rates are significantly higher than that to fluconazole. Greater kill rates are indicated by higher KR_{max} values.
[b] KR_{max} (per h) data are averages of results from three experiments.

***S. cerevisiae* screens for resistant mutants with DFD-VI-15.** The same methodology for identifying hypersensitive mutants was used for resistant mutants (described above), except that solid agar plates were replaced by 96-well plates with YPD broth containing 15 μg/ml of DFD-VI-15. This concentration was fungicidal for *S. cerevisiae* BY4743. Growth was determined at 24 and 48 h, and the resistant mutants were identified visually as those that grew in the presence of DFD-VI-15. The screen was performed in triplicate with fresh liquid cultures. The MIC_{50} of the resistant mutants was determined in YPD medium. Mutants resistant to 12 to 24 μg/ml were further analyzed phenotypically as described below.

Data analysis. FunSpec (51) was used to identify the functional categories that were represented by the mutants whose absence renders cells hypersensitive or resistant to DFD-VI-15. The corresponding list of the genes whose absence resulted in these phenotypes to DFD-VI-15 were imported into FunSpec, and, based on prior knowledge, data were integrated in the Munich Information Center for Protein Sequence (MIPS) functional categories. Cell functions were searched using the *Saccharomyces* Genome Database (SGD) Gene Ontology (GO) Term Finder. The *P* values in FunSpec represent the probability that the intersection of a given list with any functional category occurs by chance. The Bonferroni correction divides the *P* value threshold that would be deemed significant for an individual test by the number of tests conducted and thus accounts for spurious significance due to multiple testing over the categories of a database. After the Bonferroni correction, the only categories displayed are those for which the chance probability of enrichment is lower than *P*/CD, where CD is the number of categories in the selected database.

Drop plates of CAF2 and BY4743 with DFD-VI-15 under aerobic and anaerobic conditions. The sensitivities of *C. albicans* strain CAF2 and *S. cerevisiae* strain BY4743 to DFD-VI-15 were tested by plating serial dilutions of 5×10^1 to 5×10^6 cells (each in a total of 5 μl) onto YPD agar plates containing 12 (*S. cerevisiae*) or 24 (*C. albicans*) μg/ml of the compound. Yeast cells were obtained from overnight cultures grown in YPD broth at 30°C and standardized by hemocytometer counts. The growth of each strain was evaluated for sensitivity after 48 h of incubation at 35°C. Strains were also evaluated for their growth on YPG (yeast extract-peptone-glycerol) agar containing 1% yeast, 2% peptone, and 2% glycerol and in anaerobic conditions (using Anaerobic Packs) with and without DFD-VI-15.

Flow cytometry assays. (i) Intracellular ROS. Reactive oxidant species (ROS) production was determined by staining cells with the fluorescent dye DCFDA (2′-7′-dichlorofluorescein diacetate; Sigma) using a FACScan flow cytometer (488 nm; Becton, Dickinson) (3, 33). *C. albicans* SC5314 cells were grown at 30°C overnight in YPD medium. After 16 h, the pellets were collected and suspended to 1×10^6 cells in 15 ml of YPD and separated into three tubes. Two of the tubes were treated with 12 or 24 μg/ml of DFD-VI-15 for 4 h, since more cells were needed for these assays. The third tube was used as the untreated control. After 4 h, cells were collected from each condition and washed twice with phosphate-buffered saline (PBS). The pellets were suspended to 1×10^6 cells in 1 ml of PBS and treated with or without 25 μM DCFDA for 30 min at 30°C in the dark. Cell fluorescence in the absence of DCFDA was used to verify that background fluorescence was similar for treated and untreated strains. Cells that were treated with DCFDA were then collected and washed twice with PBS. Then propidium iodide (PI) was added to each sample to exclude dead cells prior to the DCDFA assays. The mean fluorescence for ROS was quantified only in live and similar-sized cells.

(ii) Mitochondrial membrane potential. Mitochondrial membrane potential was detected by staining cells with JC-1 (Cayman Chemical Company), a cationic dye used to assess variation in mitochondrial potential, using a FACScan flow cytometer (488 nm; Becton, Dickinson). JC1 monomers display a green cytoplasmic fluorescence at 525 nm, when cells are excited at 490 nm. In cells with physiological mitochondrial membrane potential, JC-1 forms complexes known as JC1 aggregates with intense red fluorescence. In cells with dysfunctional mitochondrial membrane potential, JC-1 remains in a monomeric form and displays a green fluorescence. *C. albicans* SC5314 cells that had been grown overnight at 30°C were treated with 12 and 24 μg/ml of DFD-VI-15 for 4 h. After washing, cells were treated with JC-1 according to the manufacturer's recommendations (33). JC-1 fluorescence was quantified only in live and standard-sized cells by adding 7-amino-actinomycin D (7-AAD) to exclude dead cells in assays. JC1 fluorescence at 488 nm was collected in the

TABLE 4 Activity (MIC_{50}) of BZT compounds against azole-resistant strains of *C. albicans*[a]

Strain	Mechanism of resistance[b]	MIC_{50} of BZT compounds	MIC_{50} of fluconazole	Source or reference[c]
CAF2/SC5314	Sensitive	1.6	0.2	This paper
95-68	CDR overexpression	1.6–3.2	>64	35, 59
FH5	CDR overexpression	1.6	64	35, 59
96-25	MDR + *ERG11* overexpression	1.6	32	35, 59
C17 (12–99)	CDR + MDR + *ERG11* overexpression	1.6–3.2	>64	35, 59
DSY1764	*Erg11/erg11erg3/erg3*	1.6–3.2	>128	16, 17
DSY296	*TAC1-5/TAC1-5 ERG11-5/ERG11-5*	1.6–3.2	>64	16, 17
B4	Mrr1p mutation (G878E/G878E)	1.6–3.2	12.5	39
CAAL-61	AA G307S, Y447H	1.6–3.2	>64	38
CAAL-74	AA Y132F, G448V	1.6–3.2	>64	38
CAAL-75	AA Y132H, K143R	1.6–3.2	>64	38

[a] Strain numbers, mechanisms of resistance, and MIC_{50} values are shown. The MIC_{50} values reflect the range of concentrations for all BZT compounds against all strains.
[b] AA, amino acid point mutants of *erg11*.
[c] References describe the source and/or resistance mechanisms.

TABLE 5 Activity (MIC_{50}) of BZT compounds against micafungin- and amphotericin-resistant strains of *C. albicans* and *C. glabrata*[a]

Strain[b]	Mechanism of resistance[c]	MIC_{50} of BZT compounds (μg/ml)	MIC_{50} of micafungin/AmB (μg/ml)	Source or reference[d]
C. albicans				
ATCC 90028		1.6	0.016/0.25	This paper
DSP11	AA (FKS1) S645F	3.2	2	22, 46
DSP12	AA (FKS1) S645Y	3.2	2	22, 46
DSP14	AA (FKS1) F641S	3.2	>2	22, 46
DSP15	AA (FKS1) S645P	3.2	2	22, 46
C. glabrata[e]				
RC-201		0.8–3.2	0.016/0.25	This paper
DSP16	AA (FKS2) S663P	1.6–3.2	1	22, 46
DSP17	AA (FKS1) S645P	1.6–3.2	>2	22, 46
DSP18	AA (FKS1) F659V	0.8–3.2	>2	22, 46
DSP19	AA (FKS2) S663P	1.6–3.2	>2	22, 46
DSP20	AA (FKS1) D666E	1.6–3.2	1	22, 46

[a] Strain numbers, mechanisms of resistance, and MIC_{50} values are shown. The MIC_{50} values reflect the range of MIC_{50} values for all BZT compounds against all strains.
[b] DSP 11–15, *C. albicans* strains. DSP 16–20 and strain 21230, *C. glabrata* strains.
[c] AA, point mutants of *FKS1* or *FKS2*.
[d] References that describe the source and/or resistance mechanisms are included.
[e] *C. glabrata* 21230 was also resistant to amphotericin B (56 and data not shown).

TABLE 6 Results of checkerboard experiments[a]

Compound	Interaction type (FICI)		
	Fluconazole	Micafungin	Amphotericin B
DFD-VI-15	Additive (1.0)	Additive (0.5)	Additive (0.6)
BD-I-186	Additive (1.5)	Synergy (0.3)	Additive (1.3)
DFD-V-66	Additive (1.2)	Additive (0.8)	Additive (1.0)
DFD-V-49	Additive (1.0)	Additive (1.0)	Synergy (0.25)

[a] Checkerboard experiments identify synergy. The *in vitro* interactions of the active compounds with fluconazole, micafungin, and amphotericin B were evaluated against those for *C. albicans* CAF2. The FICI (fractional inhibitory concentration) values are calculated, and the interactions are grouped. BD-I-186 has a synergistic interaction with micafungin, and DFD-V-49 has a synergistic interaction with amphotericin B. All other interactions were additive. No antagonistic interactions were observed. See text for definitions.

FL1 channel (green fluorescence) and in the FL2 channel (orange fluorescence).

RESULTS

MIC determinations using the broth microdilution method. In the course of screening for antifungal compounds in accordance with the guidelines of the CLSI standards M27-A3 and M38-A2 (broth microdilution method), four synthetic compounds belonging to the benzisothiazolinone (BZT) scaffold were discovered to have potent antifungal activity (see Fig. S1 in the supplemental material). These compounds have no structural similarity to known antifungal compounds. The compounds (DFD-VI-15, BD-I-186, DFD-V-49, and DFD-V-66) show antifungal activity against *Candida* spp., *A. fumigatus*, *C. neoformans*, and four dermatophyte species at MIC_{50} concentrations in the range of 0.8 to 12.5 μg/ml (Tables 1 and 2). Of the four BZT compounds, DFD-VI-15 and BD-I-186 have relatively better activity against all test organisms (0.8 to 3.2 μg/ml) except *A. fumigatus*. *Candida* spp., such as *C. glabrata* and *C. parapsilosis*, that often are resistant to triazoles such as fluconazole are susceptible to the BZT compounds. For all species tested except *A. fumigatus*, the compounds are fungicidal, while only DFD-V-66 displayed fungicidal activity against *A. fumigatus* (Table 1). Fungicidal activity is defined as an MFC_{50} ≤4-fold the MIC_{50} (24). Likewise, the BZT compounds are more often fungicidal against four dermatophytic fungi (Table 2).

Growth curves and time-kill experiments. Growth of *C. albicans* CAF2 was measured for 48 h in the presence or absence of DFD-VI-15 at concentrations ranging from 0 to 6.4 μg/ml (Fig. 1). Growth was inhibited in a concentration-dependent manner for DFD-VI-15 and the other BZT compounds to a similar extent (data not shown for other BZT compounds). To measure viability in the presence of compounds, time-kill experiments were done. The time-kill rate is defined as the reduction in colony count (log_{10}) divided by exposure time compared to the growth of untreated cultures (Fig. 2). Viable cell counts were determined at 2-h intervals, and the 6- to 8-h time point was used to calculate kill rates of *C. albicans* CAF2 at different concentrations of DFD-VI-15. Fungicidal activity of DFD-VI-15 was noted initially at 2 to 4 h with 3.2 to 6.4 μg/ml and then increased to a maximum kill at 12 h at both concentrations. We estimate the fungicidal concentration to be 3.2 to 6.4 μg/ml. While only DFD-VI-15 data are shown, the other BZT compounds had similar killing profiles (data not shown).

The maximum kill rates of DFD-VI-15, BD-I-186, DFD-V-66, and DFD-V-49 were compared to those of other antifungal drugs and found to be similar to those of amphotericin B and micafungin while greater than that of fluconazole (Table 3).

Activity against drug-resistant strains. Although the BZT compounds were active against a panel of fungal pathogens described above, we next screened the BZT compounds against a variety of clinical or laboratory-constructed antifungal drug-resistant strains of *C. albicans* and *C. glabrata* and, when possible, their parental strains. Strains were selected to represent some of the mechanisms that are commonly found in clinically resistant isolates (10, 16, 17, 19, 22, 34, 38, 39, 46, 56, 59) (Tables 4 and 5). The MIC_{50} values of all BZT compounds remained unchanged against *C. albicans* strains resistant to fluconazole. Similar results were found when the compounds were tested against micafungin-resistant isolates of *C. albicans* and *C. glabrata* and a single isolate of *C. glabrata* resistant to amphotericin B (Table 5). We suggest that since these strains were resistant to fluconazole, micafungin, or amphotericin B, their sensitivity may indicate unique targets for the BZT compounds.

Synergy experiments can partially resolve common or unique targets of unknown compounds. To address this issue and the data from Tables 4 and 5, synergy experiments using the standard checkerboard method were done. The BZT compounds were tested in combination with fluconazole, micafungin, or amphotericin B against *C. albicans* CAF2. Fractional inhibitory concentration index values (FICI) are indicated in Table 6. Two of the four compounds, BD-I-186 and DFD-V-49, synergized with micafungin (β-1,3-glucan synthesis inhibitor) and amphotericin B (membrane ergosterol), respectively. Synergy may indicate that each compound of a pair of compounds inhibits a different target to increase the activity of one compound.

Toxicity studies. We compared each of the four BZT com-

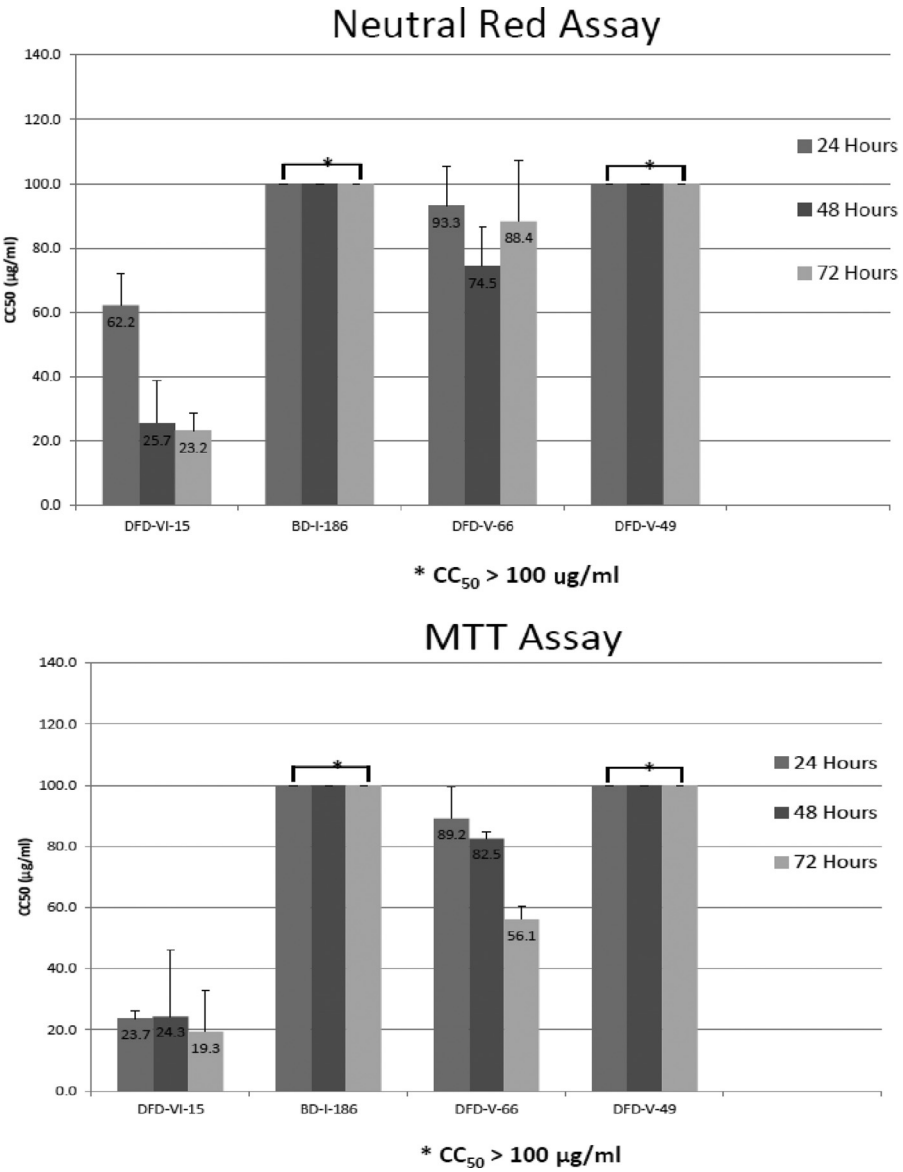

FIG 3 Cell cytotoxicity of DFD-VI-15, BD-I-186, DFD-V-66, and DFD-V-49 against the human HepG2 cell line. Viability assays were used to determine the CC_{50} concentration with neutral red (upper) and MTT (lower) stains. Assays were performed at 24, 48, and 72 h. The CC_{50}s for BD-I-186 and DFD-V-49 are 100 μg/ml, or about 50- to 60-fold higher than the MIC_{50}, for each compound. For DFD-VI-15 and DFD-V-66, the CC_{50}s were 10- to 20-fold and 30- to 50-fold greater, respectively.

pounds for toxicity *in vitro* against the HepG2 human hepatocarcinoma cell line (Fig. 3). The neutral red and MTT assays measure the viability of cells by incorporation of dye in lysosomes (neutral red) or mitochondria (MTT), respectively. Dose-response curves were done for each compound at 24, 48, and 72 h, and the concentration that resulted in 50% cell growth inhibition was determined. Of the four BZT compounds, DFD-VI-15 displayed the most significant toxicity at ~4- to 10-fold higher than the MIC_{50} for that compound, while BD-I-186 and DFD-V-49 had CC_{50} values of 100 μg/ml, or about 60-fold of the MIC_{50} of each compound (CC_{50}/MIC_{50} ratios). Less toxicity was seen with DFD-V-66 than with DFD-VI-15.

Identification of *S. cerevisiae* mutants sensitive to DFD-VI-15. DFD-VI-15-sensitive strains were identified by screening the BY4743 *S. cerevisiae* parental strain (sensitive to DFD-VI-15; MIC_{50}, 6 μg/ml) and a set of 4,700 nonessential yeast gene null mutants with 6 μg/ml of compound. In these assay conditions, hypersensitive mutants on agar plate assays appear at 1 to 5%. Two representative agar plate assays of ~96 mutants are shown in Fig. S2 in the supplemental material. Also, these concentrations were chosen for the screen since preliminary studies indicated that about 1 to 5% of mutants were hypersensitive, an amount reported by others (5). The entire library screen was done three times on YPD agar with and without compound. Strains were labeled sensitive to DFD-VI-15 if growth inhibition occurred from three mutant library screens. This analysis identified 96 strains whose gene deletion caused sensitivity to DFD-VI-15. Microtiter plate dilution assays were used to verify the hypersensitiv-

TABLE 7 Functions of selected genes deleted in the *S. cerevisae* BY4743 DFD-VI-15 hypersensitive mutants[a]

Biological process	Gene(s)
Mitochondrial morphology	*ERG6*, *ERG4*, *ERG24*, *ERG3*, *SAC1*
Mitochondrial DNA replication	*ABF2*
Mitochondrial ribosomal protein	*PET123*, *MRP51*
Mitochondrial inner membrane	*YME1*
Mitochondrial protein maturation	*ISA2*
Mitochondrial glutathione reductase	*GLR1*
Amino acid biosynthesis	*TRP1*, *ARO2*, *ILV1*, *TRP5*, *THR1*
Transcription factor, iron utilization	*AFT1*
Transcription factor, stress response	*RPN4*
Nitrogen catabolite repression	*URE2*
Zinc-regulated transcription factor	*ZAP1*
Meiotic gene regulation	*UME6*
Mitotic cell cycle spindle orientation	*LTE1*
Protein sorting, ribosome stalk	*VPS34*, *RPP1A*, *SEC66*
Chromatin remodeling	*SNF2*
tRNA synthesis	*ELP6*

[a] Functional annotation of *C. albicans* CAF2 orthologs. For *C. albicans*, *ZAP1*, *UME6*, and *VPS34* are required for hyphal development; *ZAP1* is also a regulator of biofilm formation.

ity of mutants selected on agar plate assays. Of the 96 hypersensitive mutants representing 85 annotated genes, 31 mutants were verified to have MIC_{50} values less than that of *S. cerevisiae* BY4743, <6 μg/ml. Eleven of these mutants, as defined by GO annotation, fall into a cluster represented by direct or indirect loss of mitochondrial functions ($P < 0.05$) (Table 7). The second largest category of mutants was deleted in genes encoding amino acid synthesis functions.

Identification of *S. cerevisiae* mutants resistant to DFD-VI-15. DFD-VI-15-resistant strains were identified by screening the same mutant collection with 15 μg/ml of compound, followed by a second screen in YPD broth containing 6 to 48 μg/ml. Two replicates of the entire library screen were done in 96-well microtiter plates as stated above. Three mutants showed resistance at 48 h (data not shown). The three mutants had deletions of gene *CNE1*, *HLJ1* (both 12 μg/ml), or *ACE2* (24 μg/ml). Hlj1p is a cochaperone for Hsp40p and promotes endoplasmic reticulum (ER)-associated degradation of integral membrane proteins. Cne1p is an integral ER membrane protein that is part of the quality control of glycoprotein folding (FunSpec). The Ace2p transcription factor is required for many activities in *S. cerevisiae*, *C. glabrata*, and *C. albicans*, including polarized growth, virulence, and morphogenesis and, interestingly, is a positive regulator of glycolysis (6, 30, 31, 41). We suggest that the hypersensitive and resistant mutants may reflect respiratory dysfunction in treated cells; to demonstrate this, the following experiments were done.

Drop plate experiments. The hypersensitive and resistant mutants of *S. cerevisiae* suggest that DFD-VI-15 may have activity that results in detrimental energy loss. To determine if this is correct, three types of phenotypic profiling analyses were pursued in strains treated with this compound. On YPD (glucose; glycolytic respiration) as well as YPG (glycerol; mitochondrial respiration) agar, cell growth was measured using drop plate assays in the presence or absence of compound. We found that parental strains CAF2 (*C. albicans*) (Fig. 4, upper panel) and BY4743 (*S. cerevisiae*) (Fig. 4, lower panel) were inhibited by DFD-VI-15 in aerobic cultures containing glucose (YPD) but also on glycerol (YPG), a mitochondrial substrate. Under anaerobic conditions on YPD agar, compound inhibition was not observed with *S. cerevisiae* BY4743, while *C. albicans* CAF2 was less affected than *S. cerevisiae* BY4743, which was inhibited.

ROS production is increased in CAF2 cells treated with DFD-VI-15. Mitochondrial dysfunction is associated with an increase in cell ROS (33). To assess the levels of ROS in untreated and treated cells, we used an inoculum of 10^6 cells/ml, compared to the amount in MIC assays (10^3 cells/well). Moreover, preliminary experiments were performed to check the cell viability during the assay with various concentrations of DCFDA. The flow cytometry analysis is performed only on viable cells, with propidium iodide detecting dead cells. Quantitative flow cytometry experiments were done to measure the total amount of intracellular ROS following treatment with DFD-VI-15. The DCFDA stain diffuses into the cell, is hydrolyzed into 2′,7′-dichlorofluorescein (DCFH) by esterases, and accumulates in viable cells. Treated cells had significantly higher levels of ROS than untreated cells (Table 8). GOA31, a *C. albicans* mutant strain with defects in mitochondrial function and higher ROS levels, was used as a positive-control strain to measure ROS levels and mitochondrial potential (below) as we have demonstrated previously (3, 33).

DFD-VI-15 inhibits mitochondrial membrane potential. We demonstrate that mitochondrial membrane potential decreases in *C. albicans* in the presence of DFD-VI-15 in a dose-dependent manner (Fig. 5). We were unable to measure a membrane potential in untreated or treated cultures of *S. cerevisiae* (data not shown). These data indicate that DFD-VI-15 is at least partially directed against mitochondrial functions.

DISCUSSION

The novelty of our studies starts first with the structures of active compounds, heretofore not reported (18). Further, from our current data, we have established that compounds with a BZT core structure have antifungal activity against a variety of human pathogen isolates, including dermatophytic fungi (Tables 1 and 2). The compounds are for the most part fungicidal, have kill-time ratios that compare favorably to those of amphotericin B and micafungin, and are inhibitory against a variety of clinical isolates of *Candida* spp. that are resistant to triazoles, micafungin, and amphotericin B.

We realize that the BZT compounds have only modest activity (μg/ml) against our panel of fungal pathogens, but their fungicidal activity compelled us to understand the MOA of one (DFD-VI-15). Initial experiments on MOA employed macromolecular synthesis determinations in treated and untreated cultures. However, inhibition of protein, RNA, or ergosterol synthesis by DFD-VI-15 was not observed after 1 to 3 h of treatment (data not included). Also, while from the same scaffold, unpublished data indicate that BD-I-186 may target cell separation, suggesting a different MOA.

In this paper, we placed considerable importance on chemogenomics to understand the MOA of DFD-VI-15. Chemogenomics is the application of genomics with chemistry (5, 9, 25, 27, 52) in the pursuit of target discovery. As DFD-VI-15 was significantly active against *S. cerevisiae* (MIC_{50} = 6 μg/ml, YPD broth), the

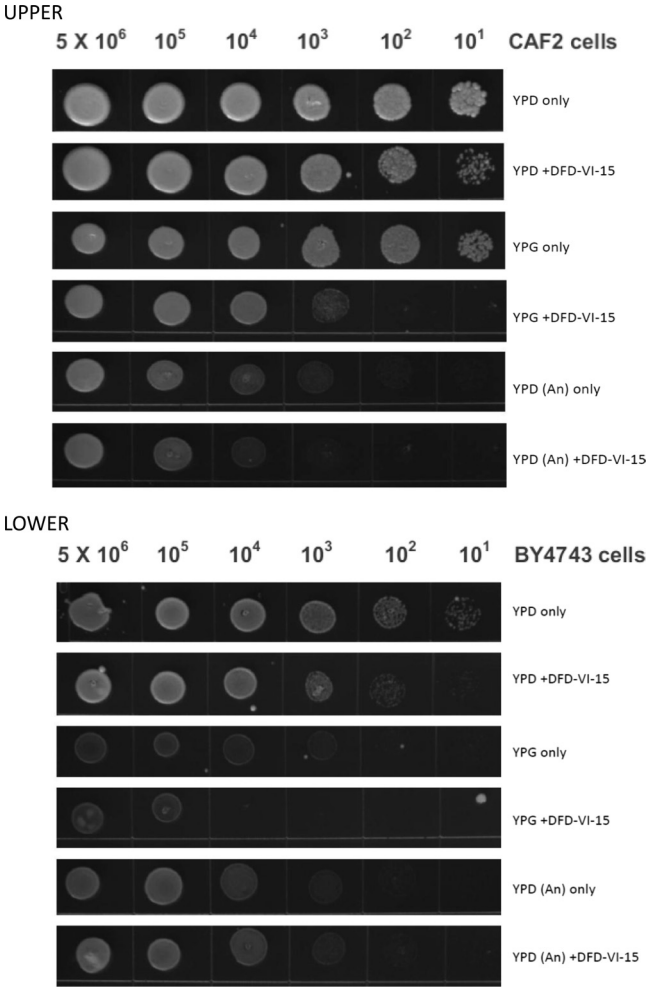

FIG 4 Drop plate assays with DFD-VI-15. Both *C. albicans* CAF2 (upper panel) and *S. cerevisiae* BY4743 (lower panel) were inhibited by the compound at 2× the MIC_{50}. The concentrations of DFD-VI-15 used were 12 and 24 μg/ml for BY4743 and CAF2, respectively. Both strains showed hypersensitivity with the compound on yeast extract-peptone-glucose (YPD) or yeast extract-peptone-glycerol (YPG) medium (aerobic respiration). The antifungal activity of the compound was much less apparent when the strains were grown anaerobically (An).

availability of mutant libraries of yeast provided an attractive approach to MOA studies. We chose initially to use the homozygous null, diploid library of ~4,700 nonessential gene mutants. The prevailing paradigm is that nontarget gene products are believed to buffer or protect (or even compensate for) an inhibited target (52). Mutants lacking these genes have a hypersensitivity phenotype. Oppositely, resistant mutants can represent the target since the lack of a target confers resistance. Susceptibility assays identified hypersensitive and resistant mutants. Cluster analysis using FunSpec and phenotype experiments have helped us construct a hypothetical MOA model.

Our screens yielded 31 mutants that were hypersensitive to DFD-VI-15, and of these mutants, 11 lacked mitochondrial or mitochondrium-related encoding genes. The next largest category of hypersensitive mutants (five) lacked genes involved in amino acid biosynthesis. Other categories include regulators of hyphal growth, protein sorting, iron utilization, chromatin remodeling, and stress adaptation. Annotation data for 27 mutants are shown in Table 7. The alignment of 11 genes associated directly or indirectly with mitochondrial functions follows that of other investigators. For example, Altmann and Westermann (2) utilized a library of 768 *S. cerevisiae* mutants lacking essential genes to identify those genes that were required for normal mitochondrial morphogenesis. Of these, 119 essential genes that clustered in essential cell pathways that included ergosterol biosynthesis, mitochondrial import and assembly, vesicular trafficking and secretion, actin cytoskeleton-dependent transport, and ubiquitin/26S proteasome-dependent protein degradation were described. Our list of genes of hypersensitive mutants includes those functional annotations that are required for normal mitochondrial morphology as described previously (2).

We also identified three mutants that were resistant to DFD-VI-15, including one lacking *ACE2*. In *C. albicans* the *ace2* mutant has been also implicated in a number of functions, and it is important to our current observations, as it is a positive regulator of glycolysis (31, 41). Genes encoding glycolytic metabolism were downregulated while respiratory genes associated with the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and ATP synthesis were upregulated in the *C. albicans ace2* null mutant (41). The resistance of the *ACE2* mutant to DFD-VI-15 may be explained by a compensatory change that elevates mitochondrial functions and, consequently, survival of treated cells. However, our phenotype data suggest that DFD-VI-15 inhibits mitochondrial respiration, as parental cells have mitochondrial defects, including increased ROS and membrane depolarization. Further, anaerobically grown cells (especially *S. cerevisiae*) are more resistant to the compound, pointing to a role for aerobic respiration as inhibited by DFD-VI-15.

We advocate mitochondria as rich sources of antifungal targets for several reasons. First, there are significant fungus- and even *Candida*-specific proteins and pathways of mitochondrial respiration. For example, most fungi, but not *S. cerevisiae*, have an alternative oxidase (AOX) pathway and a parallel pathway (PAR) not found in mammalian cells (1). Most, but not all, of the complex I proteins of the electron transport chain (ETC) are highly conserved. Second, mitochondria are essential for energy production and, consequently, many cell functions. Therefore, inhibition of fungus-specific proteins should affect numerous cell activities. Third, published data indicate that inhibition of respiratory complexes and the AOX pathway by antimycin and benzohydroxamate (BHAM), respectively, convert caspofungin-resistant strains of *C. parapsilosis* to a hypersensitivity phenotype (13, 61). Fourth,

TABLE 8 ROS measurements[a]

Strain and treatment	Mean fluorescence	SD	*P* value[b]
SC5314, untreated	13.6	1.1	
SC5314, 12 μg/ml DFD-VI-15	18.1	0.8	0.005*
SC5314, 24 μg/ml DFD-VI-15	19.6	1.2	0.003*
GOA31	24.9	1.2	

[a] The mean fluorescence of DCFDA was compared between nontreated and treated (12 and 24 μg/ml of DFD-VI-15) cells of *C. albicans* SC5314 and GOA31. There was significantly higher fluorescence in treated strains than in untreated strains. These data show that there is a higher accumulation of intracellular ROS upon treatment with DFD-VI-15. Strain *C. albicans* GOA31, which is known to overproduce ROS following deletion of its *GOA1*, was used as a positive control (33).
[b] *P* value compared to untreated SC5314 calculated by unmatched *t* test. *, statistically significant ($P < 0.01$).

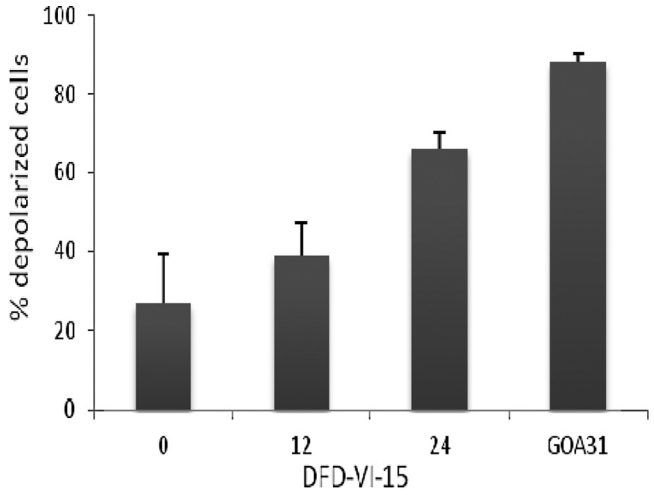

FIG 5 Mitochondrial membrane potential was detected by staining cells with JC-1, a cationic dye used to assess variation in mitochondrial potential, using a FACScan flow cytometer (488 nm; Becton, Dickinson). A dose-dependent increase in depolarization was seen at 12 and 24 μg/ml of compound. GOA31 is a *C. albicans* mutant that has a depolarized mitochondrial membrane (33). This strain was included as a positive control. 7-AAD (7-amino-actinomycin D) was used to exclude dead cells so that membrane potential was assessed only in viable cells.

inhibitors of mitochondrial targets are even suggested for the treatment of human diseases such as type 2 diabetes, certain cancers, and neurodegenerative diseases (28, 57, 63). Exploitation of mitochondrial targets is compelling, especially given the lack of new antifungal targets.

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