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Real-Time Imaging of the Azole-Class of Antifungal Drugs in Live Candida Cells

Raphael I. Benhamou^{#a}, Maayan Bibi^{#b}, Kfir B. Steinbuch^a, Hamutal Engel^c, Maayan Levin^a, Yael Roichman^a, Judith Berman^{b,*}, Micha Fridmana^{a,*}

^aSchool of Chemistry, Raymond & Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv, 6997801, Israel

^bDept. of Molecular Microbiology & Biotechnology, School of Molecular Cell Biology and Biotechnology, George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, 6997801, Israel

^cBLAVATNIK CENTER for Drug Discovery, Tel Aviv University, Tel Aviv, 6997801, Israel

[#] These authors contributed equally to this work.

Abstract

Azoles are the most commonly used class of antifungal drugs, yet where they localize within fungal cells and how they are imported remain poorly understood. Azole target lanosterol 14 α -demethylase, a cytochrome P450, encoded by *ERG11* in *Candida albicans*, the most prevalent fungal pathogen. We report the synthesis of fluorescent probes that permit visualization of antifungal azoles within live cells. Probe **1** is a dansyl dye-conjugated azole and probe **2** is a Cy5-conjugated azole. Docking experiments indicated that each of the probes can occupy the active site of the target cytochrome P450. Measuring MIC of the new dye-conjugated azoles revealed that probe **1**, Like the azole drug fluconazole, is not effective against a mutant that lacks the target cytochrome P450. In contrast, the azole drug ketoconazole and probe **2** retained some antifungal activity against mutants lacking the target cytochrome P450, implying that both act against more than one target. Unexpectedly, both fluorescent azole probes co-localized with the mitochondria, as determined by fluorescence microscopy with MitoTracker dye, both in wild-type and in a mutant that should not bind azoles effectively. Thus, these fluorescent probes are useful molecular tools that can lead to detailed information about the activity and localization of the important azole class of antifungal drugs.

Keywords

Candida; Antifungal agents; Azole drugs; Fluorogenic probes

Introduction

Fungal infections present an increasing medical challenge in the West, as well as in regions with limited health care availability. A large number of cases of oral thrush and/or

*Corresponding Authors: Micha Fridman, mfridman@post.tau.ac.il, +972-3-6408687. Judith Berman, jberman@post.tau.ac.il, +972-52-5842031.

esophageal fungal infections are associated with HIV/AIDS infections each year, and the mortality rate of HIV-associated invasive fungal diseases likely exceeds that of tuberculosis or malaria. *Candida albicans* and *Candida glabrata* are among the most frequently treated opportunistic fungal pathogens, with high crude and high attributable mortality rates.^{1,2}

The repertoire of antifungal drug classes and drug targets are limited because of the evolutionary similarity between fungal and human cells. During the last three decades, members of the azole class of antifungal drugs, and fluconazole (FLC, Figure 1) in particular, have been the first-line treatment for invasive fungal disease. Low cost, accessibility and oral bioavailability have kept azoles as the drug of choice for treating most fungal infections in HIV/AIDS patients in sub-Saharan Africa.^{2,3} FLC can be administered orally and is the preferred azole for intravenous administration.^{4–6} Ketoconazole (KET, Figure 1) is a racemic mixture of *cis*-enantiomers used for treatment of both topical and systemic infections.

Resistance to azoles is an increasing clinical concern⁷ and non-*albicans* *Candida* species, such as *C. glabrata*, are becoming more prevalent due, at least in part, to higher levels of intrinsic resistance and/or to rapid mechanisms by which they acquire resistance to azole drugs.⁸ Azoles are thought to inhibit lanosterol 14 α -demethylase, a cytochrome P450 (cytochrome P450DM) necessary for the biosynthesis of ergosterol, an essential component of the yeast cell membrane that is analogous to cholesterol in mammalian cells.^{9–12} Understanding how resistance is acquired requires an understanding of how the drug enters the cell, where the drug localizes and how it affects cellular metabolism. Despite decades of broad clinical use, fundamental questions about azole antifungal uptake and localization within fungal cells remain unanswered. Answering these questions will help guide the development of next-generation azole-based antifungal agents, thereby ensuring continued clinical efficacy of this important class of drugs.

In recent decades, fluorescent labeling methods and fluorescent bioactive probes have become powerful tools for medical and biological research.^{13–18} Here we report the synthesis and characterization of two azole-based fluorescent probes, and evaluate their suitability for the study of the azole-class of antifungal drugs. One of the probes exhibits properties like FLC and the other appears to be more akin to KET. We provide novel localization data that demonstrates the utility of the probes for studies of drug uptake and cellular localization mechanisms.

Results and Discussion

Design and synthesis of fluorescent antifungal azole probes

All azoles contain a conserved core structure composed of a meta-di-halogenated phenyl ring and a triazole or imidazole ring (Figure 1, red). A third molecular segment varies significantly in size and in atomic composition (Figure 1, black). We reasoned that installation of a fluorescent dye as the third segment onto the conserved core structure would allow the synthesis of inherently fluorescent azole-derived probes that retain biological activities similar to those of azole antifungal drugs (Scheme 1).

Two fluorescent dyes that differ significantly in their excitation and emission spectra, dansyl (ex: ~340 nm; em: ~520 nm) and Cy5 (ex: ~670 nm; em: ~690 nm) were used to design fluorescent azole probes **1** and **2**, respectively (Scheme 1).¹⁹ To synthesize the fluorescent azole, we first generated racemates **1a** and **2a** from 2,4-difluoro- α -(1*H*-1,2,4-triazolyl)acetophenone as previously reported.²⁰ The dansyl-based azole probe **1** was prepared by coupling of intermediate **1a** with dansyl chloride and was obtained in 53% isolated yield (Scheme 1A). The Cy5-based Probe **2** was prepared through a click reaction between intermediate **2a** and *N*-pentynyl-Cy5 (Np-Cy5)²¹ and was obtained in 44% isolated yield (Scheme 1B).

Docking of the fluorescent probes into the catalytic domain of the target cytochrome P450DM

To test if probes **1** and **2** can reside in the catalytic domain of cytochrome P450DM as do the azole antifungal drugs, we docked the two fluorescent azole probes into the crystal structure of the target enzyme from *S. cerevisiae* using the Induced Fit model (Figure 2).¹⁰ Probe **1** was docked into the crystal structure of cytochrome P450DM in complex with FLC (Figure 2A).¹⁰

Like FLC, probe **1** exhibited the ability to bind in the catalytic domain of cytochrome P450DM, with the 1,2,4-triazole ring coordinated with the heme iron atom. The rod-shaped structures of probe **2** and of KET were docked into the crystal structure of cytochrome P450DM in complex with the rod-shaped azole drug itraconazole (Figure 2B). Like itraconazole, the 1,2,4-triazole ring of probe **2** and KET is coordinated with the heme iron atom. Interestingly, superposition of the structures of itraconazole, and the docked structures of KET and probe **2** indicated that the elongated segments of all three compounds are similarly aligned along the pocket that binds lanosterol, the substrate of cytochrome P450DM (Figure 2B).²² Docking of both the *R* and *S* enantiomers of probes **1** and **2** suggested that their interactions with the target cytochrome P450DM take place through a similar binding mode, with variations occurring as a result of the differences in orientation of their tertiary alcohol, 2,4-difluorophenyl ring and fluorescent dye. The calculated Glide Scores suggested that the *R* and *S* enantiomers of probe **1** bind to the target protein with similar affinities (-9.956 kcal/mol, -7.620 kcal/mol respectively, Table S1, supporting information). The *S*-enantiomer of probe **2** binds only slightly better than the *R*-enantiomer (-13.172 kcal/mol and -10.758 kcal/mol, respectively). Thus, *in silico* analysis supports the idea that probe **1** has properties analogous to FLC and probe **2** has properties similar to those of KET and that both enantiomers of each probe can readily occupy the catalytic domain of the target protein, thereby justifying the use of racemates rather than the preparation of enantiomerically pure compounds.

Comparison between the antifungal activities of probes **1** and **2** to those of azole drugs FLC and KET

Antifungal activity profiles of probes **1** and **2** were compared to those of FLC and KET by broth double dilution assays,²³ providing minimal inhibitory concentrations (MICs) for a panel of *C. albicans* and *C. glabrata* isolates (Strains A-K, Table 1). The free dye reagents

Np-Cy5 and dansyl chloride that was hydrolyzed to dansyl acid (Dan-OH) were used as controls.

The activity spectrum of FLC and probe **1** was similar for all isolates, with probe **1** MIC values one to two double dilutions higher than those of FLC (Table 1). Dan-OH, the free fluorescent dye reagent used to generate probe **1**, showed no antifungal activity against the tested strains (MICs > 64 µg/mL), indicating that the antifungal activity of probe **1** does not result from independent cytotoxic activity of the dansyl dye segment.

Several of the strains in the tested *Candida* panel were selected because they are azole resistant. Both KET and probe **2** displayed antifungal activity against all the *C. albicans* and *C. glabrata* strains in the tested panel, including those that are FLC resistant. For example, strain D (*C. albicans* DSY296) carries hyperactive alleles of *TAC1*, that drive expression of efflux pump genes and lead to increased efflux of FLC.^{24,25} As expected, strain D was highly resistant to both FLC and probe **1** and further supports the idea that, like FLC, azole probe **1** is effluxed efficiently by the ABC transporters, especially if those transporters are up-regulated by a hyperactive Tac1 transcription factor. By contrast, strain D did not exhibit resistance to either ketoconazole or probe **2**.

A potentially confounding issue was that the independent Np-Cy5 dye, incorporated in probe **2**, had inherent antifungal activity, with MIC values of 4 - 16 µg/mL against all of the tested strains. However, the antifungal activity of Np-Cy5 was significantly lower than that of probe **2** against the strains in the panel (Table 1), suggesting that the antifungal activity of probe **2** cannot be due to the Cy5 segment alone. Rather, the azole core likely drives the antifungal activity of this probe, with an additional contribution from the Cy5 segment.

Antifungal activity against cytochrome P450DM knockout strains

To further test the suitability of probe **1** and probe **2** for the study of azole antifungal drugs, we measured their activity against a mutant lacking copies of both the *ERG11* and *ERG3* genes (Table 2) compared to the parental lab strain SN152 from which it was constructed.²⁶ *ERG11* encodes the cytochrome P450DM target of azole drugs and is essential for aerobic growth when *ERG3*, which encodes a C-5 sterol desaturase, is functional. Thus, to test the requirement for the Erg11 P450DM target, we used *erg11 erg3* mutants, which are viable despite lacking the azole target cytochrome P450DM. As a negative control, we used another derivative of parental strain SN152 that lacked both copies of *ERG2*, which encodes the C-8 sterol isomerase and thus involved in ergosterol biosynthesis. *erg2* mutant is not inhibited by the azole-class of antifungals. Importantly, neither FLC nor probe **1** inhibited the growth of the *erg11 /erg3* mutant (MICs > 64 µg/mL). This is consistent with the assumption that the Erg11 cytochrome P450DM, is targeted by probe **1**, as well as by FLC. Furthermore, FLC and probe **1** retained antifungal activity against the *erg2* mutant strain, consistent with the assumption that C-8 sterol isomerase is not the target of either FLC or probe **1**. Thus, probe **1** and FLC exhibited very similar antifungal activities.

Unexpectedly, both KET and probe **2** retained residual antifungal activity against the *erg11 /erg3* mutant, with MIC values 32-fold and 256-fold higher than that of the parent strain, respectively. The antifungal activity of KET and probe **2** against the *erg2*

control strain and the parental strain were indistinguishable, with identical MIC values (Table 2). These results are consistent with the assumption that cytochrome P450DM is not the only drug target for either KET or probe **2** in *C. albicans*. Interestingly, the antifungal activity of Np-Cy5 dye was 64-fold lower than that of probe **2** against the parental strain, and yet was similar to that of probe **2**, against the *erg11 erg3* mutant strain. These results indicate that the antifungal activity of probe **2** was not due to its Cy5 segment alone and suggest that the remaining activity of probe **2** against the *erg11 erg3* strain may be due to the Cy5 moiety.

At high concentrations (100 µg/mL), KET has been reported to cause direct membrane damage to *C. albicans* cells, as measured by methylene blue uptake.^{27,28} Other studies, using lower KET concentrations, did not detect this membrane effect.^{29,30} Since Cy5 can be chemically defined as a cationic amphiphile, and since cationic amphiphiles have membrane permeabilizing activity, we asked if either probe **2** or KET might permeabilize *C. albicans* cell membranes. No effect of either of these compounds in membrane damage was observed, by following the permeability of propidium iodide into *C. albicans* SC5314 cells incubated with concentrations 8-fold and 80-fold higher than the MIC of KET and probe **2** over a two hour time course (See Fig.S1 section 8, in the Supporting Information). Thus, the antifungal activity in addition to P450DM inhibition is not due to increased cell membrane permeability. Furthermore, we do not know if, in addition to inhibition of cytochrome P450DM, KET and probe **2** have similar or distinct additional activities.

In-vitro mammalian cytotoxicity of the fluorescent azole probes

To determine whether probes **1** and **2** could be used to study antifungal activity in cultures of mammalian cells, we evaluated their cytotoxicity in cultures of mouse embryonic fibroblast (MEF) 3T9 cells and human embryonic kidney (HEK) 293 cells, using FLC as a negative control. Both FLC and probe **1** had no significant cytotoxic effect on either of the mammalian cell lines ($IC_{50} \gg 64 \mu\text{g/mL}$, data shown in section 9 in the Supporting Information). This indicates that probe **1** will be particularly suitable for the study of the azole class of antifungals in co-culture experiments containing mammalian cells. In contrast, probe **2**, like Np-Cy5, was toxic to both the MEF3T9 and HEK293 cell lines (IC_{50} values 6.83 ± 2.68 and $9.06 \pm 4.15 \mu\text{g/mL}$, respectively). Thus, the use of probe **2** will be dose-limited in experiments involving mammalian cells.

Real-time live-cell fluorescent imaging

Fluorescence microscopy of *C. albicans* (SC5314) was used to detect the accumulation of probe **1** and probe **2** in *C. albicans* cells. Lab strain SC5314 (Strain A, Table 1) was susceptible to FLC, KET, probe **1** and probe **2**. Fluorescence localization was monitored for 120-minutes of drug exposure at 30°C. Shortly after exposure to the fluorescent probes, fluorescence was evident inside the cells and exhibited a distinctive distribution pattern reminiscent of mitochondria. Consistent with this, both probes **1** and **2** co-localized with the mitochondrion-specific dye MitoTracker Green with Pearson correlation values³¹ of 0.83 and 0.72, respectively, after 120 min of incubation with the probes. This pattern did not change over the 120 min incubation period (Figure 3) and supports the idea that probes **1** and **2** localize primarily within mitochondria in the first hours following drug uptake. As a

control, we monitored the localization of the Dan-OH and Np-Cy5 fluorescent dye segments used to synthesize probes **1** and **2**. No significant levels of Dan-OH dye were detected within cells throughout the experiment, indicating that mitochondrial localization of probe **1** did not result from its dansyl dye segment (Figure 3F). The Np-Cy5 dye was detectable through the cytoplasm and in several cell compartments at very early time points (Figure 4B), but the dynamics of Np-Cy5 uptake and localization were very different from those of probe **2**. Specifically, Np-Cy5 entered and then left the cell much more rapidly than probe **2**, suggesting that the free dye crosses the yeast cell membrane through passive diffusion (Sections 10 and 11 in the Supporting Information).

The slower uptake and longer retention of probe **2** uptake may be dependent upon an active process such as facilitated diffusion thought to import azole antifungals.³² In contrast, intense staining of the cytoplasm and mitochondria by the Np-Cy5 was observed shortly after the addition of the dye to fungal cells and almost disappeared after 120 min (Figure 4B, 4D respectively). The localization of both probes to the mitochondria and the different localization of the dye precursors (no localization for Dan-OH and non-specific localization of Np-Cy5) are consistent with the idea that azole antifungals localize to mitochondria during the first hours of drug exposure.

We then tested the localization of both probes in cells of *C. albicans* DSY296 (Strain D, table 1). This azole-resistant strain carries a homozygous mutation in *ERG11* (*ERG11-5/ERG11-5*) encoding a G464S substitution that confers FLC resistance, and is thought to affect the ability of the Erg11 protein to bind azoles.^{9,33} In DSY296, as in wild-type SC5314 cells, both probes **1** and **2** co-localized with the mitochondrion-specific dye MitoTracker Green after 120 min of incubation with Pearson correlation values of 0.63 and 0.85, respectively (See Figure S7, section 12 in the Supporting Information file). These results suggest that mitochondrial localization of azoles is independent of their interaction with cytochrome P450DM.

Notably, both probes **1** and **2** localized to mitochondria despite having fluorescent moieties that differ both in size and atomic composition. This suggests that the core molecular segment common to all azoles is responsible for localization to the mitochondria. We propose that many or all azole antifungals may localize to mitochondria, at least early after entry into the cell. Connections between mitochondrial dysfunction, respiratory activity and efflux levels have been reported to affect azole resistance in both *C. glabrata* and *C. albicans*.^{34–36} Moreover, mitochondrial respiration is required to produce heme and to maintain the activity of many of the enzymes in the ergosterol biosynthesis pathway,^{37,38} yet most enzymes in the ergosterol biosynthesis pathway localize primarily to the endoplasmic reticulum.^{39,40} Whether they also localize to mitochondria is not known. Importantly mitochondria are thought to affect anti-fungal drug responses, at least in part, because of their role in membrane lipid homeostasis.⁴¹ Clearly, how the mitochondrial localization of azole antifungals might affect ergosterol metabolism requires further investigation.

Conclusion

We designed, synthesized and visualized the cellular localization of two fluorescent antifungal azole probes, dansyl azole probe **1** and Cy5 azole probe **2** in *C. albicans*. Docking computations suggested that both the *R* and the *S* enantiomers of both probes can bind in the catalytic domain of cytochrome P450DM, the major target for the azole class of antifungal drugs. The spectrum of antifungal activity against *C. albicans* and *C. glabrata* of probe **1** resembled that of FLC and appeared to target only the Erg11 cytochrome P450DM. The antifungal activities of KET and of probe **2** were significantly reduced when cytochrome P450DM was deleted from the yeast, indicating that P450DM is a target for both these azoles. Interestingly, KET and probe **2** exhibited antifungal activity against *C. albicans* and *C. glabrata* isolates that were highly resistant to FLC. This implies that both KET and probe **2** have at least one target in addition to Erg11. Both probes **1** and **2** primarily localized to, and accumulated in, mitochondria. This provides the first visual evidence that azole antifungals may be active against mitochondrial targets. Thus, the two fluorescent probes reported herein have the potential to facilitate the collection of detailed information about the biological properties of the important azole class of antifungal drugs.

Experimental Section

Docking computations

Probe **1** was docked into the structure of cytochrome P450DM from *S. cerevisiae* in complex with FLC (PDB 4ZDZ).⁴² Probe **2** and KET were docked into the crystal structure of cytochrome P450DM from *S. cerevisiae* in complex with itraconazole (PDB 4ZDY).⁴² FLC and itraconazole were used as the centroids to generate grids for docking. The molecules were first docked using the Glide SP method⁴³, in which a flexible ligand is docked to the rigid receptor binding site. These computations indicated that probe **2** cannot be docked to the binding site due to steric clashes. When the flexibility of the protein was taken into account according to the Induced-Fit (IFD) model⁴², docking of both probes was successful. In all docking computations the interaction between the N4 of the 1,2,4-triazole ring of the azole ligand was used as a constrain. Notably, this was the single only constraint imposed. All computations were performed using the Schrödinger software, release 2016-2, small-molecule drug discovery Suite (Schrödinger, LLC, New York, NY, 2016) on Windows and Linux platforms. The following Schrödinger modules were used: Protein Preparation Wizard⁴⁴, LigPrep, Glide^{43,45}, Prime⁴⁶, IFD⁴². All modules were accessed via the Maestro graphical interface. Analysis of a large number of possible binding poses of the molecules in the binding site of the target showed that many of the conformations of the bound ligands had high target binding affinity and are in good agreement with the binding mode of the original bounded ligands (Itraconazole and FLC). The Glide Score was used to estimate binding affinity of the ligands in complex with the target and is a modified molecular mechanics energy function and IFD Scores were calculated according to the Glide scoring function^{43,45} and the prime conformational refinement.⁴⁶ For every ligand the IFD score was used to rank the poses, and only the poses with the highest scores are presented in the Table S1 (section 6 in the Supporting Information).

Preparation of stock solutions

Probes **1** and **2** were dissolved in anhydrous ethanol (96%) to final concentration of 5 mg/mL. The antifungal agents fluconazole (FLC) and ketoconazole (KET) were purchased from Sigma Aldrich. FLC was dissolved in anhydrous ethanol to final concentration of 5 mg/mL, and KET was dissolved in DMSO to final concentrations of 5 mg/mL.

Fungal strains

The yeast strains *C. albicans* SC5314 (A), *C. albicans* ATCC 90028 (B), *C. albicans* ATCC 96901 (E), *C. albicans* ATCC 64214 (G) and *C. parapsilosis* ATCC 90018 (H) were purchased from the ATCC. The yeast strain *C. albicans* DSY294 (C)⁴⁷ and *C. albicans* DSY296 (D)⁴⁷ was provided by Dominique Sanglard (Lausanne University), *C. albicans* ATCC P-87 (F)⁴⁸ was obtained from David Soll (University of Iowa), *C. glabrata* 11-304 (I)⁴⁹ and *C. glabrata* 11-064 (K)⁴⁹ were provided by Ronen Ben-Ami (Tel Aviv University), *C. glabrata* ATCC 2001 (J)⁵⁰ was provided by Jane Usher (Exeter University), *C. albicans* SN152 (parental strain, L)²⁶, *C. albicans* SN152 (*erg11* Δ *erg3*, M)²⁶ and *C. albicans* SN152 (*erg2*, N)²⁶ were provided by Benjamin Vincent and Susan Lindquist (Massachusetts Institute of Technology).

Minimal inhibitory concentration broth dilution assay

All strains were tested using the broth double dilution method in 96-well plates (Corning) and grown in RPMI 1640. Starter cultures were incubated for 24 h (37 °C, 5% CO₂, aerobic conditions) and then diluted 1:100 into fresh medium. Tested compounds were added to the RPMI 1640 media to form the mother liquor (32 μ L stock solution in 1218 μ L of RPMI 1640) at the starting concentration of 128 μ g/mL. Next, 100 μ L of serial double dilutions of the tested compounds in RPMI 1640 (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μ g/mL) were prepared in flat-bottomed 96-well microplates (Corning). Control wells with no compounds and wells without yeast cells containing each tested concentration of the compounds (blanks) were also prepared. An equal volume (100 μ L) of yeast suspensions in RPMI 1640 was added to each well for a final volume of 200 μ L. Final ethanol concentrations ranged from 0.0012% to 1.3%. The final inoculum was between 5×10^4 CFU/mL and 5×10^5 CFU/mL.⁵¹ After incubation for 24 h at 37 °C in 5% CO₂, MTT (50 μ L of a 1 mg/mL solution in H₂O) was added to each well followed by additional incubation at 37 °C for 2 h. Results were confirmed in two independent experiments, and each concentration was tested in triplicate.

Flow cytometry for analysis of cell viability using propidium iodide

Cells were grown in 3ml YPAD (yeast peptone dextrose medium plus 40 mg/L adenine and 80 mg/L uridine; Sigma) with shaking at 30°C in tubes overnight. Cultures were diluted 1:100 and incubated for two more hours at 30°C. Two sets of experiments were performed for each concentration; tested drugs and probes were added for 2h with shaking at 30°C to a final concentration of 10 μ g/mL and 50 μ g/mL for FLC, probe **1**, and Dan-OH; a final concentration of 1 μ g/mL and 10 μ g/mL were added to KET, Probe **2** and Np-Cy5. Then 10 μ L from the cultures were added to 80 μ L of TE 50:50 buffer (50mM TrisHCl pH 8.0, 50mM EDTA) and 10 μ L of propidium iodide (PI) stain to a final PI concentration of 1

mg/mL (P4170 SIGMA ALDRICH). Flow cytometry data were collected from 25,000-35,000 cells per time point by using B2 laser excitation (excitation at 488 nm and emission at 614/50 nm) on a MACSQuant flow cytometer. Analysis was performed using FlowJo 8.7 software.

Mammalian cells cytotoxicity assay

Cells were grown in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin in a humidified chamber of 95% air, 5% CO₂ at 37 °C. HEK 293 or 3T9 MEF cells were plated into 96-well microtiter plates (48 x 10³ cells per well) and allowed to adhere for 24 h. The cells were then treated with solutions containing the tested compounds (10 µL per well) at various concentrations ranging from 0.5 to 64 µg/mL and incubated for 24 h. Cell viability was determined by the MTT assay as follows: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium at a final concentration of 0.25 mg/mL, followed by 2h of incubation. The medium was then aspirated and DMSO (100 µL per well) was added. The plates were read on a Tecan Spectra Fluor microplate reader at 570 nm and a reference wavelength of 630 nm. Each compound was tested in triplicate, and the results were averaged from at least three independent experiments.

Cell staining

Cells were grown to log phase in liquid YPAD (yeast peptone dextrose medium plus 40 mg/L adenine and 80 mg/L uridine; Sigma) with shaking at 25 °C in a 125-ml Erlenmeyer flask. Cells from a log phase culture were warmed to 30 °C and the tested probes were added to a final concentration of 10 µg/mL for probe **1** and 1 µg/ml for probe **2**. After 60 min of incubation, MitoTracker Green FM (M7514, ThermoFisher Scientific) was added to a final concentration of 10 nM (first diluted in DMSO), and cells were incubated for additional 60 min at 37 °C. Cells were kept in the dark for a total of 120 min.

Live cell imaging

Cells were washed and resuspended in SDC prior to being imaged on glass slides covered with glass coverslips. Cells treated with probe **1** were imaged on a MORE imaging system (TILL photonics GmbH) with an Olympus UPlanApo 100X 1.3 NA oil immersion objective. The bandpass filters sets used to image probe **1** were excitation 390/40 nm and emission 520/20 nm. MitoTracker was imaged with excitation 485/20 nm and emission 530/25 nm. Cells treated with probe **2** were imaged on a Nikon Ti microscope equipped with a Plan Apo VC 100x Oil objective and a Zyla 5.5 sCMOS camera (Andor) using NIS elements Ar software. The bandpass filters sets used to image probe **2** were excitation 585 nm and emission 630/75 nm. In experiments with probe **2**, MitoTracker was imaged using excitation at 470 nm and emission at 525/50 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FLC	Fluconazole
KET	Ketoconazole
Np-Cy5	<i>N</i> -pentynyl-Cy5
Dan-OH	Dansyl acid

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Figure 1.
Members of the azole class of antifungal drugs.

Figure 2.

A. Docked structure of the *S*-enantiomer of probe **1** (yellow) superimposed on the crystal structure of a complex between FLC (red) and *S. cerevisiae* cytochrome P450DM (PDB ID: 4ZDZ). **B.** Docked structures of the *R*-enantiomer of probe **2** (blue), (*R,S*) enantiomer of KET (green) superimposed on the crystal structure of a complex between the (*R,S*) enantiomer of itraconazole (orange) and *S. cerevisiae* cytochrome P450DM (PDB ID: 4ZDY). Protein residues are shown as grey ribbons with the heme prosthetic group in pink. All computations were performed using Schrödinger software, release 2016-2 on Windows and Linux platforms.

Figure 3.

Localization of probe 1, probe 2 and the two free dyes (Dan-OH and Np-Cy5) in live *C. albicans* SC5314 cells after 120min. Probe 1 (A-D), Dan-OH (E-H), probe 2 (I-L) and Np-Cy5 (M-P). MitoTracker Green FM stain C+G+K+O. Differential interference contrast microscopy images A+E+I+M. Merged images D+H+L+P.

Figure 4.

Localization of probe 2 and Np-Cy5 in live *C. albicans* SC5314 cells. Np-Cy5 (A-D) and probe 2 (E-H). Np-Cy5 after 60 min (A+B) and after 120 min (C+D). Probe 2 after 60 min (E+F) and after 120 min (G+H).

Scheme 1.
Synthesis of fluorescent antifungal azole probes.

Table 1
Antifungal activities of parent azoles, dyes, and fluorescent probes.

Yeast strain	Compound MIC [$\mu\text{g/mL}$]					
	FLC	1	Dan-OH	KET	2	Np-Cy5
A. <i>C. albicans</i> SC5314	1	2	>64	0.125	0.125	4
B. <i>C. albicans</i> ATCC 90028	1	2	>64	0.25	0.5	8
C. <i>C. albicans</i> DSY294	0.5	2	>64	0.25	0.25	8
D. <i>C. albicans</i> DSY296	>64	>64	>64	0.125	1	4
E. <i>C. albicans</i> ATCC 96901	>64	>64	>64	1	1	4
F. <i>C. albicans</i> ATCC P-87	0.25	1	>64	0.125	0.125	8
G. <i>C. albicans</i> ATCC 64214	>64	>64	>64	8	4	8
H. <i>C. parapsilosis</i> ATCC 90018	1	2	>64	0.0625	0.125	4
I. <i>C. glabrata</i> 11-304	32	32	>64	4	0.5	8
J. <i>C. glabrata</i> ATCC 2001	>64	64	>64	4	0.5	16
K. <i>C. glabrata</i> 11-064	64	>64	>64	0.5	2	16

All MICs were determined using the broth double-dilution method starting from a concentration of 64 $\mu\text{g/mL}$. Cells were grown in RPMI 1640 medium at 37°C under CO₂ for 24h. Each concentration was tested in triplicate, and results were confirmed by two independent sets of experiments.

Table 2
Antifungal activities of parent azoles dyes and fluorescent probes against ergosterol biosynthesis mutant strains.

Compounds	MIC [$\mu\text{g/mL}$]			
	<i>C. albicans</i> SN152			
	Parental	<i>erg11</i>	<i>erg3</i>	<i>erg2</i>
FLC	0.25	>64		0.5
Probe 1	2	>64		2
Dan-OH	>64	>64		>64
KET	0.0625	16		0.0625
Probe 2	0.0625	2		0.0625
Np-Cy5	4	2		4

All mutant strains were deleted for both copies of the relevant genes.²⁶ MICs were determined using the broth double-dilution method starting from a concentration of 64 $\mu\text{g/mL}$. Cells were grown in RPMI 1640 medium at 37°C with 5% CO₂ for 24h. Each concentration was tested in triplicate, and results were confirmed by two independent sets of experiments.