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Piperazinyl quinolines as chemosensitizers to increase fluconazole susceptibility of *Candida albicans* clinical isolates

Willmen Youngsaye[a], **Benjamin Vincent**[b,c], **Cathy L. Hartland**[a], **Barbara J. Morgan**[a,1], **Sara J. Buhrlage**[a,2], **Stephen Johnston**[a], **Joshua A. Bittker**[a], **Lawrence MacPherson**[a], **Sivaraman Dandapani**[a], **Michelle Palmer**[a], **Benito Munoz**[a,*], **Luke Whitesell**[b], **Susan Lindquist**[b,d], and **Stuart L. Schreiber**[a,e,*]

[a]Chemical Biology Platform and Probe Development Center, Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge, MA 02142, USA

[b]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

[c]Microbiology Graduate Program, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139, USA

[d]Department of Biology and Howard Hughes Medical Institute, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139, USA

[e]Howard Hughes Medical Institute, Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, MA 02142, USA

Abstract

The effectiveness of the potent antifungal drug fluconazole is being compromised by the rise of drug-resistant fungal pathogens. While inhibition of Hsp90 or calcineurin can reverse drug resistance in *Candida*, such inhibitors also impair the homologous human host protein and fungal-selective chemosensitizers remain rare. The MLPCN library was screened to identify compounds that selectively reverse fluconazole resistance in a *Candida albicans* clinical isolate, while having no antifungal activity when administered as a single agent. A piperazinyl quinoline was identified as a new small molecule probe satisfying these criteria.

Keywords

Candida albicans; Fluconazole; Antifungal; Chemosensitizer; Molecular Libraries Probe Production Center; Network (MLPCN)

Acquired drug resistance by medically relevant microorganisms poses a grave threat to human health and has enormous economic consequences.[1–3] Fungal pathogens present a particular challenge because they are eukaryotes and share many of the same mechanisms that support the growth and survival of the human host cells they infect. While contemporary antifungal medications such as fluconazole remain effective, the usefulness of such drugs is compromised by either dose-limiting host toxicity or the frequent emergence of high-grade resistance.[2,3]

*Corresponding authors. Tel.: +1 617 714 7225; fax: +1 617 714 8969 (B.M.); tel.: 1 617 714 7080; fax: 1 617 714 8969 (S.L.S), bmunoz@broadinstitute.org (B. Munoz), stuart_schreiber@harvard.edu (S.L. Schreiber).
[1]Present address: Goodwin Proctor LLP, 53 State Street, Boston, MA, 02109, USA
[2]Present address: Cancer Biology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA, 02215, USA

Supplementary Material
Experimental protocols for cellular assays and for the preparation of **1** can be found, in the online version, at doi:

The opportunistic fungus *Candida albicans* preferentially invades immunocompromised individuals and is responsible for numerous cutaneous, mucosal, and systemic blood-borne infections annually.[4] The azole antifungal fluconazole is often prescribed to control such infections, but fluconazole's fungistatic nature and emerging resistance are beginning to detract from its effectiveness. Typically, a daily regimen of 100 mg is sufficient to treat infections, but dosages as high as 800 mg/day can be ineffective against fluconazole-resistant *C. albicans*.[5]

Chemosensitizing *Candida albicans* to fluconazole is one approach to combat the emerging resistance of this pathogen. A limited number of small molecules have demonstrated modest potential in this arena,[6–11] and the proteins Hsp90 and calcineurin appear integral to some of the resistance pathways utilized by *Candida*.[12] This tentative progress towards stemming the increasing fluconazole resistance of *Candida* prompted us to screen the National Institutes of Health Molecular Libraries Probe Production Centers Network (NIH-MLPCN) compound collection with the goal of identifying small molecules that act as fungal-selective chemosensitizers and could be used to probe the various antifungal resistance mechanisms of *Candida*.

A high throughput screen of ~300,000 compounds evaluated growth inhibition of the *C. albicans* clinical isolate CaCi-2[5] in the presence of a sub-lethal concentration of fluconazole (Figure 1, PubChem AID 1979).[13] 1,893 compounds exhibited >75% inhibition when dosed at 9.5 μM, of which 622 possessed IC_{50} values less than 1 μM when tested in a dose-response assay.

An orthogonal screen evaluated the efficacy of these 622 hits in combination with fluconazole against a more resistant *C. albicans* clinical isolate CaCi-8,[5,13] selecting for compounds that were active with IC_{50}'s below 50 μM. At this stage, 403 compounds were identified as chemosensitizers of both CaCi-2 and CaCi-8.

To remove hits with undesirable activity profiles, two counterscreens were incorporated into the late stages of the screening campaign. The first employed murine 3T3 fibroblasts to assay non-selective mammalian cell toxicity while reevaluating CaCi-2 *in the absence* of fluconazole identified inherently fungitoxic substances. 296 of 403 candidates successfully passed both counterscreens, and the piperazinyl quinoline **1** (Figure 1) was selected for further investigation as a potential probe.

A number of analogs structurally related to **1** were prepared and evaluated for their ability to reverse fluconazole resistance in the *C. albicans* test strains. Two critical intermediates **5** and **6** were prepared by reacting excess piperazine with either 4-chlorobenzoic acid or 4,7-dichloroquinoline (Scheme 1). Amide coupling of piperazinyl quinoline **5** with different carboxylic acids afforded analogs **7**. Similarly, acylated piperazine **6** was appended to various aryl chlorides and bromides to provide analogs **8**.

Upon their preparation, the resulting collection of analogs was tested for their ability to increase fluconazole susceptibility in CaCi-2 and CaCi-8. The fungi were incubated at 37 °C for 48 hours with the test compound and 8 μg/mL fluconazole before growth inhibition was assessed by Alamar blue fluorometry. Geldanamycin, a non-selective Hsp90 inhibitor, was used as a control for growth inhibition (100% inhibition at 10 μM).[12] The different analogs were also screened against mammalian fibroblasts and CaCi-2 in the absence of fluconazole to identify substances with intrinsic toxicity or antifungal effects. In the absence of fluconazole, none of the analogs showed any appreciable activity against CaCi-2 (IC_{50} = 15–26 μM) and were non-toxic to fibroblasts as well (IC_{50} = 21–26 μM).

The chemosensitizing properties of select 4-chlorobenzamide analogs **7** on CaCi-2 and CaCi-8 are presented in Table 1. The initial hit **1** proved to be an effective chemosensitizer of both fungal strains (IC_{50} = 0.7 and 1.3 μM, respectively) but suffered from poor solubility. Attempts to incorporate alternative *para*-substituents typically lowered the effectiveness of the resulting compounds as demonstrated with **7a–c**. Similarly, relocation of the chloro substitutent to the *meta* or *ortho* positions (*e.g.* **7d–e**) was not tolerated, although the 3,4-dichloro variant **7f** displayed levels of activity comparable to the original hit.

Amides derived from alkanoic acids were significantly more soluble than **1** but were less effective chemosensitizers. Analogs bearing linear (**7h**), branched (**7i**), and cyclic (**7j–k**) alkyl chains were partial growth inhibitors of CaCi-2 at micromolar concentrations. However, the cycloalkane amides **7j–k** displayed greater effectiveness against the more resistant CaCi-8 strain than their acyclic counterparts (**7h–i**). Ureas such as **7l** were prepared from reacting **6** with different isocyanates and showed only moderate growth inhibition against CaCi-2 when dosed with fluconazole. Sulfonamides did not increase fluconazole susceptibility of either CaCi-2 or CaCi-8 (data not shown).

Several heteroaromatic replacements of the 4-chlorophenyl ring were also prepared. As observed with the alkanamides, compounds such as **7m–o** were more soluble in PBS solution but possessed lower potencies relative to **1**. Despite its poor aqueous solubility, the 4-chlorophenyl amide remained the most effective functional group among the several dozen variants evaluated, and its efficacy in the cellular assays suggests **1** may possess a high binding affinity for its target.

Next, the role of the 7-chloroquinoline fragment was investigated, and several representative analogs that were prepared are summarized in Table 2. Initially, alternatives to chlorine were introduced into the ring system, and the specific sites of substitution were varied as well. Analogs **8a–d** represent some of the permutations considered, but none were able to match the effectiveness of **1**. In addition, this series of compounds did little toimprove solubility. Ultimately, our efforts to modify the quinoline system were unfruitful; the 7-chloro derivative still offered the greatest cellular potency against both CaCi-2 and CaCi-8.

In order to circumvent the steep SAR associated with the quinoline scaffold, a series of nitrogen heterocycles were explored as surrogates. The various replacements often resulted in large increases in solubility but these gains were offset by diminished activity. Truncating the quinoline system to the analogous pyridines (**8e–g**) essentially negated all cellular activity. Similarly, related heterocycles such as pyridazine **8h**, pyrimidine **8i**, and pyrazine **8j** were marginal inhibitors of *C. albicans* growth. Bicyclic systems such as **8k** and **8l** showed weak inhibition against both *Candida* strains, and this activity trend was also observed for the smaller, monocyclic imidazole and thiazole counterparts (**8m–n**). The 4-chlorobenzene derivative **8o** was one of several phenyl analogs prepared; compounds of this class were ineffective growth inhibitors.

Compound **1** was determined to be the most effective chemosensitizer of *C. albicans* CaCi-2 and CaCi-8 in cellular assays with IC_{50} values of 0.7 and 1.3 μM, respectively. Aqueous solubility remains a liability for this compound, and all attempts to increase solubility reduced cellular activity. **1** had no discernible effect on CaCi-2 in the absence of fluconazole, and murine 3T3 fibroblasts showed no response up to 26 μM. To provide insight into possible modes of action, **1** was evaluated in yeast reporter assays for potential inhibitory activity against Hsp90-dependent and calcineurin-dependent signaling pathways. Inhibition of these pathways has been linked to lower fluconazole resistance in *Candida*,[12] but compound **1** did not affect either mechanism (IC_{50} >26 μM), suggesting that it may exploit an alternative pathway to reverse fluconazole resistance in *C. albicans*. Target

identification via resistance studies are ongoing and will hopefully uncover novel fungal stress response pathways that could further yield additional therapeutic targets for future generations of antifungals.

The current work evaluated over 300,000 compounds of the NIH-MLPCN collection to identify agents capable of reversing fluconazole resistance in clinical isolates of *Candida albicans*. After triage through a series of counterscreens and an orthogonal assay, 296 substances with the ability to sensitize two *Candida albicans* clinical isolates towards fluconazole treatment were identified. Piperazinyl quinoline **1** was selected for further SAR investigation and optimization efforts. As a result of these synthetic studies, compound **1** emerged as the most effective chemosensitizer, able to achieve full growth inhibition of both CaCi-2 (97–100% inhibition) and the more resistant CaCi-8 (97–100% inhibition) at approximately 1 μM. In addition, it was determined that **1** was not inherently toxic or fungicidal and did not act *via* two established resistance paths. This compound has been registered with NIH Molecular Libraries Program (probe ML 189) and is available upon request.[14]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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13. The fluconazole MIC for CaCi-2 and CaCi-8 was determined to be 2 μg/mL and 8 μg/mL, respectively. For the CaCi-2 and CaCi-8 growth inhibition assays, a fixed fluconazole concentration of 8 μg/mL was used. In CaCi-2, this corresponded to a baseline growth inhibition of 60–80% and was sufficient to suppress the trailing growth associated with this strain.
14. Compound **1** (probe ML 189) is also available from commercial vendors.

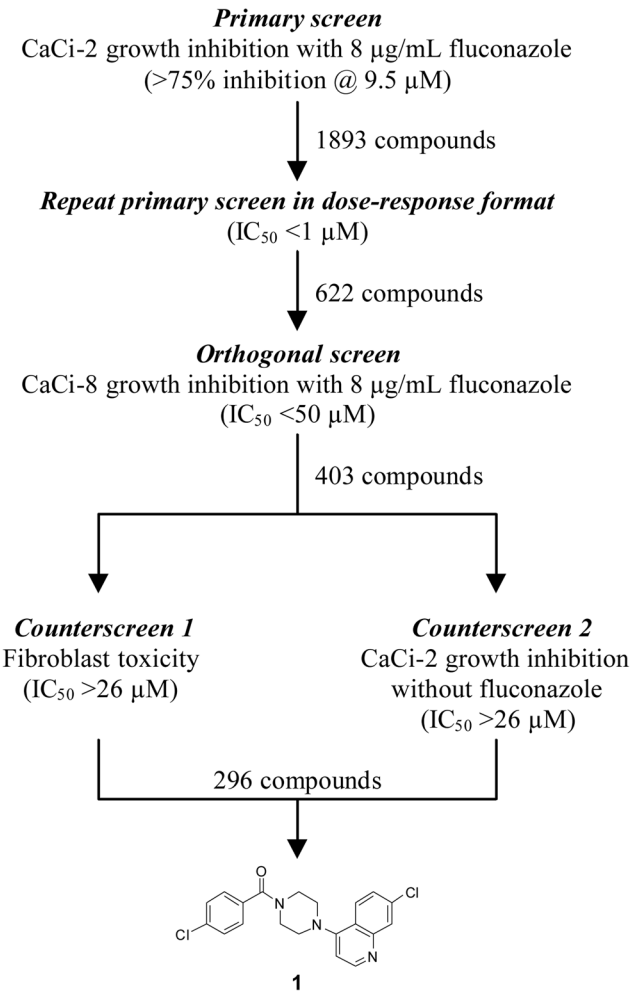
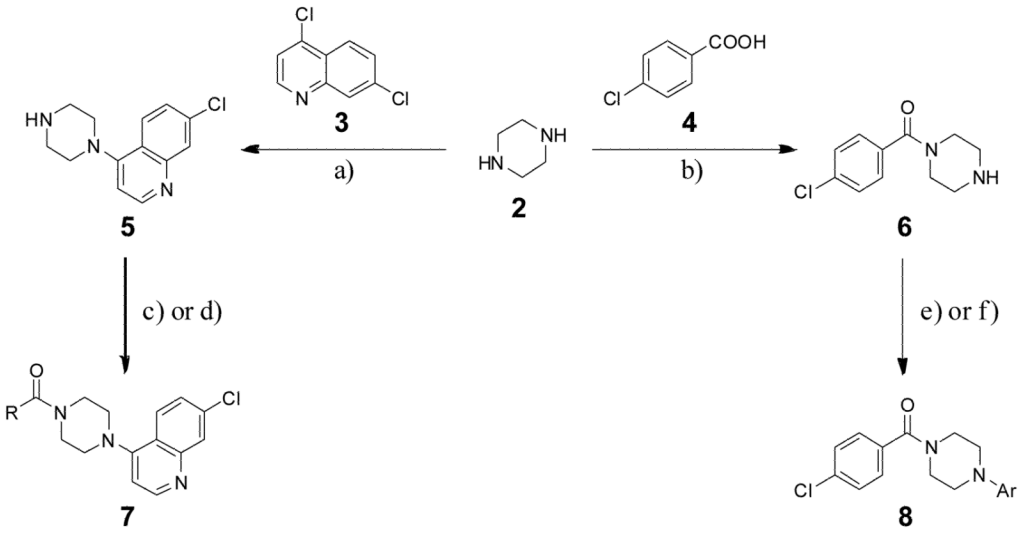


Figure 1.

Summary of HTS campaign of the MLPCN ~300,000 compound collection. The selection criterion for each assay is given in parentheses

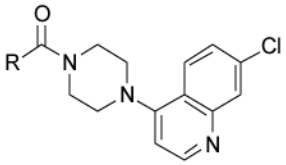


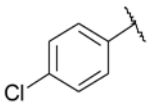
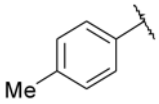
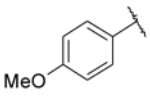
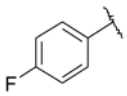
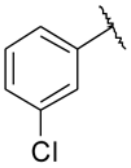
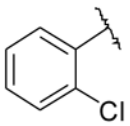
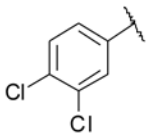
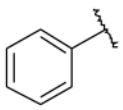
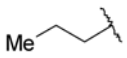
Scheme 1.

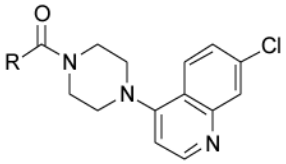
Synthesis of analogs. Reagents and conditions: a) Et_3N, 130 °C; b) EDCI, DMAP, CH_2Cl_2; c) R-CO_2H, EDCI, DMAP, CH_2Cl_2; d) RNCO, CH_2Cl_2; e) ArCl, Et_3N, 130 °C; f) ArBr, NaOt-Bu, 15 mol% BINAP, 5 mol% $Pd_2(dba)_3$, toluene, 80 °C.

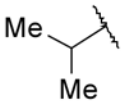
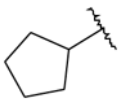
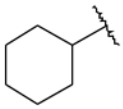
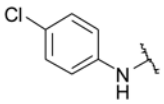
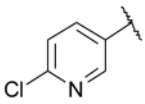
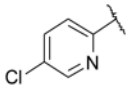
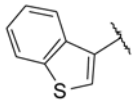
Table 1

Activity of benzamide replacements in the presence of fluconazole[a]



Compound	R	IC_{50} (μM)[b]	
		CaCi-2	CaCi-8
1		0.7 ± 0.5	1.3 ± 1.6
7a		2.3 ± 1.0	3.1 ± 2.8
7b		5.3 ± 1.1	16.8 ± 9.3
7c		3.6 ± 2.3	4.2 ± 2.7
7d		4.4 ± 2.1	7.8 ± 5.6
7e		7.3 ± 3.2	14.1 ± 7.2
7f		1.0 ± 0.6	2.1 ± 1.8
7g		4.4 ± 3.1	6.1 ± 4.4
7h		6.3 ± 4.4	15.0 ± 11.6



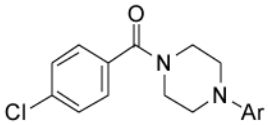
Compound	R	IC_{50} (μM)[b]	
		CaCi-2	CaCi-8
7i		7.2 ± 3.8	>26.0
7j		3.6 ± 2.4	5.3 ± 4.1
7k		1.7 ± 1.1	4.0 ± 2.7
7l		4.2 ± 2.2	2.3 ± 2.5
7m		6.7 ± 3.2	10.9 ± 9.0
7n		4.9 ± 2.3	4.1 ± 3.0
7o		1.9 ± 0.9	4.0 ± 3.0

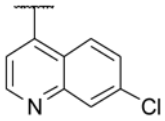
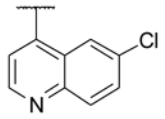
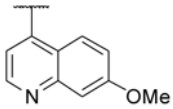
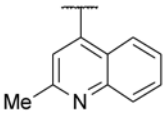
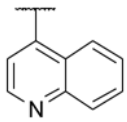
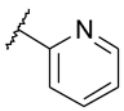
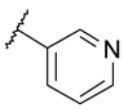
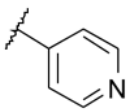
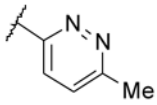
[a] CaCi-2 and CaCi-8 cells were incubated at 37 °C for 48 hours with test compound and 8 μg/mL fluconazole.

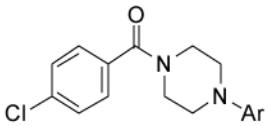
[b] Average of at least three runs

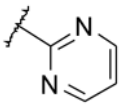
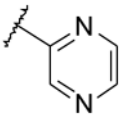
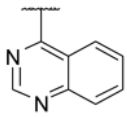
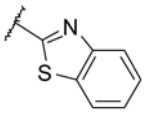
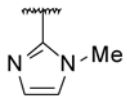
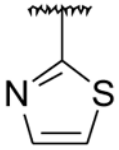
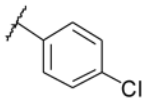
Table 2

Activity of select quinoline analogs in the presence of fluconazole[a]



Compound	Ar	IC_{50} (μM)[b]	
		CaCi-2	CaCi-8
1		0.7 ± 0.5	1.3 ± 1.6
8a		2.4 ± 1.9	6.3 ± 6.1
8b		4.8 ± 2.8	13.7 ± 4.7
8c		7.3 ± 4.9	>26.0
8d		5.7 ± 4.2	14.7 ± 8.2
8e		>10.0	24.0 ± 3.5
8f		7.3 ± 4.9	>26.0
8g		7.3 ± 4.9	>26.0
8h		8.5 ± 6.0	20.6 ± 10.8



Compound	Ar	IC_{50} (μM)[b]	
		CaCi-2	CaCi-8
8i		7.1 ± 5.8	18.1 ± 10.9
8j		9.0 ± 3.5	22.2 ± 7.7
8k		4.8 ± 2.2	11.0 ± 9.0
8l		5.5 ± 4.9	10.8 ± 13.2
8m		6.2 ± 5.7	>26.0
8n		6.9 ± 5.9	18.9 ± 10.5
8o		8.7 ± 3.9	19.0 ± 21.4

[a] CaCi-2 and CaCi-8 cells were incubated at 37 °C for 48 hours with test compound and 8 μg/mL fluconazole.

[b] Average of at least three runs