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BRIEF ARTICLE

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High-Throughput-Screening-Based Identification and Structure−Activity Relationship Characterization Defined (*S*)-2-(1-Aminoisobutyl)-1-(3-chlorobenzyl)benzimidazole as a Highly Antimycotic Agent Nontoxic to Cell Lines

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Supporting Information

ABSTRACT: Novel nontoxic (*S*)-2-aminoalkylbenzimidazole derivatives were found to be effective against *Candida* spp. at low micromolar concentrations using high-throughput screening with infected HeLa cells. A collection of analogues defined the chemical groups relevant for activity. The most active compound was characterized by transcriptional analysis of the response of *C. albicans* Sc5314. (*S*)-2-(1-Aminoisobutyl)-1-(3-chlorobenzyl)benzimidazole had a strong impact on membrane biosynthesis. Testing different clinically relevant pathogenic fungi showed the selectivity of the antimycotic activity against *Candida* species.

INTRODUCTION

In the treatment of invasive fungal infections several therapeutic problems remain, in particular toxicity, variable drug bioavailability, lack of oral or intravenous preparations, and significant drug interactions for some agents.[1] Previously rare fungal species emerge, and moreover, most known strains develop resistance against marketed antifungals.[2] Invasive aspergillosis and candidosis still account for high rates of nosocomial infections and mortality, in particular for immunocompromised patients. Amphotericin B remains a standard therapy for many life threatening mycoses despite its high nephrotoxicity.[3] Ketoconazole is more and more replaced for its low selectivity and hepatic side effects.[4] Numerous triazole based antimycotic therapeutics are continuously optimized and introduced in clinical practice.[1a,5] Generally, the azole class of antifungals acts by binding to the active site of 14-lanosterol demethylase (14α-sterol demethylase or P-450$_{DM}$) inhibiting the biosynthesis of the fungal cell-wall component ergosterol.[6] However, a gradual rise in triazole resistance[7] and reproductive and hepatic toxicity of triazole based antifungals is observed. The ability of triazoles to inhibit CYP-dependent enzymes raises concerns about triazole effects on hormone synthesis and drug metabolism.[8] Therefore, we also included compound collections of the more favorable imidazole type for our antifungal HTS approach.

RESULTS AND DISCUSSION

Activity−Selectivity Assay for Antifungal Screening. An assay has been set up allowing the identification of a potent antifungal compound and the determination of its tolerability by human cells in one step. Among various cell lines tested (Caco-2, HeLa, A431, and A459), HeLa cells proved to show robust and reproducible growth behavior and to be susceptible to *C. albicans* infections.[9] All accessible potential in vitro targets of the pathogen and the host were covered simultaneously, because as readout, the survival of infected host cells was measured directly instead of detecting growth retardation of the microorganism or inhibition of enzymatic functions of the pathogen.[10] Thus, critical compounds that show cytotoxicity or are unable to enter the host and/or pathogen cells in general could be excluded at an early stage.[11]

The collection of 30 000 individual synthetic compounds employed as a first basis was previously well characterized to be composed of 99.7% leadlike structures.[12] Subsequently further combinatorial compound collections comprising more than 70 000 individuals have been investigated.

The HeLa cell line was simultaneously incubated with *C. albicans* in the presence of the individual compounds (∼20 μM) for 5 days. After 18 h of incubation viability of HeLa cells was analyzed microscopically and after 5 days quantified using fluorescein diacetate (FDA) which is metabolized to a colorimetric detectable fluorophore by vital cells. Additionally, individual wells were visually scanned for growth of *C. albicans*. Thus, a fluorescence signal of >40% per well in comparison to the positive control (untreated HeLa cells ∼100%) and negative control

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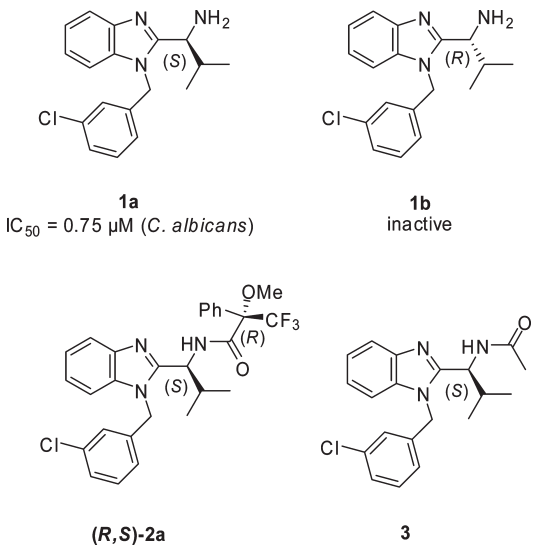


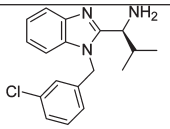
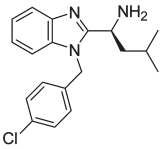
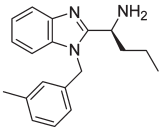
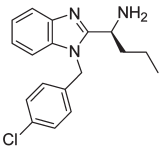
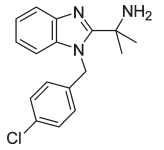
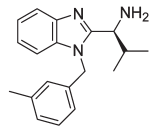
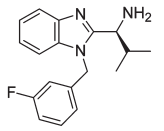
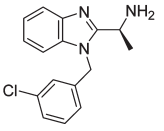
Figure 1. Hit compound **1a**, MTPA-derivative (*R*,*S*)-**2a**, (*R*)-enantiomer **1b**, and *N*Ac derivative **3**.

(untreated infected HeLa cells ~0%) was the prerequisite for a hit compound and indicated growth inhibition of *C. albicans* without significantly impairing the vitality of the host cells. The total hit rate of the HTS assay amounts to 1:1000 compounds (0.1%) and thus was found to be in a typical and acceptable range for a robust homogeneous assay. The primary hit compounds belonged to different classes of heterocyclic compounds, but only the benzimidazoles were promising in terms of high activity and low cytotoxicity. All primary hit compounds were resynthesized, and dose related responses were determined. The antimycotic activity was measured as half maximal inhibitory concentration IC_{50}. Half maximal cytotoxic concentrations were determined as CC_{50}. The resulting selectivity index (SI) was measured as the ratio CC_{50}/IC_{50}.

Chemistry and Strategy for SAR Studies. Among the initial hits from the primary HTS the 1,2-disubstituted benzimidazole **1a** (Figure 1) showed high antifungal activity and low cytotoxicity (IC_{50} = 0.75 μM, CC_{50} = 97.5 μM) resulting in a high selectivity index (CC_{50}/IC_{50}) SI = 130 (Table 1). The N-acetylated analogue **3** and the (*S*)-enantiomer **1b** were completely inactive. The integrity of the stereochemistry has been confirmed by derivatization to the respective Mosher's amide **2a** (Figure 1). Obviously, the potency of **1a** entirely depended on the configuration similar to that described for the dioxolan-based antifungal drugs ketoconazole and itraconazole which are still administered to patients as racemic mixtures of differently active cis/trans isomers.[13,14] Next we constructed compound collections of (*S*)-2-aminoalkylbenzimidazoles by systematically varying three regions of the core structure without affecting the essential (*S*)-configuration and without changing the free primary amino group. The synthesis procedures of **1a** and analogues are summarized in Scheme 1.

To delineate the structural requirements essential for biological activity, we altered the substitution pattern of **13**−**15** on the benzimidazole core (Figure 2). Trifluoromethyl, chloro, and hydrogen substituents as R^1 were introduced via nitroanilines **5a**−**c**, leading to the scaffolds **13** (R^1 = 5-CF_3), **14** (R^1 = 5,6-diCl), and **15** (R^1 = H). The further diversification of the benzylic R^2 group consisted of halo, methyl, and methoxy substitution on the aromatic ring, whereas only minor modifications in the aliphatic residue R^3 were made (**13*Xx***, **14*Xx***, **15*Xx***). The latter was restricted to various aliphatic α-amino acids **9a**−**f** to diversify the R^3 group. Therefore, a focused collection of 90 benzimidazoles ($R^1 \times R^2 \times R^3$) = (3 × 5 × 6) was synthesized (Figure 2). The results of the bioassay show that **13**−**15** with 5-CF_3 and 5,6-diCl substitution of the benzimidazole moiety are inactive. Modifications of the benzylic substitution pattern in the R^2 group are tolerated without a general loss of antifungal activity. Interestingly, a variation of the aliphatic R^3 branch is also tolerated in a relatively broad range. Further modifications resulted in a loss of the antifungal activity (see Supporting Information). The purely aliphatic substituted compounds **15*Xa*** and **15*Xf*** showed high antifungal activity almost in the range of that

Table 1. In Vitro Antifungal Activities (IC_{50}), Cytotoxicity (CC_{50}), and Resulting Selectivities (SI) of Selected Compounds 15*Xx* against *Candida albicans*

Compound	Structure	IC_{50}	CC_{50}	SI
1a = **15*Da***		0.75 μM	97.5 μM	130
15*Ab*		4 μM	36 μM	9
15*Cd*		2 μM	30 μM	15
15*Ad*		2 μM	28 μM	14
15*Ae*		5 μM	50 μM	10
15*Ca*		3.3 μM	10.6 μM	3.2
15*Ea*		2.2 μM	20.6 μM	9.4
15*Df*		7.8 μM	19.3 μM	2.5

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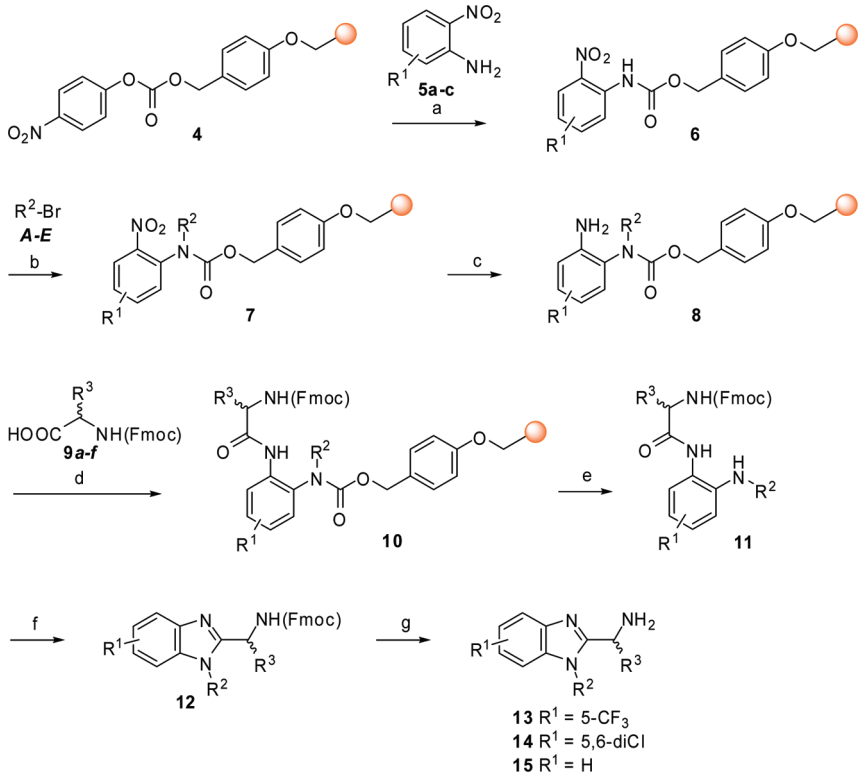
Scheme 1[a]



[a] Reagents and conditions: (a) 3 equiv of NaH, DMF, 2.5 equiv of nitroaniline **5a**−**c**, 12 h; (b) 5 equiv of alkyl bromide **A**−**E**, 8 equiv of LiO^{t}Bu, THF/DMSO (1:1, v/v), 12 h; (c) 1 M $SnCl_2$ in DMF, 12 h; (d) 2.5 equiv of Fmoc-amino acid **9a**−**f**, 5 equiv of DIEA, 2.5 equiv of PyBrOP, NMP, 12 h; (e) 25% TFA/DCM, 1 h; (f) HOAc, reflux, 12 h; (g) 5% piperidine, DCM, 2 h.

of amphotericin B (IC_{50} = 0.5 μM), the reference compound for antifungal treatment.

Since none but hydrogen substitution of **15*Xx*** on the benzimidazole core was connected to activity, we performed a "scaffold hop" to the corresponding non-benzo-anellated 2-aminoalkylimidazoles which resulted in a complete loss of antimycotic activity. Selected structural benzoanellated analogues of **15*Da*** = **1a** and their respective activities are summarized in the supplement including respective IC_{50}, CC_{50}, and SI. Particularly the linkage to the phenyl substituent and the position and nature of the halogen therein are crucial for activity. The essential benzene ring in the benzimidazole core acts most likely as a hydrophobic interaction site. The highest activity and selectivity was determined for the initial hit **15*Da*** which in turn was studied further. Only the 3-fluorobenzyl substituted analogue **15*Ea*** showed comparable activity (Table 1).

Identification of the Potential Molecular Target by Transcriptional Profiling. To identify the mode of action of **15*Da***, transcriptional profiling was performed. For this purpose, the *C. albicans* strain Sc5314 was incubated in rich medium in the presence or absence of **15*Da*** (IC_{50} = 0.75 μM). The cultures were analyzed using DNA microarrays as described elsewhere.[9] A significant number of genes that were found to be up-regulated at least 2.0-fold belong to the Erg pathway, are connected to cell wall biogenesis, or are potential membrane proteins. This indicates that the novel (*S*)-2-aminoalkylbenzimidazole identified has a strong impact on membrane biosynthesis via the ergosterol pathway, similar as this has been shown earlier for the azoles.[15] In contrast to the antifungal benzimidazole benomyl or the anthelmintic mebendazol, **15*Da*** does not affect microtubules.[9]

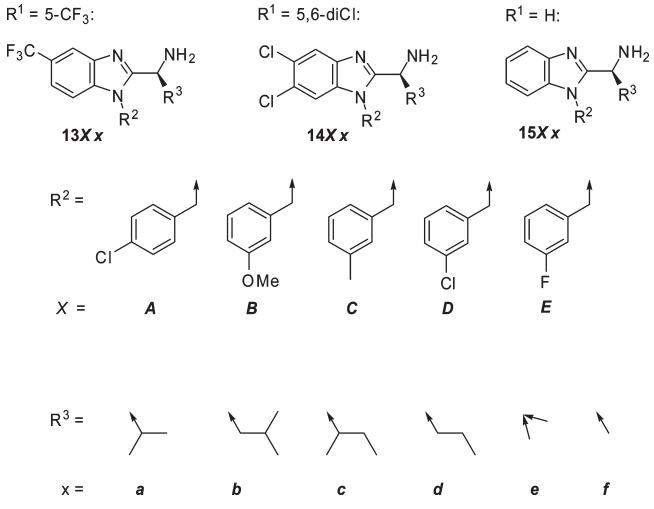


Figure 2. Compound collection **13*Xx***, **14*Xx***, **15*Xx***.

Determination of Minimal Inhibitory Concentrations (MIC) and Comparison to a Reference Antifungal Compound with Proven Activity. To compare the results from the activity−selectivity assay to standard MIC determination, the MICs of **15*Da*** and related compounds were assessed using the M27 standard protocol, which included a comparison of the activities to a reference compound with proven activity (CLSI Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard M27-A3).[16] MICs were

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Table 2. Activity of Five Selected EMC Compounds and Drug References on a Set of 21 Test Strains[a]

test strain	test compd, MIC (μg/mL)					
	15*Cf*	**15*Df***	**15*Ea***	**15*Ca***	fluconazole	**15*Da***
ATCC Control Strains						
C. albicans DSMZ 11949	8	4	16	16	2	2
C. glabrata ATCC 90030	64	8	8	0.25	0.125	0.125
C. parapsilosis 22019	128	4	16	1	1	2
C. parapsilosis 90018	128	4	16	1	0.25	0.5
Issatchenkia orientalis ATCC 6258	128	4	16	1	0.25	0.5
Clinical Isolates						
C. glabrata AN 8626	64	4	16	0.125	4	0.125
C. glabrata CG 7	64	8	64	32	4	2
C. albicans CA20	1	0.125	0.125	0.125	0.125	0.125
C. albicans CA21	64	0.125	0.125	0.5	0.125	0.125
C. albicans MY 2902/2008	32	0.5	16	32	0.5	16
C. albicans SCS 71865 L	128	8	16	32	0.125	64
C. albicans Jg 32570	128	128	128	128	128	16
C. albicans AM2001/0007	64	8	4	16	0.5	0.25
C. albicans AN 1699	128	16	16	64	8	32
C. albicans AN 1994	128	16	2	32	128	16
C. tropicalis AN 1946	128	8	32	8	128	4
C. albicans AN 562	128	16	16	32	1	16
C. albicans VB 1723	128	1	4	32	0.5	0.25
C. albicans AN 3156	128	16	16	32	8	16
C. parapsilosis AN 5485	128	8	16	2	0.25	1
C. parapsilosis AV 7675	128	128	128	128	2	8
av MIC [μg/mL] (geometric mean) for 21 strains	70.66	5.38	10.42	6.78	1.49	2.00
av MIC [μg/mL] (geometric mean) for 16 clinical isolates	72.88	5.66	9.51	11.31	2.18	2.83

[a] *C. albicans* DSMZ 11949, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019, *C. parapsilosis* 90018, and *Issatchenkia orientalis* ATCC 6258 are standard reference strains for in vitro susceptibility testing of yeast. The 16 clinical isolates were previously cultured from specimens of infected patients and selected at random for this study.

measured for type strains, and significant clinical isolates of several *Candida* spp. (Table 2), *C. albicans* DSMZ 11949, *C. glabrata* ATCC 90030, *C. parapsilosis* 22019, *C. parapsilosis* 90018, and *Issatchenkia orientalis* ATCC 6258 were used as reference control strains. Fluconazole was used as a control for antifungal activity of the test compounds. The average minimal inhibitory concentration (MIC_{av}) of **15*Da*** for a set of 16 strains was 2.83 μg/mL, which is within the same concentration as the one of fluconazole (MIC_{av}, 2.18 μg/mL) (Table 2). Interestingly, three of the clinical isolates (*C. albicans* Jg 32570, *C. albicans* AN 1994, and *C. tropicalis* AN 1946) were resistant to fluconazole, but **15*Da*** showed antifungal activity.

CONCLUSION

In summary, a high throughput based screening approach allowed the simultaneous in vitro determination of cytotoxicity in host cells and antifungal activity of a compound against *Candida* spp. Novel nontoxic antimycotic (*S*)-2-aminoalkylbenzimidazoles were identified as antifungal substances, and a structure−activity based characterization was performed including the reduction of the active benzimidazole core to the corresponding nonactive imidazole scaffold. Interestingly, only the (*S*)-stereoisomers of 2-aminoalkylbenzimidazoles were found to be efficacious. By transcription profiling analysis of the response of *Candida albicans* SC5314, (*S*)-2-(1-aminoisobutyl)-1-(3-chlorobenzyl)benzimidazole **15*Da*** indicated a strong impact on membrane biosynthesis pathways of the pathogen. These results give rise to our assumption that primarily the inhibition of the fungal cytochrome P450 oxidase-mediated synthesis of ergosterol is the putative mode of action of (*S*)-2-aminoalkylbenzimidazoles.

Furthermore, we examined the activity against a set of 21 fungal strains including different type strains and significant clinical isolates of *Candida* spp. and confirmed the selective antimycotic activity of (*S*)-2-aminoalkylbenzimidazoles. The most active compounds showed potencies equal to that of fluconazole. Three *Candida* strains that are resistant to fluconazole showed a better susceptibility to **15*Da***. Consequently, the novel nontoxic (*S*)-2-aminoalkylbenzimidazoles represent promising candidates for future developments in antimycotic drug discovery.

EXPERIMENTAL SECTION

Chemistry. General Methods. The (*S*)-2-aminoalkylbenzimidazoles were prepared by solid-phase chemistry (Scheme 1). The purity of the key compounds was > 95% (HPLC-MS, RP-18, GromSil 80, ODS-7 pH, 4 μm, 40 mm × 2 mm; gradient, eluent A (0.1% aqueous TFA) to eluent B (0.1% TFA in acetonitrile) over 8 min; flow rate 0.6 mL/min; MS: Waters Micromass ZQ quadrupole mass spectrometer).

Exemplary Synthesis of (*S*)-2-(1-Aminoisobutyl)-1-(3-chlorobenzyl)benzimidazole (15*Da*). Immobilized 2-nitrophenylcarbamate **6** was synthesized from *o*-nitroaniline **5** and *p*-nitrophenyl carbonate Wang resin **4** using sodium hydride as base in dry DMF. Alkylation with 3-chlorobenzyl bromide and lithium *tert*-butoxide and subsequent reduction with 1 M stannous chloride dihydrate in DMF led to 3-chlorobenzyl-(2-aminophenyl)carbamate Wang resin **8*D***. Acylation of **8*D*** with Fmoc-valine **9*a*** and PyBroP as coupling reagent in DMF was followed by cleavage of **10*Da*** from the polymeric support with 25% TFA in DCM. The collection of lyophilized crude **11*Da*** was cyclized for 16 h in neat glacial acetic acid at 80 °C to produce (*S*)-2-[1-(*N*-Fmoc-amino)isobutyl]-1-(3-chlorobenzyl)benzimidazole **12*Da*** which was purified by flash chromatography. Removal of the Fmoc protecting group led to (*S*)-2-(1-aminoisobutyl)-1-(3-chlorobenzyl)benzimidazole **15*Da*** which was purified by flash chromatography. Yield: 121.8 mg (89%). $C_{18}H_{20}ClN_3$, M = 313.83 g/mol. Purity: 98% (HPLC−ESI-MS, 214 nm), $[M + H]^+$ = 314.6 m/z. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.88 (d, 1H, Ar-*H*, ^3J = 7.6 Hz), 7.62−7.49 (m, 3H, Ar-*H*), 7.33−7.26 (m, 2H, Ar-*H*), 7.18−7.13 (m, 2H, Ar-*H*), 5.66 (dd, 2H, Ar−C*H*$_2$, ^2J = 17.0 Hz), 4.75 (d, 1H, C*H*-NH$_2$, ^3J = 8.7 Hz), 2.60 (m, 1H, C*H*-(CH$_3$)$_2$), 1.17 (d, 3H, CH(C*H*$_3$), ^3J = 6.1 Hz), 0.49 (d, 3H, CH(C*H*$_3$), ^3J = 6.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.9, 148.7, 135.5, 135.1, 132.1, 130.8, 129.5, 127.4, 127.1, 125.3, 124.7, 116.3, 112.4, 70.8, 51.7, 48.7, 32.3, 18.9, 18.7.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis protocols, ^{1}H NMR and ^{13}C NMR data, purification details, yields, purities by HPLC−MS and activity−selectivity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CC, cytotoxic concentration; CYP, cytochrome P450; DCM, dichloromethane; FDA, fluorescein diacetate; Fmoc, fluorenylmethoxycarbonyl; Caco-2, HeLa, A431, and A459, human cell lines; HTS, high-throughput screening; MIC, minimal inhibitory concentrations; PyBroP, bromo-tris-pyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reverse phase high performance liquid chromatography; SI, selectivity index; SAR, structure−activity relationship; TFA, trifluoroacetic acid

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