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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL ARTICLE

Amide- and bis-amide-linked highly potent and broadly active antifungal agents for the treatment of invasive fungal infections-towards the discovery of pre-clinical development candidate FC12406

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Abstract

Most fungal infections are common, localized to skin or mucosal surfaces and can be treated effectively with topical antifungal agents. However, while invasive fungal infections (IFIs) are uncommon, they are very difficult to control medically, and are associated with high mortality rates. We have previously described highly potent bis-guanidine-containing heteroaryl-linked antifungal agents, and were interested in expanding the range of agents to novel series so as to reduce the degree of aromaticity (with a view to making the compounds more drug-like), and provide broadly active high potency derivatives. We have investigated the replacement of the central aryl ring from our original series by both amide and a bis-amide moieties, and have found particular structure-activity relationships (SAR) for both series', resulting in highly active antifungal agents against both mold and yeast pathogens. In particular, we describe the in vitro antifungal activity, absorption, distribution, metabolism and elimination (ADME) properties, and off-target properties of FC12406 (34), which was selected as a pre-clinical development candidate.

Keywords Antifungal · amide · bis-amide · guanidine

Introduction

Most fungal infections are common, localized to skin or mucosal surfaces and can be treated effectively with topical agents. While invasive fungal infections (IFIs) are more uncommon, they are very difficult to control medically, and are associated with high mortality rates, especially in immunosuppressed patients [1]. The two most common fungal species associated with IFIs are *Aspergillus fumigatus* and *Aspergillus flavus*. Importantly, advances in aggressive clinical practices in cancer therapy, and solid organ and hematological transplants have created growing patient populations subjected to extended timeframes of immunosuppression which in turn increases their susceptibilities to life-threatening invasive infections [2–5]. The most common IFI is invasive candidiasis, and is the fourth leading healthcare associated blood stream infection in the US, reaching close to 50% mortality rates [6, 7]. Invasive aspergillosis is also a cause of high mortality in immunocompromised patients with only one-year survival rates of 59% and 25% among solid organ [8] and stem cell transplant recipients [4], respectively. Invasive pulmonary aspergillosis leads to acute respiratory distress syndrome and high mortality in severe influenza patients [9] and a similar clinical progression has been reported recently in intensive care COVID-19 patients [10, 11].

There are only three classes of antifungal agents widely used to treat IFIs; polyenes (e.g. amphotericin B), azoles (e.g. fluconazole, itraconazole, isavuconazole, posaconazole, and voriconazole) and echinocandins (e.g. caspofungin, micafungin, and anidulafungin). All classes target key components of the fungal cell membrane or cell wall,

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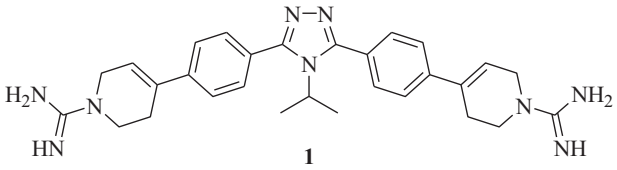
namely ergosterol (polyenes and azoles) or β-(1,3,)-D-glucan (echinocandins), respectively [12]. The limited spectrum of antifungal agents coupled with important shortcomings in their use, including modest success rates in reducing high mortality rates, emerging resistance, especially to the azole and echinocandin classes, detrimental drug interactions, and toxicity liabilities for amphotericin B and several azoles, make the current antifungal armamentarium very vulnerable. Therefore, there is an urgent need for the development of new antifungal agents that are mechanistically distinct from current antifungals to effectively treat these dangerous infections and reduce the potential for appearance of cross resistance.

We have developed a series of non-peptide analogs of the host defense proteins (HDP mimics, smHDPs) as antimicrobial agents that have distinct advantages over peptides for pharmaceutical use [13, 14]. Our approach is to recapitulate the biological and structural properties of HDPs into oligomeric backbones without trying to duplicate the dimensional structures of the peptides, but rather by creating small structurally constrained cationic compounds. The smaller size and improved metabolic stability of smHDPs optimizes their pharmacokinetic and tissue distribution properties over peptidic HDPs and, with the selection of appropriate building blocks, smHDPs can be produced economically. Furthermore, synthetic chemistry provides considerably greater chemical space beyond amino acids to fine-tune structures for enhanced antimicrobial and safety properties.

A number of structurally distinct smHDPs have shown potent activity against *Candida* spp in vitro and in vivo. Minimal inhibitory concentrations (MICs) against *Candida albicans* and non-albicans *Candida* species [15, 16] ranged between 0.5 and 8 μg/mL and selectivity indices were >100-fold over mammalian cells. Several of the compounds were also rapidly fungicidal versus *C. albicans* at 2 – 4x over the MIC showing > $2\log_{10}$ reductions in colony forming units (cfus) within 2–5 h of exposure [16]. Selected compounds were highly efficacious in mouse models of oral candidiasis after a single dose applied topically to the infected tongue [17] and in a disseminated candidiasis model measuring kidney burdens 24 h after a single subcutaneous dose [18]. These animal results indicate that suitable safety and absorption, distribution, metabolism, and elimination (ADME) properties can be incorporated into smHDPs for in vivo administration and activity. Many of the smHDPs described above had limited activity against *Aspergillus* spp. and other molds, so we have embarked on an optimization program to improve the breadth of activities against fungal pathogens.

Recently, we described bis-guanidine smHDPs with a range of substitutions on heteroaryl central rings between bis-aryl rings and terminal guanidines [19]. Our investigations revealed that a 1,2,3,6-tetrahydropyridine was a superior central linker between the aryl rings and the guanidines. In particular, compound **1**, Fig. 1, was found to be non-cytotoxic, as well as highly potent and broadly active against *Candida, Aspergillus* and *Fusarium* spp. [19]. These compounds were significantly more potent against multiple fungal pathogens than other guanidine-containing antifungal agents including bis-guanidine pyrrole derivatives [17], bis-guanidine peptidomimetics of lactoferrin [20], macrocyclic analogs of guazatine [21, 22], the aryl-guanidine abafungin [23] and mono-aminoguanidine analogs described by Miyazaki et al. [24]. More generally, a wide range of guanidine-containing agents have been described as having antifungal [25] and antimicrobial [26] effects, indicative of the potential of guanidine-containing derivatives to serve as potential drugs.



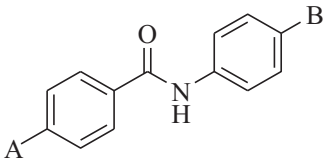
1

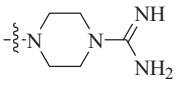
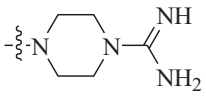
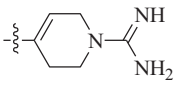
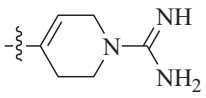
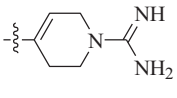
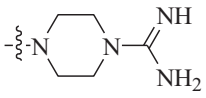
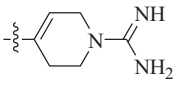
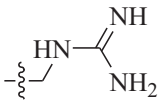
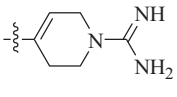
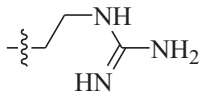
Fig. 1 The lead compound from the heteroaryl-linked series

Results and discussion

In seeking alternate scaffolds for our antifungal agents, we were cognizant that there was a high degree of aromaticity in our heteroaryl-linked analogs such as **1**, Fig. 1 [19]. With this in mind, we initially chose to replace the central aromatic ring with an amide bond, and embarked upon the synthesis of novel compounds. The first compound made was the bis-piperazine derived compound **2**, Table 1. This compound was found to show good potency against four of the six mold strains tested against, and suggested that this would be a productive series to pursue further. We have separately described the use of the 1,2,3,6-tetrahydropyridine as a linker of value in the production of antifungal agents [19], and incorporated this moiety in the amide series, generating derivative **3**, which showed excellent activity against all six of the mold strains, but was found to have higher than acceptable cytotoxicity in NIH3T3 cells (41 μM) (we tested our compounds for cytotoxicity in both NIH3T3 and HepG2 cells). Compared to amphotericin B, **3** was more potent against all six of the strains tested, and showed superior or equivalent potency as posaconazole against the same six strains tested. Hybridization of the 1,2,3,6-tetrahydropyridine and the piperazine resulted in analog **4**, which was reasonably potent but not as active as **3**. Wishing to vary the distance between the two

Table 1 N-phenyl benzamide containing analogs



Cmpd	A	B	MIC (μg/mL) determined in the ATCC strains listed					
			A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
amphotericin B			3.13	3.13	3.13	3.13	1.56	3.13
posaconazole			0.78	0.78	>12.5	1.56	1.56	0.78
2			0.78	0.78	0.78	0.78	>100	>100
3			0.05	0.024	<0.012	<0.012	0.39	0.78
4			0.05	0.050	0.10	0.05	3.13	6.25
5			0.05	0.20	0.05	0.05	>12.5	>12.5
6			0.20	0.39	0.05	0.05	3.13	12.5

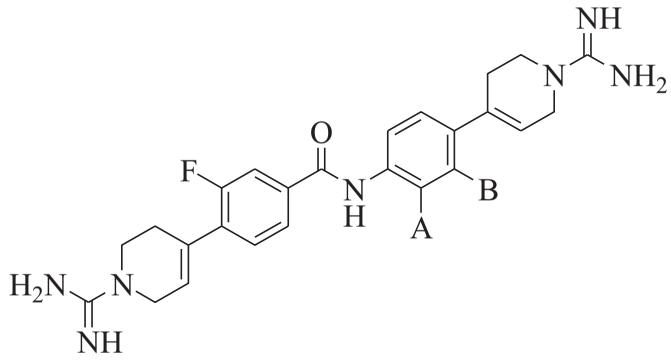
MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus*.

guanidine moieties, combinations of the 1,2,3,6-tetrahydropyridine on the left-hand side with alternate alkyl linkers on the right-hand side were assembled, and two of these, **5** and **6**, were found to possess good potency. Overall, it was clear to us that the optimal template for antifungal activity was with the 1,2,3,6-tetrahydropyridine as the linker on each side, and we focused our efforts in this area.

Focusing on the 1,2,3,6-tetrahydropyridine linked analogs, we next investigated substitutions on the aryl rings. We determined that a 3-fluoro-substitution on the left-hand aryl ring **7**, was well tolerated, Table 2, and chose to utilize this scaffold for investigations in to the substitution preferences for the right-hand aryl ring. Mono-methylation gave **8** and **9**, for which we felt that overall the 3-position substitution, **9**, was preferable. Focusing on this position, we incorporated F (**10**), Cl (**11**), and OMe (**12**), with the methoxy compound (**12**) being particularly interesting to us due to its improved aqueous solubility (1210 μg/mL).

Based on the excellent potencies achieved with a number of the phenyl analogs, we next chose to investigate heteroaryl ring substitution in this system. On the left-hand

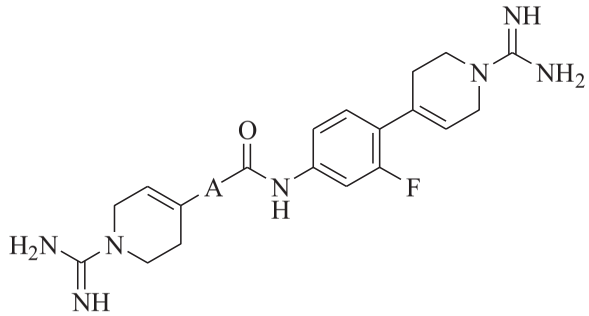
Table 2 SAR around the N-phenyl-3-fluorobenzamide series

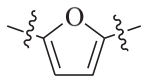
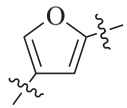
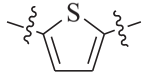
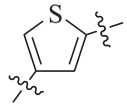


		MIC (μg/mL) determined in the ATCC strains listed						
Cmpd	A	B	*A. fla.* 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
7	H	H	0.05	0.05	<0.012	0.024	0.39	1.56
8	Me	H	0.10	0.20	0.05	0.05	0.39	6.25
9	H	Me	0.10	0.20	0.024	0.05	0.39	3.13
10	H	F	0.10	0.05	<0.012	0.05	0.39	1.56
11	H	Cl	0.20	0.20	<0.012	0.05	0.39	3.13
12	H	OMe	0.20	0.20	0.05	0.10	0.39	6.25

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

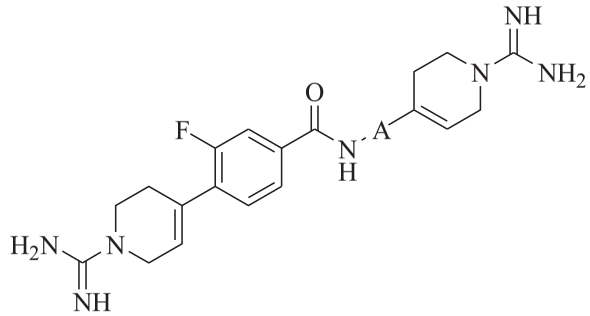
Table 3 Left-hand side aryl derivatives

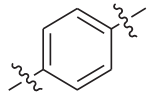
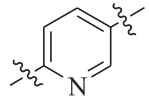
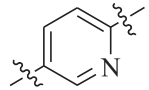


Cmpd	A	MIC (μg/mL) determined in the ATCC strains listed					
		A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
13		0.39	0.20	0.20	0.20	1.56	>25
14		0.024	0.024	<0.012	<0.012	0.20	0.78
15		0.024	<0.012	<0.012	<0.012	0.20	1.56
16		0.39	0.39	0.05	0.10	0.39	1.56

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

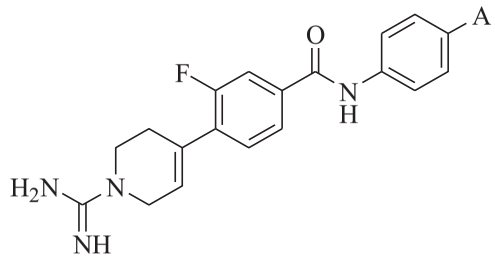
Table 4 Right-hand side pyridyl analogs

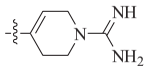
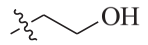
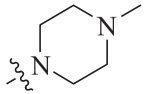
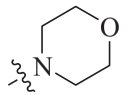


Cmpd	A	MIC (μg/mL) determined in the ATCC strains listed					
		A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
7		0.05	0.05	<0.012	0.024	0.39	1.56
17		0.05	0.024	<0.012	0.024	0.20	0.39
18		0.10	0.05	0.024	0.024	0.39	1.56

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

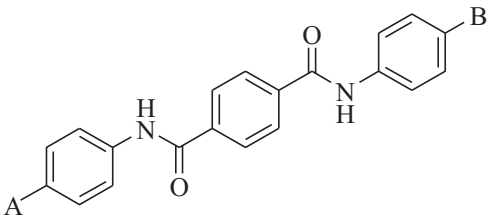
Table 5 Right-hand side guanidine replacements

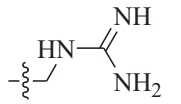
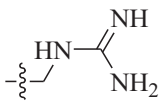
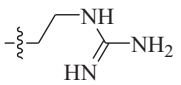
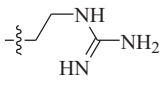
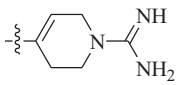
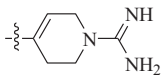
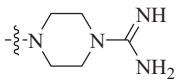
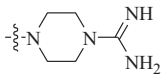
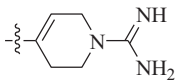
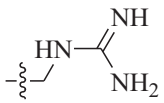


Cmpd	A	MIC (μg/mL) determined in the ATCC strains listed					
		A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
7		0.05	0.05	<0.012	0.024	0.39	1.56
19		>25	>25	>25	>25	>25	>25
20		>25	>25	>25	>25	>25	>25
21		>25	>25	>25	>25	>25	>25

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

Table 6 Initial bis-amide containing analogs



Cmpd	A	B	MIC (μg/mL) determined in the ATCC strains listed					
			A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F. sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
22			1.56	6.25	0.39	0.39	>25	>25
23			6.25	25	3.13	0.78	25	>25
24			0.39	0.20	0.39	0.39	>25	>25
25			0.78	0.39	0.39	0.39	>25	>25
26			<0.024	<0.024	<0.024	<0.024	<0.024	1.56

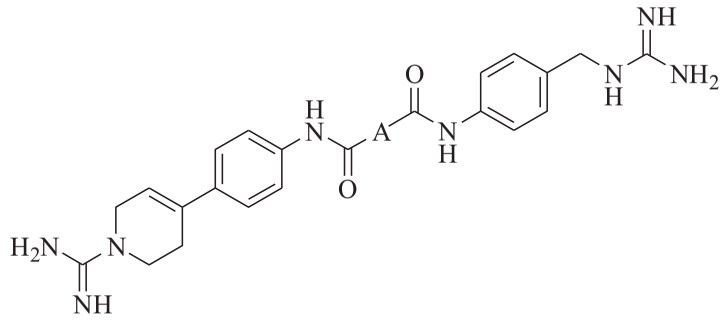
*MIC*Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus*.

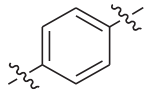
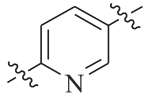
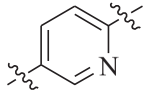
side, 2,5-disubstituted furan **13** showed good activity, Table 3, while the 2,4-disubstituted furan derivative **14** showed exceptional potency against all six strains. The equivalent thiophene compounds were also synthesized, **15** and **16**, and in particular the 2,5-disubstituted analog, **15**, like **14**, displayed excellent potency. On the right-hand side, two pyridyl derivatives were synthesized, with 2-pyridyl **17** showing high potency against all six strains, Table 4, while 3-pyridyl **18** was slightly less active, but still highly potent.

We were also interested in the possibility of replacing one or both of the guanidine moieties with alternate groups so as to increase the functional group diversity of our derivatives, and with this in mind a select number of analogs were made as derivatives of **7**, Table 5. The guanidine replacements were selected to include hydrogen-bond donors and hydrogen-bond acceptors. Unfortunately, none of the derivatives **19, 20**, or **21** were found to possess any antifungal activity, indicating that two guanidine functional groups were necessary for antifungal activity.

Having found that amide-linked analogs have shown good antifungal activity, *vide supra*, we sought to expand the scope of our derivatives further, so as to discover a new series with different properties, and embarked upon the synthesis of novel bis-amide containing compounds. We initially synthesized symmetrical alkyl linked guanidines, with either a methylene or ethylene linker, **22** and **23**, Table 6, as the bis-amide containing analogs were perceived to provide a greater distance between the two guanidine

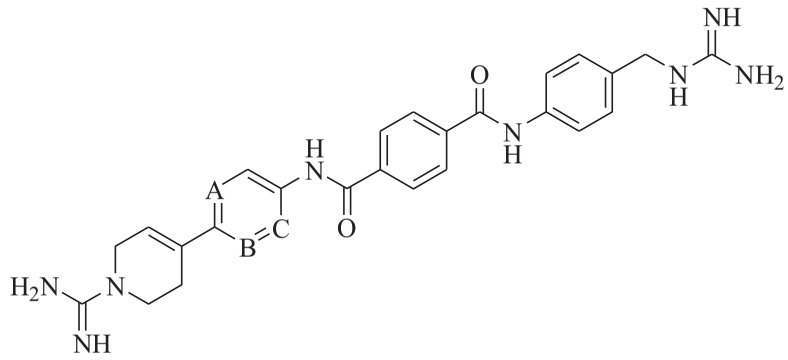
Table 7 Central pyridyl bis-amide analogs



Cmpd	A	MIC (μg/mL) determined in the ATCC strains listed					
		A. fla. 3631	*A. fum*. 3626	*F. fal*. 3636	*F.sol*. 58877	*M. cir*. 26579	*M.ram*. 90286
26		<0.024	<0.024	<0.024	<0.024	<0.024	1.56
27		<0.024	<0.024	<0.024	<0.024	0.10	0.78
28		<0.024	<0.024	<0.024	<0.024	0.10	0.78

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus*.

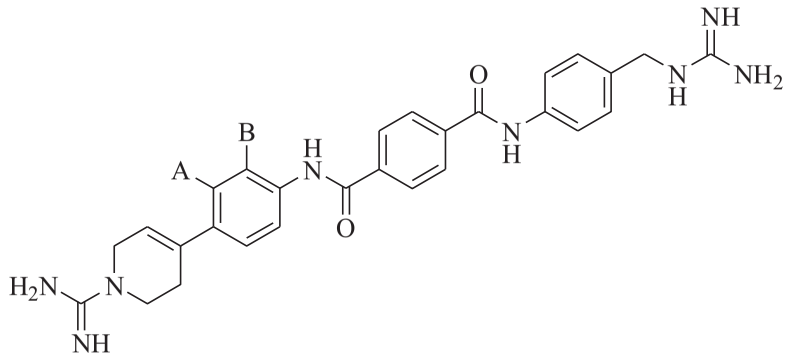
Table 8 Central pyridyl bis-amide analogs



Cmpd	A	B	C	MIC (μg/mL) determined in the ATCC strains listed					
				A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F.sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
26	CH	CH	CH	<0.024	<0.024	<0.024	<0.024	<0.024	1.56
29	CH	CH	N	0.10	0.024	<0.024	<0.024	0.39	0.78
30	CH	N	N	0.024	<0.012	<0.012	<0.012	0.78	3.13
31	N	CH	N	0.20	0.10	0.10	0.10	0.39	0.78

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

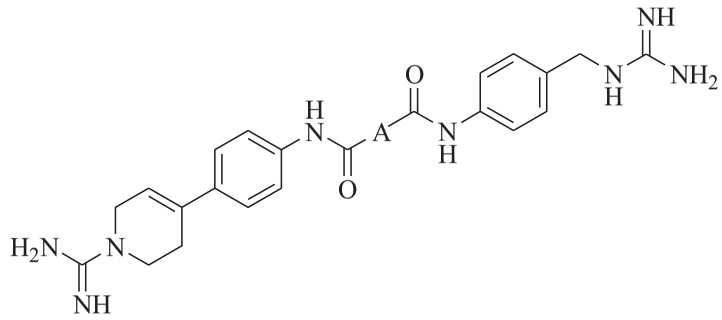
Table 9 Central pyridyl bis-amide analogs

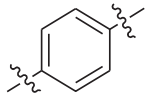
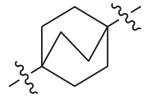
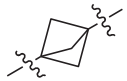


Cmpd	A	B	MIC (μg/mL) determined in the ATCC strains listed					
			A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F.sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
26	H	H	<0.024	<0.024	<0.024	<0.024	<0.024	1.56
32	OMe	H	0.78	0.39	0.78	0.39	0.78	3.13
33	H	OMe	1.56	12.5	0.78	0.39	>25	>25

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

Table 10 Saturated central ring analogs



Cmpd	A	MIC (μg/mL) determined in the ATCC strains listed					
		A. fla. 3631	*A. fum.* 3626	*F. fal.* 3636	*F.sol.* 58877	*M. cir.* 26579	*M. ram.* 90286
26		<0.024	<0.024	<0.024	<0.024	<0.024	1.56
34		0.10	0.10	<0.024	0.05	0.78	3.13
35		0.20	0.20	0.10	0.10	0.39	3.13

MIC Minimal Inhibitory Concentration determined by standardized procedures specified in CLSI M38-A3, *ATCC* American Type Culture Collection. Cmpd: Compound. *A. fla.: Aspergillus flavus. A. fum.: Aspergillus fumigatus. F. fal.: Fusarium falciforme. F. sol.: Fusarium solani. M. cir.: Mucor circinelloides. M. ram.: Mucor ramosissimus.*

Table 11 In vitro ADME and off-target activities for **14**

Stability ($t_{1/2\,min}$); mouse liver microsomes[a]	% binding (at 1 μM); mouse plasma proteins[a]	% inhibition (at 10 μM); CYP450 enzymes[a]			% inhibition (at 10 μM); hERG QPatch[b]
		1A2	2C19	3A-T	
>60	98.3	0	2.2	23	15.2

[a]All studies were done according to standardized procedures at Absorption Systems (Exton, PA, USA).

[b]hERG inhibition studies were conducted at Reaction Biology (Malvern, PA, USA) in triplicate in an automated Q-Patch assay under standard conditions.

Table 12 In vitro ADME and off-target activities for **34**

Stability ($t_{1/2\,min}$); mouse liver microsomes[a]	% binding (at 1 μM); mouse plasma proteins[a]	% inhibition (at 10 μM); CYPP450 enzymes[a]			% inhibition (at 10 μM); hERG QPatch[b]
		1A2	2C19	3A-T	
>60	89.4	0	28.6	24.4	5.3

[a]All studies were done according to standardized procedures at Absorption Systems (Exton, PA, USA)

[b]hERG inhibition studies were conducted at Reaction Biology (Malvern, PA, USA) in triplicate in an automated Q-Patch assay under standard conditions

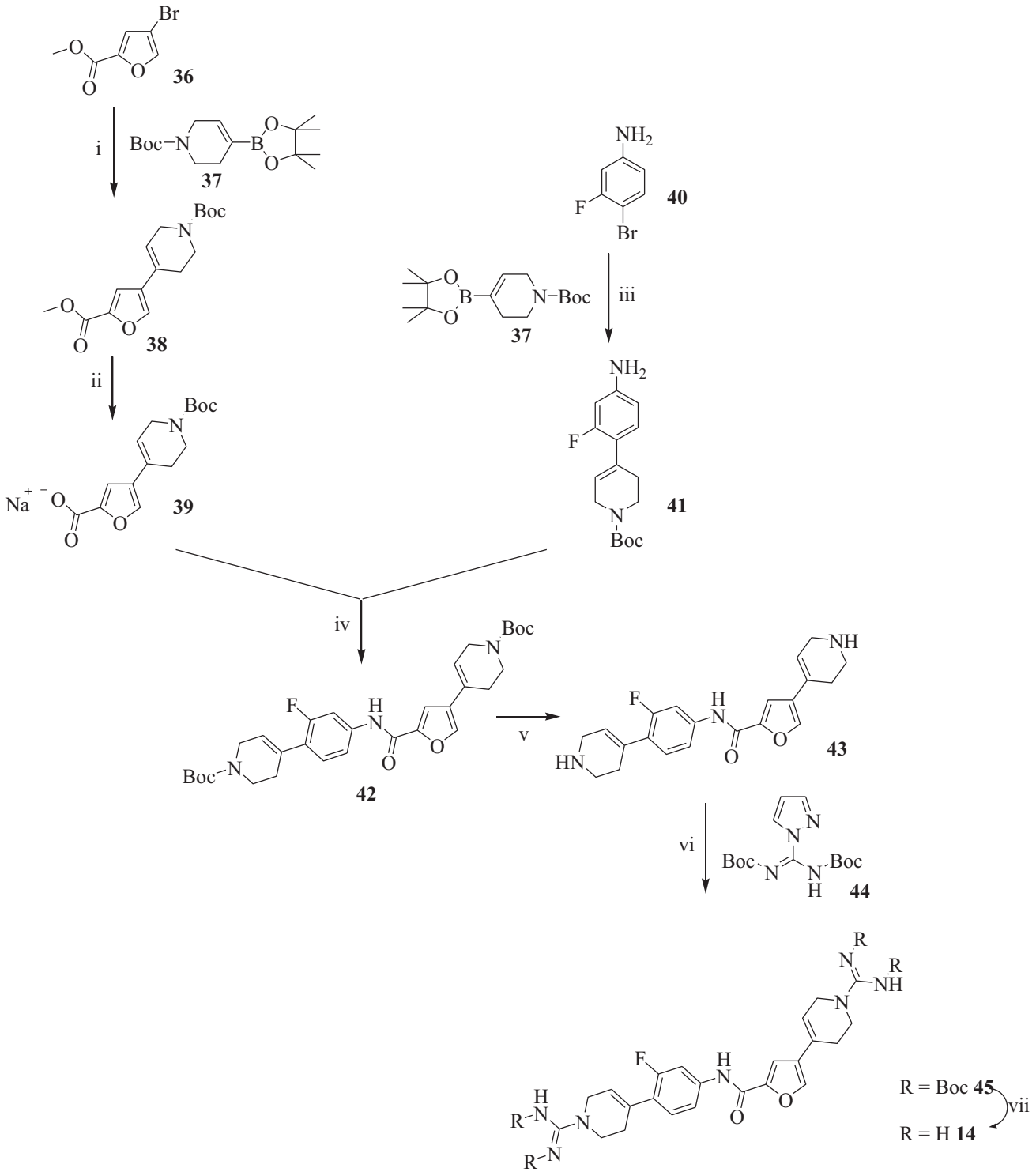
moieties, which we believed to be an important parameter. Of these, compound **22** displayed superior antifungal activity, though far lower than we desired. We have separately described the use of the 1,2,3,6-tetrahydropyridine as a linker of value in the production of antifungal agents, [19], and incorporated this moiety in to the bis-amide linked series, generating derivative **24**, which showed improved activity against four of the mold strains. An alternate connector between the guanidine and aryl ring that was investigated was the piperazine, which gave **25**, and this compound was less active than **24**. Hybridization of the methylene linker from **22** and the 1,2,3,6-tetrahydropyridine linker from **24** resulted in the unsymmetrical **26**, which was highly potent against all six strains. Compound **26** was found to possess higher cytotoxicity than we would prefer (152–170 μM), has high mouse plasma protein binding (>99%) and modest aqueous solubility at pH 7.4 (171 μg/mL). With this information in hand, we moved forward seeking to improve the in vitro properties and cytotoxicity, while maintaining the high potency of **26**.

One method we have sought to utilize for the improvement in aqueous solubility is via the incorporation of an additional basic, saltable, nitrogen into the series. With this in mind, we synthesized two pyridyl derivatives, as variations on the central aromatic ring, Table 7. Both compound **27** and **28** were highly potent, and it was found that while **27** had higher cytotoxicity (CC_{50}: 3T3 > 117 μM, HepG2 > 117 μM), and apparently lower solubility, compound **28** displayed appreciably lower cytotoxicity (CC_{50}: 3T3 > 1171 μM, HepG2 739 μM). Unfortunately, it was shown that **28** had low aqueous solubility at pH 7.4, of only 4.5 μg/mL, and as a result of this information the series was deprioritized.

Heteroaryl left-hand ring analogs of **26** were also synthesized, Table 8. Pyridyl derivative **29**, pyridazine **30**, and pyrazine **31** were all highly potent. The cytotoxicity of all three analogs were superior to **26** [**29** (CC_{50}: 3T3 544 μM, HepG2 353 μM), **30** (CC_{50}: 3T3 231 μM, HepG2 326 μM), and **31** (CC_{50}: 3T3 > 585 μM, HepG2 585 μM)], however the aqueous solubilities of **29, 30**, and **31**, were all noticeably lower than for **26**, although **30** and **31** were two and four times respectively more soluble than **29**.

Seeking to improve the aqueous solubility of compound **26**, we investigated methoxy substitution on the left-hand aryl ring, Table 9. Methoxy was chosen as a group capable of disrupting planarity in the system, and hence improving the solubility [27], and also improving the aqueous solubility of the analogs by virtue of its polarity. Methoxy substitution adjacent to the 1,2,3,6-tetrahydropyridine provided **32**, which was reasonably potent, while substitution next to the amine, **33**, gave a derivative that was less potent. Due to the relatively lowered antifungal activity compared to **26**, neither **32** nor **33** was considered worthwhile to pursue further.

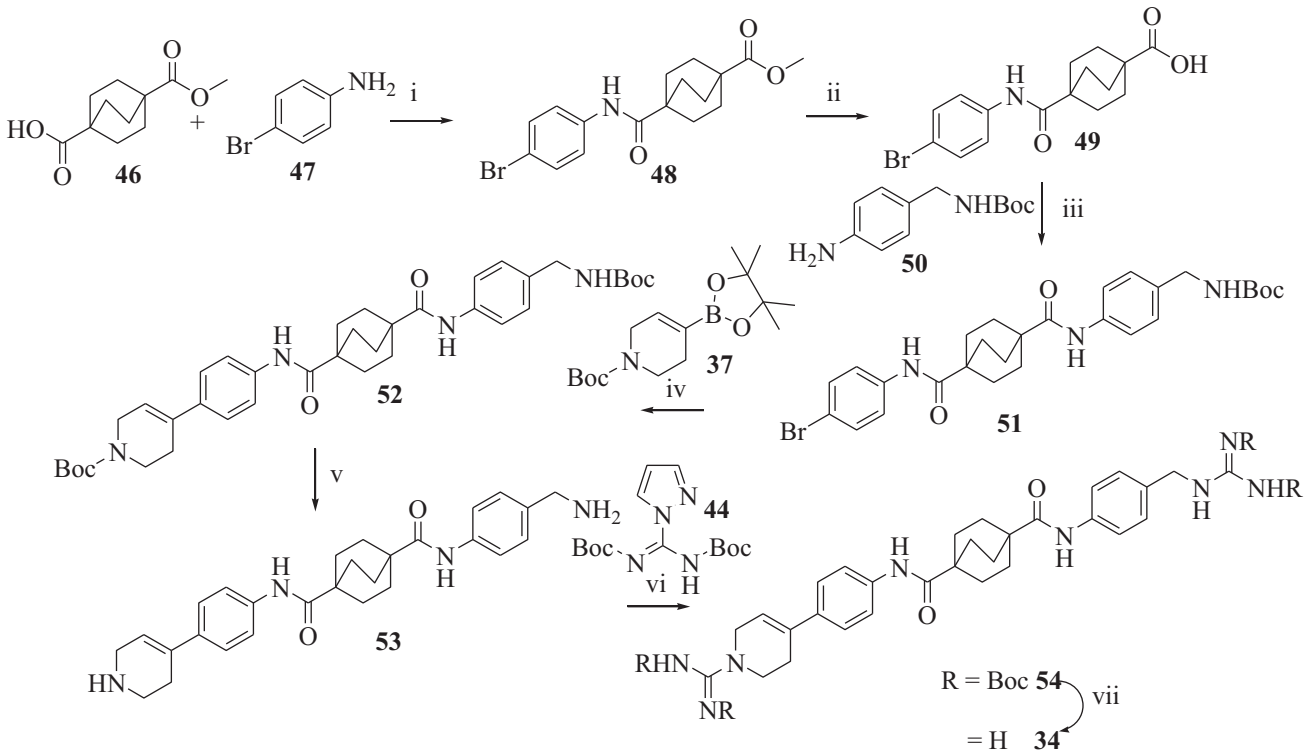
An alternate way that we sought to improve the physical properties of the derivatives was via incorporation of saturation of the central ring, so as to make the compounds less aromatic, more three-dimensional, and more drug-like [28]. We have previously found in the symmetrical bis-amide linked series, that the central phenyl ring could be replaced by a trans-substituted cyclohexane while maintaining potency-data not shown. For the new analogs we chose to use the bicyclco[2,2,2]octane and bicyclo[1,1,1]pentane rings- rings that contain no chiral center, and are also known to be phenyl isosteres [29]. Bicyclo[2,2,2]octane derivative **34** displayed



Scheme 1 (i) $Pd(dppf)Cl_2$ • DCM, 2 M Na_2CO_3, 1,4-dioxane, 80 °C, 7 h, 61%; (ii) 1 N NaOH, MeOH - THF, 40 °C,16 h, 98%; (iii) $Pd(dppf)Cl_2$ • DCM, 2 M Na_2CO_3, 1,4-dioxane, 80 °C, 16 h, 90%; (iv) HATU, $EtNiPr_2$, DMF, 20 °C, 16 h, 58%; (v) TFA, DCM, 20 °C, 3 h, Quantitative; (vi) $EtNiPr_2$, DMF, 20 °C, 16 h, 72%; (vii) TFA, DCM, 20 °C, 3 h, Quantitative Synthesis of **14**

excellent potency against five of the six strains, and bicyclo[1,1,1]pentane analog **35** was also highly potent, Table 10. We were delighted to find that both compounds possessed far lower mouse plasma protein binding than **26** (89 and 83% respectively), and **35** also has five times higher aqueous solubility than **26** (770 µg/mL).

Comparing the different amide-linked analogs, we were most interested in compound **14**, and chose to investigate



Scheme 2 (i) HATU, NEt_3, DMF, 20 °C, 16 h, 85%; (ii) NaOH, 1,4-dioxane, water, 80 °C, 20 h, 90%; (iii) HATU, NEt_3, DMF, 20 °C, 16 h, 53%; (iv) $Pd(OAc)_2$, S-Phos, K_2CO_3, 1,4-dioxane, water, 95 °C, 16 h, 34%; (v) TFA, CH_2Cl_2, 20 °C, 16 h, 93%; (vi) NEt_3, DMF, 35 °C, 16 h, 46%; (vii) TFA, CH_2Cl_2, 20 °C, 16 h, 30% Synthesis of **34**

this compound further. Susceptibility to **14** was tested against a clinical isolate of *Candida albicans*, and the MIC (50% growth inhibition) was 0.78 µg/mL, indicative of broad antifungal activity against both mold and yeast pathogens. Cytotoxicity assays measuring 50% inhibition of metabolic activity (CC_{50}) following 24-h exposures in mouse NIH3T3 fibroblasts and human HepG2 liver cells showed **14** had low cytotoxic CC_{50} values of 68 and 129 µM, respectively, for selectivity indices (CC_{50}/MIC) that ranged over 150-fold. With this data in hand, we chose to move **14** forward into in vitro ADME and off-target studies (Table 11). Compound **14** was stable in mouse liver microsomes (>60 min half-life), had moderately high protein binding in mouse plasma (98.3%), showed little inhibition of three cytochrome P-450 (CYP) enzymes at 10 µM (0 to 23%), and had no meaningful hERG activity at 10 µM in an automated patch clamp assay (15.2%). The aqueous solubility of **14** at pH 7.4 was found to be 58 µg/mL. Unfortunately, when **14** was tested in a three-day mouse maximum tolerated dose (MTD) study, the MTD was determined to be only 5 mg/Kg.

From the different bis-amide linked analogs previously described, we felt that compound **34** was overall the most interesting, and elected to carry out additional studies on this derivative. Susceptibility to **34** was tested against a clinical isolate of *Candida albicans*, and the MIC (50% growth inhibition) was 0.2 µg/mL, showing that this compound has broad antifungal activity against both mold and yeast pathogens. Cytotoxicity assays measuring 50% inhibition of metabolic activity (CC_{50}) following 24-h exposures in mouse NIH3T3 fibroblasts and human HepG2 liver cells showed **34** had low cytotoxic CC_{50} values of 439 and 253 µM, respectively, for selectivity indices (CC_{50}/MIC) that ranged over 80-fold. With this data in hand, we chose to move **34** forward into in vitro ADME and off-target studies (Table 12). Compound **34** was stable in mouse liver microsomes (>60 min half-life), had moderately high protein binding in mouse plasma (89.4%), showed little inhibition of three CYP enzymes at 10 µM (0 to 29%), and had low hERG activity at 10 µM in an automated patch clamp assay (5.3%). The aqueous solubility of **34** at pH 7.4 was found to be 46 µg/mL. When **34** was tested in a three day mouse maximum tolerated dose study, we were delighted to find that the MTD for this compound was 40 mg/Kg.

A representative scheme for the synthesis of the amide-linked analogs is exemplified by the synthesis of **14**, Scheme 1, [30]. Starting from commercially available bromo-furan **36**, Suzuki coupling with **37**, followed by saponification of the ester gave the sodium salt **39**. Separately, Suzuki coupling of bromoaniline **40** with **37**

provided **41** in high yield. Amide coupling of fragment **39** with fragment **41**, mediated by HATU, produced amide **42** in good yield. Bis-Boc removal on **42** using trifluoroacetic acid (TFA) gave a quantitative yield of bis-amine **43**. Guanylation of **43** using the commercially available guanylating agent **44** provided **45** in good yield, and final step deprotection using TFA gave a quantitative yield of **14**.

For the bis-amide linked analogs, the synthesis of **34** is exemplary, and is shown in Scheme 2 [30]. Starting from commercially available bicyclooctane carboxylic acid **46**, an amide coupling was carried out with 4-bromo-aniline **47** to give the mono-amide **48**. Saponification of ester **48** with sodium hydroxide gave acid **49**, and HATU-mediated amide coupling with the requisite aryl amine provided **51**. Suzuki coupling of **51** with commercially available boronate ester **37** gave **52**, and bis-Boc-removal using TFA provided bis-amine **53** in high yield. Reaction of **53** with guanylating agent **44** gave the bis-guanidine **54**, and final step deprotection using TFA provided **34**.

Conclusions

We have shown that amide- and bis-amide-linked bis-guanidine-containing smHDPs are highly potent, and display broad-spectrum antifungal activities which are comparable or superior to current clinically utilized antifungal agents. For the amide-linked series, a range of substitutions have been shown to be tolerated on the amide-linked rings, and heteroaryl rings are also in general well tolerated in the system. Investigations into the linker between the guanidine and aryl rings showed that the 1,2,3,6-tetrahydropyridine was the optimal linker on both sides of the molecules. In the bis-amide-linked series, it has been shown that asymmetry, with the incorporation of one methylene-linked guanidine and one 1,2,3,6-tetrahydropyridine-linked guanidine was important for high antifungal activity. In this series, replacement of the central aromatic ring with saturated rings was well tolerated. In particular, compound **34** was found to be highly potent, broadly active, non-cytotoxic, and to have good in vitro ADME and off-target properties. As a result of the overall profile of the compound, **34** (FC12406) was selected as a pre-clinical development candidate.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

References

1. Brown GD, Denning DW, Levitz SM. Tackling human fungal infections. Science. 2012;336:647 https://doi.org/10.1126/science.1222
2. Perfect JR. The antifungal pipeline: a reality check. Nat Rev Drug Discov. 2017;16:603–16. https://doi.org/10.1038/nrd.2017.46
3. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis. 2010;50:1091–1100. https://doi.org/10.1086/651263
4. Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, et al. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dise. 2010;50:1101–11. https://doi.org/10.1086/651262
5. Rüping MJ, Vehreschild JJ, Cornely OA. Patients at high risk of invasive fungal infections: when and how to treat. Drugs. 2008;68:1941–62. https://doi.org/10.2165/00003495-200868140-00002
6. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis. 2016;62:e1–50. https://doi.org/10.1093/cid/civ933
7. Antibiotic Resistance Threats in the Unites States. 2019 Centers for Disease Control and Prevention; 2019. Available from https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf. Accessed 2/6/2023
8. Webb BJ, Ferraro JP, Rea S, Kaufusi S, Goodman BE, Spalding J. Epidemiology and clinical features of invasive fungal infection in a US health care network. *Open Forum Infect. Dis*. 2018 Aug (Vol. 5, No. 8, p. ofy187). US: Oxford University Press. https://doi.org/10.1093/ofid/ofy187
9. Schauwvlieghe AF, Rijnders BJ, Philips N, Verwijs R, Vanderbeke L, Van Tienen C. et al. Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study. Lancet Respir Med. 2018;6:782–92. https://doi.org/10.1016/S2213-2600(18(30274-1.
10. Mohamed A, Rogers TR, Talento AF. COVID-19 associated invasive pulmonary aspergillosis: diagnostic and therapeutic challenges. J Fungi. 2020;6:115 https://doi.org/10.3390/jof6030115
11. Silva LN, de Mello TP, de Souza Ramos L, Branquinha MH, Roudbary M, Dos Santos AL. Fungal infections in COVID-19-positive patients: a lack of optimal treatment options. Curr Top Med Chem. 2020;20:1951–7. https://doi.org/10.2174/1568026620220091711010
12. Arastehfar A, Lass-Flörl C, Garcia-Rubio R, Daneshnia F, Ilkit M, Boekhout T, et al. The quiet and underappreciated rise of drug-resistant invasive fungal pathogens. J Fungi. 2020;6:138 https://doi.org/10.3390/jof6030138
13. Scott RW, Tew GN. Mimics of host defense proteins; strategies for translation to therapeutic applications. Curr Top Med Chem. 2017;17:576–89.
14. Tew GN, Scott RW, Klein ML, DeGrado WF. De novo design of antimicrobial polymers, foldamers, and small molecules: from

discovery to practical applications. Acc Chem Res. 2010;43:30–9. https://doi.org/10.1021/ar900036b
15. Hua J, Yamarthy R, Felsenstein S, Scott RW, Markowitz K, Diamond G. Activity of antimicrobial peptide mimetics in the oral cavity: I. Activity against biofilms of Candida albicans. Mol oral Microbiol. 2010;25:418–25. https://doi.org/10.1111/j.2041-1014.2010.00590.x
16. Ryan LK, Freeman KB, Masso-Silva JA, Falkovsky K, Aloyouny A, Markowitz K, et al. Activity of potent and selective host defense peptide mimetics in mouse models of oral candidiasis. Antimicrob Agents Chemother. 2014;58:3820–7. 110.1128/AAC.02649.13
17. Jana GH, Jain S, Arora SK, Sinha N. Synthesis of some diguanidino 1-methyl-2, 5-diaryl-1H-pyrroles as antifungal agents. Bioorg Med Chem Lett. 2005;15:3592–5. https://doi.org/10.1016/j.bmcl.2005.05.080
18. Menzel LP, Chowdhury HM, Masso-Silva JA, Ruddick W, Falkovsky K, Vorona R, et al. Potent in vitro and in vivo antifungal activity of a small molecule host defense peptide mimic through a membrane-active mechanism. Sci Rep. 2017;7:4353 https://doi.org/10.1038/s41598-017-04462-6
19. Baugh SDP, Chaly A, Weaver DG, Pelletier JC, Thanna S, Freeman KB, et al. Highly potent, broadly active antifungal agents for the treatment of invasive fungal infections. Bioorg Med Chem Lett. 2021;33:127727 https://doi.org/10.1016/j.bmcl.2020.127727
20. Stensen W, Turner R, Brown M, Kondori N, Svendsen JS, Svenson J. Short cationic antimicrobial peptides display superior antifungal activities toward candidiasis and onychomycosis in comparison with terbinafine and amorolfine. Mol Pharm. 2016;13:3595–3600. https://doi.org/10.1021/acs.molpharmaceut.6b00654
21. Dreassi E, Zizzari AT, D'Arezzo S, Visca P, Botta M. Analysis of guazatine mixture by LC and LC–MS and antimycotic activity determination of principal components. J Pharm Biomed Anal. 2007;43:1499–506. https://doi.org/10.1016/j.jpba.2006.10.029
22. Manetti F, Castagnolo D, Raffi F, Zizzari AT, Rajamaki S, D'Arezzo S, et al. Synthesis of new linear guanidines and macrocyclic amidinourea derivatives endowed with high antifungal activity against Candida spp. and Aspergillus spp. J Med Chem. 2009;52:7376–9. https://doi.org/10.1021/jm900760k
23. Borelli C, Schaller M, Niewerth M, Nocker K, Baasner B, Berg D, et al. Modes of action of the new arylguanidine abafungin beyond interference with ergosterol biosynthesis and in vitro activity against medically important fungi. Chemotherapy. 2008;54:245–59. https://doi.org/10.1159/000142334
24. Nakada-Motokawa N, Miyazaki T, Mizuta S, Tanaka Y, Hirayama T, Takazono T, et al. Design and synthesis of a class of compounds that inhibit the growth of fungi which cause invasive infections. ChemistrySelect. 2020;5:1140–5. https://doi.org/10.1002/slct.201904380
25. Baugh SDP. Guanidine-containing antifungal agents against human-relevant fungal pathogens (2004–2022)—a review. J Fungi. 2022;8:1085 https://doi.org/10.3390/jof8101085
26. Kim SH, Semenya D, Castagnolo D. Antimicrobial drugs bearing guanidine moieties: a review. Eur J Med Chem. 2021;216:113293 https://doi.org/10.1016/j.ejmech.2021.113293
27. Ishikawa M, Hashimoto Y. Improvement in aqueous solubility in small molecule drug discovery programs by disruption of molecular planarity and symmetry. J Med Chem. 2011;54:1539–54. https://doi.org/10.1021/jm101356p
28. Lovering F, Bikker J, Humblet C. Escape from flatland: increasing saturation as an approach to improving clinical success. J Med Chem. 2009;52:6752–6. https://doi.org/10.1021/jm901241e
29. Subbaiah MA, Meanwell NA. Bioisosteres of the phenyl ring: recent strategic applications in lead optimization and drug design. J Med Chem. 2021;64:14046–128. https://doi.org/10.1021/acs.jmedchem.1c01215
30. Full synthetic methods for the synthesis of all compounds described can be found in the supporting information.