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Quinazolinone fungal efflux pump inhibitors. Part 3: (*N*-methyl)piperazine variants and pharmacokinetic optimization

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Abstract—Further structure–activity relationships of a novel series of fungal efflux pump inhibitors with respect to potentiation of the activity of fluconazole against strains of *C. albicans* and *C. glabrata* over-expressing ABC-type efflux pumps are systematically explored. Rat protein binding and pharmacokinetics of selected analogues are reported.

The incidence of systemic fungal infections due to organisms that are not susceptible to commonly used antifungal agents such as fluconazole continues to increase, and the association of this phenomenon with overproduction of multidrug transporters of the ABC and Major Facilitator superfamilies is now well established.[1,2] We have previously reported the discovery of a series of inhibitors of efflux pumps in *Candida* spp. exemplified by MC-5805 (**1**, Fig. 1).[3] Structure–activity relationships for in vitro potentiation of fluconazole (FLU) versus *Candida albicans* and *Candida glabrata* for 3-(*N*′-methyl-piperazinyl)quinazolinones of this class have also been described.[4] These studies demonstrated that incorporation of an *N*-methyl-piperazine motif into early screening hits broadened the spectrum of inhibition of ABC-type transporters to include all clinically relevant *Candida* spp., and culminated in the identification of **2** as a 3-(*N*-methyl-piperazinyl)quinazolinone with optimal activity for the potentiation of fluconazole in vitro.

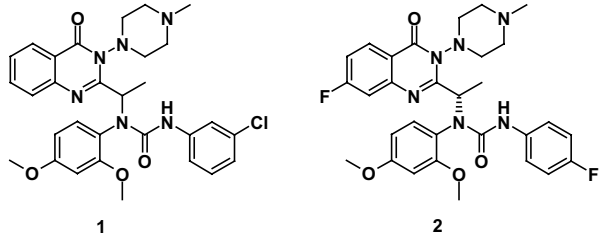

Figure 1. Prototypical leads.

Herein the results of further studies of analogues varying the *N*-methyl-piperazine moiety are reported, both in terms of their in vitro activity and with regard to pharmacokinetics in rats.

All but one of the analogues reported were synthesized by acylation of the piperazine that is the product of the route shown in Scheme 1.

The route developed for the synthesis of earlier analogues[4] was adapted to allow for facile variation of piperazine substituents by demethylation of the piperazine and protection prior to functionalization of the 2-ethyl substituent on the quinazolinone. Acylations were accomplished using conventional reagents.

Keywords: Quinazolinone; Antifungal; Efflux; EPI; Efflux pump inhibitor; Synergy; Fluconazole; Candida.
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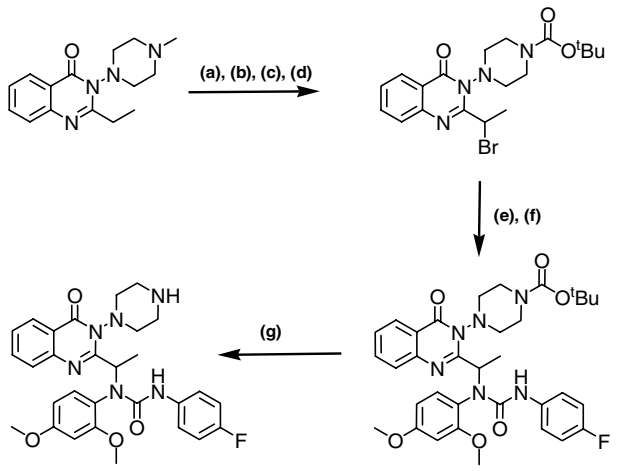


Scheme 1. Reagents and conditions: (a) $ClCO_2CH(Cl)Me$, $PhCH_3$, 95 °C, 38%; (b) 6N HCl, 80 °C; (c) BOC_2O, THF, 45 °C; (d) Br_2, NaOAc, AcOH, 50 °C, 80% over 3 steps; (e) 2,4-dimethoxyaniline, EtOAc, reflux; (f) 4-fluorophenylisocyanate, $ClCH_2CH_2Cl$, rt, 84%; (g) TFA, CH_2Cl_2, rt, 94%.

The piperidine derivative **5** was made by the route reported earlier,[3] using 1-aminopiperidine instead of 1-amino-4-methylpiperazine.

New compounds were initially synthesized as isomeric mixtures via the racemic bromide. The single isomer **32** was generated using lactic acid (instead of propionic acid) as the ultimate starting material, by an analogous route to that previously described.[3]

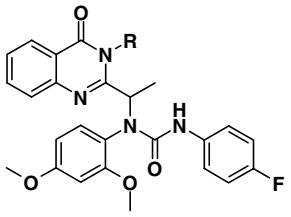
The activity of analogues was conveniently expressed as the minimum potentiation concentration (MPC_8), which is the lowest concentration achieving an 8-fold reduction in FLU MIC. An initial survey of the activity of variants of the *N*-methyl-piperazine moiety is summarized in Table 1.

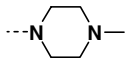
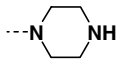
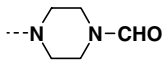
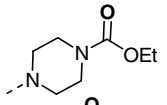
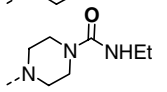
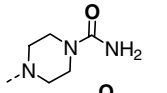
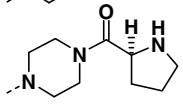
Both the des-methyl analogue **4** and the piperidine **5** are significantly less active than **3**, but the activity is restored in amide or carbamate derivatives, exemplified by **6** and **7**. The urea variant **8** of the ethyl carbamate also shows good activity, but removal of the ethyl group (as in **9**) causes a reduction in potency. These data demonstrate the benefit of incorporating an acyl motif as an H-bond acceptor on the piperazine 4-position for retaining activity against both *C. albicans* and *C. glabrata*. Unfortunately, this tactic removes the basic entity that provides the solubility benefit in **1** relative to earlier leads.

It is also notable that in analogues **3**–**9** the SAR in *C. glabrata* parallels that in *C. albicans*. In the proline derivative **10**, by contrast (and unlike in earlier screening hits,[3]) the activity in *C. glabrata* is retained selectively. This intriguing profile, coupled with the solubility benefit arising from the re-introduction of a basic entity, led us to explore related analogues in more detail (Table 2).

Once again it is evident that in order to achieve good potency against *C. albicans*, a strong H-bond acceptor

Table 1. Variants of the *N*-methyl-piperazine moiety in **1**



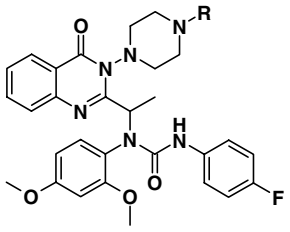
Compound	R	MPC_8 (μg/mL)	
		C. albicans	*C. glabrata*
3		4	4
4		>32	32
5		>32	>32
6		2	4
7		0.25	0.5
8		1	1
9		8	16
10		>32	4

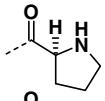
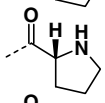
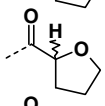
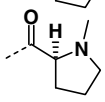
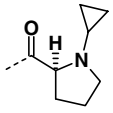
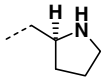
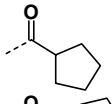
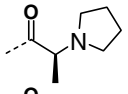
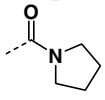
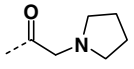
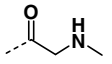
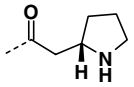
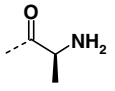
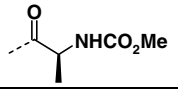
distal to the piperazine is required, and that a basic nitrogen (particularly a primary or secondary amine) is disadvantageous even in analogues where this requirement is fulfilled. Thus, the most potent analogues against both organisms are the amide **16** and urea **18**. Even in *C. glabrata*, where a basic nitrogen is better tolerated, the activity is optimal in neutral compounds.

Accepting this design constraint, we were attracted to the profile of the alanine derivative **23**, which exhibited good activity against both organisms while retaining reasonable physical characteristics, and explored closely related variants of this compound (Table 3).

Activity is only marginally dependent upon the alanine stereochemistry, but branching at this position is required (**25** vs **23**/**24**). As the lipophilicity increases, the activity improves (**26**–**29**). The combination of the urea motif with the methyl carbamate again gives better potency (**30**), with *C. albicans* showing greater sensitivity to the stereochemical disposition of the hydrophilic entity (**31**). Overall, we concluded that **23** represents the preferred piperazine substituent, based upon its potency, balanced spectrum, and reasonable lipophilicity.

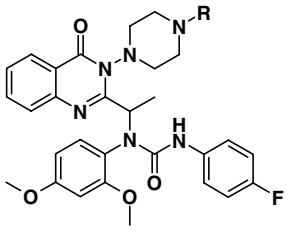
Table 2. Variants of **10**

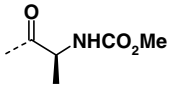
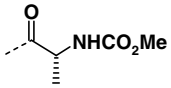
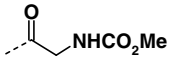
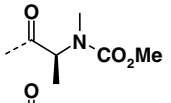
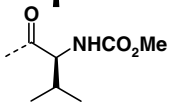
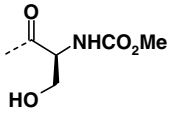
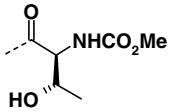
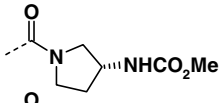
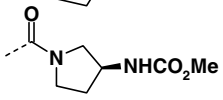


Compound	R	MPC_8 (μg/mL)	
		C. albicans	*C. glabrata*
10		>32	4
11		>32	2
12		0.5	0.125
13		32	2
14		1	0.25
15		32	2
16		0.25	0.125
17		8	0.25
18		0.0625	<0.03
19		4	1
20		>32	4
21		>32	0.5
22		>32	4
23		2	0.25

In parallel with measurement of fluconazole potentiation in vitro, pharmacological assays expected to be predictive of pharmacokinetic profiles were also performed and are summarized in Table 4.

Table 3. Non-basic variants of **10**



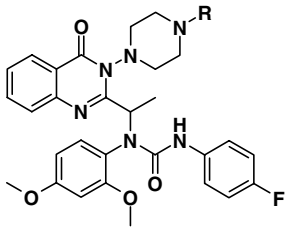
Compound	R	MPC_8 (μg/mL)	
		C. albicans	*C. glabrata*
23		2	0.25
24		4	0.5
25		>32	2
26		0.5	0.5
27		1	2
28		32	8
29		2	2
30		0.25	0.5
31		4	0.25

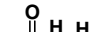
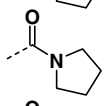
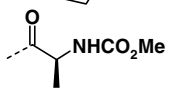
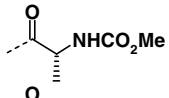
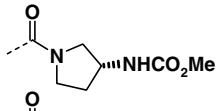
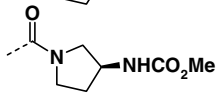
The protein binding of the analogues measured was in a reasonable range, but the sensitivity to metabolic degradation varied widely. The more lipophilic (and more active) compounds (**18**, **30**) tended to be less stable upon microsomal incubation. Notably, the rate of degradation was sensitive to the stereochemistry of the side chain (**23** vs **24**; **30** vs **31**).

The superior activity and pharmacological properties of the (L)-alanine carbamate in **23** were combined with the structural features of other regions of the lead that had previously been shown to be optimal,[4] culminating in the synthesis of **32**. The in vitro and in vivo characteristics of this compound are compared with those of the original lead **1** in Table 5.

The increased potency and improved pharmacokinetic profile of **32** over **1** translated into better efficacy as a

Table 4. Rat serum protein binding and hepatocyte stability data for selected analogues



Compound	R	Protein binding[a] (%)	Hepatocyte stability[a] (% remaining after 24 h)
3		74	18
10		77	77
18		ND	0
23		82	39
24		82	7
30		ND	4
31		ND	23

ND, not determined.
[a] See Serum protein binding and hepatocyte stability.

potentiator of the activity of fluconazole in vivo. These experiments will be the subject of a future publication.

In this and the previous studies,[3,4] the absolute structural requirements for satisfactory efflux pump inhibition leading to potentiation of the antifungal activity of fluconazole in vitro by 3-(piperazinyl)quinazolinones such as **1** have been outlined and optimized. By carefully modulating the hydrophilicity of the piperazinyl substituent, it proved possible to identify potent analogues with a balanced spectrum of activity in clinically relevant *Candida* spp., combined with reasonable protein binding and metabolic stability. These studies culminated in the synthesis of **32**, an excellent tool for studies into the feasibility of potentiation of the activity of fluconazole in animal models of fungal infection.

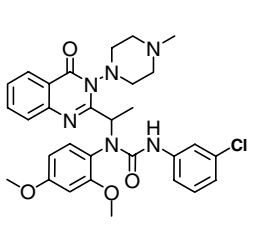
Chemicals. All analogues were purified by reverse-phase MPLC using a Phenomenex Synergy Hydro-RP 50×21.2 mm column, eluting with MeCN/water (10–100%, ramped over 20 min) at 20 mL/min, and tested as the TFA or methanesulfonic acid salt; no precipitation was observed at the test concentrations employed for assessment of antifungal activity. The structural identity of each compound was confirmed by ^{1}H NMR and MS.

The purity of the single isomer **32** was assessed as >95% by chromatography using a Chiralpack AD 250×4.6 mm column, eluting with 30% 2-propanol in hexanes.

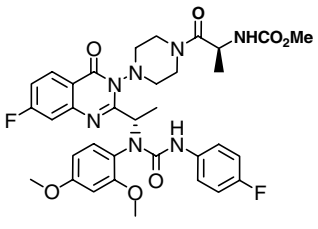
In vitro potentiation of fluconazole. FLU MICs for *C. albicans* strain YEM15 (over-expressing both CDR1 and CDR2) and *C. glabrata* strain YEM19 (over-expressing both CgDR1 and CgDR2) were measured in the presence and absence of varying concentrations of efflux pump inhibitor (checkerboard format). Drugs were tested in RPMI 1640 according to CLSI reference methods.[5] None of the analogues displayed intrinsic antifungal activity (MIC > 32 μg/ml). Not all of the analogues reported in this study were assessed in full checkerboard format; an abbreviated method of evaluation involved testing varying concentrations of efflux pump inhibitor in the presence of fixed concentrations of FLU equivalent to 1/8 and 1/32 MIC. This method was validated on a set of eight analogues by comparison with the MPC_8 values derived from full checkerboard studies; the results were identical.

Serum protein binding and hepatocyte stability. Serum protein binding was measured by adding compounds

Table 5. In vitro and in vivo profiles of **1** and **32**



1



32

Compound	MPC_8 (μg/mL)		Rat protein binding (%)	Rat CL (L/h/kg)	Rat CL_{free}(L/h/kg)
	C. albicans	*C. glabrata*			
1	1	4	98	5	225
32	0.5	0.0625	87	7.5	60

to fresh rat serum at three concentrations then separating bound from unbound by ultrafiltration using an Amicon Centrifree Micropartition device. Unbound filtrates were assayed using LC/MS/MS.

Freshly isolated rat hepatocytes attached to collagen coated 24-well plates were purchased from Cedra Corporation (Austin, TX) and maintained in nutrient culture medium. Dosing solutions of each compound were prepared at 1 and 5 μg/mL, and added to duplicate cultures. At 3, 6, and 24 h after the start of exposure, samples of the culture medium were taken and added to methanol, vortexed, and centrifuged. The supernatant was quantified using an LC/UV system.

Pharmacokinetic studies in rats. Serum pharmacokinetics were evaluated in male Sprague-Dawley rats (3 per compound) with implanted femoral and jugular vein cannulas. Compounds were administered by bolus injection via the femoral vein cannula at dose of 5 mg/kg. Blood samples were collected for up to 8 h from the jugular vein cannula and were assayed for concentration of compound using LC/MS/MS. Total clearance (CL) values were determined using a two-compartment pharmacokinetic model.

References and notes

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