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Quinazolinone fungal efflux pump inhibitors. Part 2: In vitro structure–activity relationships of (*N*-methyl-piperazinyl)-containing derivatives

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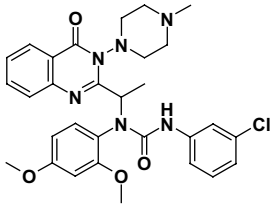
Abstract—Structure–activity relationships of a novel series of fungal efflux pump inhibitors with respect to potentiation of the activity of fluconazole against strains of *Candida albicans* and *Candida glabrata* over-expressing ABC-type efflux pumps are systematically explored.

1. Introduction

The design and execution of a screening strategy for the discovery of inhibitors of efflux pumps in *Candida albicans* has recently been described.[1] From these efforts two main chemical series emerged: natural products of the milbemycin type,[2,3] and synthetic compounds based upon a quinazolinone core. Screening hits in the latter series showed exquisite selectivity for the inhibition of an ABC-type pump in *C. albicans* (CDR1); the lack of activity against azole-resistant *Candida* spp. (particularly *C. glabrata*), and the very limited aqueous solubility gave cause for concern about the viability of the series as a starting point for optimization. However, exploratory chemistry showed that the introduction of a basic substituent gave a dramatic improvement in aqueous solubility and increased the spectrum of inhibition to include *Candida* spp. other than *C. albicans*[4] These studies culminated in the discovery of MC-5805 (**1**, Fig. 1) and stimulated a more thorough exploration of the structural requirements for activity elsewhere in the molecule.



1

Figure 1. MC-5805 (**1**).

We now report the structure–activity relationships for in vitro potentiation of fluconazole (FLU) versus *C. albicans* and *C. glabrata* for 3-(*N*′-methyl-piperazinyl)quinazolinones of this type.

2. Chemistry

New compounds were initially synthesized as racemates using routes analogous to that used for **1**, which is shown in Scheme 1. The enantiomers of **2** were

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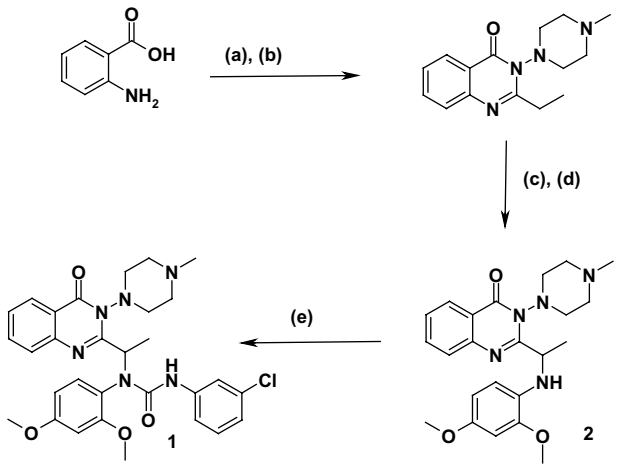


Scheme 1. Racemic synthesis. Reagents and conditions: (a) $(EtCO)_2O$, 70 °C, forming the isatoic anhydride; (b) 1-amino-4-methylpiperazine, AcOH, 70 °C; (c) Br_2, AcOH, NaOAc; (d) 2,4-dimethoxyaniline, *N*-methylpyrrolidinone, 80 °C; (e) 3-chlorophenylisocyanate, 1,2-dichloroethane.

separated by chiral HPLC, facilitating the preparation of enantiomerically pure final products.

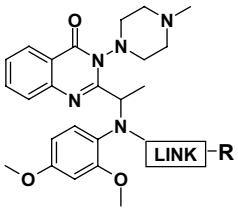
The synthesis of all variants of the quinazolinone core started from the requisite anthranilic acid. Displacement of the bromide by 2,4-dimethoxyaniline leading to **2** required unusually forcing conditions, indicative of significant steric hindrance. Although the yield of **2** was satisfactory (85%), it was lower for less nucleophilic reagents (e.g., 2,4-difluoroaniline, 48%) and the reaction failed altogether for (3-trifluoromethylsulfonyl)aniline. Variants of the (3-chlorophenyl)urea were made by reaction of the aniline **2** with the appropriate electrophile. Thus for the carbamate **13**, sulfonamide **14** and the amide **16** the aniline was treated with (4-chloro)phenylchloroformate, (4-chloro)phenylsulfonyl chloride and (4-chloro)benzoyl chloride, all in the presence of triethylamine; for the acylsulfonamide **15**, treatment with (4-chloro)phenylsulfonyl isocyanate in the absence of base provided the desired material.

3. Structure–activity relationships

The relative 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. Initially, variants of the 3-chlorophenyl urea moiety were examined, and Table 1 displays the results for representative examples.

The lack of activity of **2** confirmed the requirement for some sort of derivative of the free aniline, and the inactivity of the alkyl urea **3** illustrates the need for an aromatic substituent. A clear preference for *meta*- or *para*- over *ortho*-substitution is evident among the chloro-substituted phenyl ureas **1**, **4** and **5**; the latter is even less active than the unsubstituted analogue **6**, and this observation leads us to presume that the presence of the 2-substituent might drive a change in the local

Table 1. Variants of the 3-chlorophenyl urea moiety in **1**



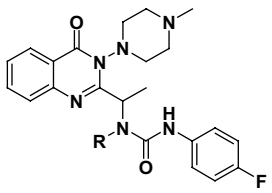
No.	Link	R	MPC_8 (μg/mL)	
			C. albicans	*C. glabrata*
1	CONH	(3-Cl)Ph	1	4
2	H	—	>32	>32
3	CONH	iPr	>32	>32
4	CONH	(4-Cl)Ph	2	1
5	CONH	(2-Cl)Ph	>32	>32
6	CONH	Ph	16	16
7	CONH	(4-F)Ph	4	4
8	CONH	(4-Me)Ph	16	4
9	CONH	(4-OMe)Ph	16	2
10	CONH	(3-CN)Ph	32	32
11	CONH	(3-Cl)-6-Pyridazinyl	16	8
12	CONH	2-Benzthiazolyl	2	16
13	CO_2	(4-Cl)Ph	32	32
14	SO_2	(4-Cl)Ph	32	>32
15	$CONHSO_2$	(4-Cl)Ph	>32	>32
16	CO	(4-Cl)Ph	4	>32
17	CO	(2-Cl)Ph	4	>32

conformation, forcing the aryl ring out of co-planarity with the urea linkage. Overall, the 3- or 4-halo-substituted compounds **1**, **4** and **7** show the best balance of potency and activity against both *Candida* spp.

It proved difficult to find a heterocyclic replacement for the phenyl ring: even the relatively conservative pyridazine isostere was significantly less active (cf. **11** vs **4**), and although other more lipophilic alternatives such as the benzthiazole **12** showed good activity against *C. albicans*, the *C. glabrata* potency was reduced.

A brief survey of alternative linkers demonstrated that the urea was preferred. A carbamate retained the balanced activity versus *C. albicans* and *C. glabrata* but was significantly less potent (cf. **13** vs **4**), and neither sulfonamide **14** nor the acylsulfonamide **15** proved an adequate surrogate. Amide **16** gave good *C. albicans* potency, but was inactive against *C. glabrata*. In contrast to the SAR of the ureas, the 2-chlorophenyl amide **17** is as active as the 4-chloro isomer **16**. This is, in fact, unsurprising, as the conformational restriction of tertiary benzamides such as these (wherein the aromatic ring is forced out of the plane of the amide for steric reasons, even the parent phenyl case) is well known;[5] this merely reinforces the argument that a conformational change accounts for the disparate activity of the urea **5**. Nonetheless, the overall shape of analogues containing an amide link clearly differs significantly from the ureas, and the fact that *C. albicans* activity is seen in both indicates either that the binding site of this particular pump is flexible or that more than one binding site may exist. Similarly, the divergent SAR across *Candida* spp. indi-

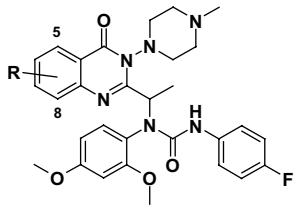
Table 2. Variants of the 2,4-dimethoxyphenyl moiety in **1**



No.	R	MPC_8 (μg/mL)	
		C. albicans	*C. glabrata*
7	(2,4-diOMe)Ph	4	4
18	Me	>32	>32
19	Ph	>32	>32
20	(2-OMe)Ph	>32	>32
21	(4-OMe)Ph	8	32
22	(3-OMe)Ph	16	>32
23	(2,4,6-triOMe)Ph	>32	>32
24	(3,4-diOMe)Ph	>32	>32
25	(3,4,5-triOMe)Ph	8	>32
26	(2,4-diF)Ph	8	32
27	(2,4-diMe)Ph	8	>32
28	(2,4-diOCH_2*c*Pr)Ph[a]	4	2
29	3-(2,6-diOMe)pyridyl	16	32

[a] *c*Pr = cyclopropyl.

Table 3. Variants of the quinazolinone core in **1**



No.	R	MPC_8 (μg/mL)	
		C. albicans	*C. glabrata*
7	H	4	4
30	5-F	8	8
31	6-F	1	2
32	7-F	0.5	1
33	6,7-diF	0.125	0.5
34	6-Cl	0.25	2
35	7-Cl	0.125	0.5
36	6-Me	0.5	4
37	6-OMe	0.5	2
38	8-OMe	8	8
39	6-OH	16	1
40	6-$OCOCH_2CH_3$	4	4
41	6-OCH_2CONH_2	>32	>32
42	6-$NHSO_2CH_3$	16	32
43	6-$NHCOCH_3$	>32	>32
44	7-CN	0.5	2
45	7-CO_2Me	32	2
46	7-CO_2H	>32	>32
47	7-Aza	32	32
48	8-Aza	>32	>32

cates either that the binding sites of the pumps differ or that the mode of binding of the inhibitors is different (or both). Overall, we regarded the (4-fluoro)phenyl urea **7** as the preferred substituent for this region of the lead, based upon its potency, balanced spectrum and reasonable lipophilicity.

We next examined the requirements for activity around the dimethoxyphenyl residue. The results for key analogues are displayed in Table 2.

The structural requirements for activity at this position are stringent. Alkyl substituents such as **18** were completely devoid of activity, as was the simple phenyl analogue **19**. Among methoxy substituted phenyl derivatives, there is a clear and very strong preference for the parent 2,4-disubstitution pattern; removal of either of these (**20**, **21**) leads to dramatic loss in activity. Similarly, all other mono- or tri-substituted variants (**22**–**25**) are much less active. Not only is the 2,4-disubstitution pattern important for activity, but the need for alkoxy substituents is highlighted by the relative inactivity of **26** and **27**. However, the size of the alkoxy groups is also important; although **28** potentiated FLU satisfactorily in the strains shown, it exhibited mild intrinsic antifungal activity versus *C. albicans* (MIC 32 μg/mL) and *S. cerevisiae* (data not shown). Finally, it proved possible to retain modest activity by replacing the phenyl ring with a suitably substituted pyridyl surrogate **29**, although we regard this as a rather conservative change, given the inductive effect of the flanking substituents on the heteroatom.

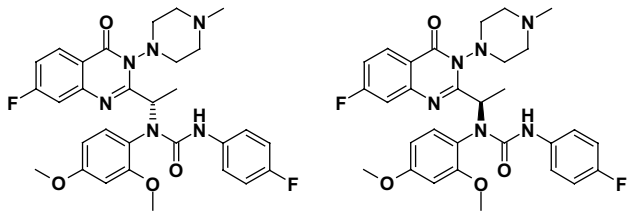
Given the extreme rigidity of this portion of the lead[4] (the aryl ring is forced out of the plane of the urea by the adjacent quinazolinone), it is unlikely that the observed SAR is due to any change in conformational bias; rather, we assume that the affinity is driven by a π-stacking interaction that requires a lipophilic, electron-rich aromatic ring, together with possible hydrogen bond(s) to the substituents.

Having established requirements for activity in the two isolated phenyl rings, we turned our attention to the quinazolinone itself, for which results are shown in Table 3.

The presence of a halo substituent at the 6- or 7-positions of the quinazolinone (**30**–**35**) increased potency against both yeasts, and small alkyl or alkoxy substituents at the 6-position (**36**, **37**) also improved activity, particularly versus *C. albicans*; the 8-methoxy analogue **38**, however, was slightly less active. Wishing to reduce the lipophilicity of the lead as much as possible, we explored more polar substituents at the 6-position and were gratified to find some residual activity in phenol **39**. The less polar propionate derivative **40** restored the potency, but the α-acetamide **41** was not active. The weak activity of sulfonylanilide **42** and the lack of activity of the (more polar) acetanilide **43** provide further evidence of this trend.

The 7-cyano derivative **44** proved quite active, but its isolation was complicated by a marked tendency towards hydrolysis of the quinazolinone amide bond. Ester **45** was considerably less active, and unsurprisingly, acid **46** showed no activity at all.

Table 4. In vitro activity of enantiomers of **32**



49 **50**

No.	Concn required for 50% pump inhibition in *S. cerevisiae* (μg/mL)[a]				MPC_8 (μg/mL)	
	CDR1	CDR2	CgCDR1	CgCDR2	*C. albicans*	*C. glabrata*
49	0.5	16	2	32	0.25	0.5
50	>32	>32	>32	>32	>32	>32

[a] See Materials and methods.

In keeping with the general requirement for lipophilicity in the quinazolinone portion of the pharmacophore, two analogues in which the carbacyclic portion of the bicycle were replaced by more polar heterocycles (**47**, **48**) were inactive.

The activity of one of the most potent racemic analogues above (**32**) was shown to reside in the (*S*)-enantiomer **49**. Like **1**, **49** is selective for the inhibition of CDR1 in *C. albicans* and CgCDR1 in *C. glabrata* (Table 4).

4. Summary and conclusions

In this and the previous study,[4] the absolute structural requirements for satisfactory efflux pump inhibition in vitro by 3-(*N*′-methyl-piperazinyl)quinazolinones such as **1** have been outlined. All three aromatic entities are necessary, and the optimal linkers are shown to be those present in the original lead. A halo substituent at the 3- or 4-position of the phenyl urea is preferred, and there is a strong requirement for 2,4-dialkoxy substitution on the other phenyl ring. Significant enhancements in potency were achieved through the introduction of halo substituents on the quinazolinone ring. It proved impossible to incorporate significantly more polar substituents anywhere on the three aromatic residues without significantly compromising efflux pump inhibitory activity.

Future publications will describe the SAR for fungal efflux pump inhibition of variants of the *N*-methyl piperazine residue, and the means by which the pharmacokinetics of the series were optimized.

5. Materials and methods

5.1. 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 enantiomers of **2** were isolated by chromatography using a Chiralpack AD 250 × 4.6 mm column, eluting with 30% 2-propanol in hexanes at 1 mL/min. Absolute stereochemistry was assigned by analogy with relative retention times of the enantiomers of **1**.[4]

5.2. 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 NCCLS reference methods.[6] The in vitro potency of the inhibitor is expressed as the lowest concentration achieving an 8-fold reduction in FLU MIC (minimum potentiation concentration, or MPC_8). All the compounds reported had no intrinsic antifungal activity (MIC > 32 μg/mL) with the exception of **28** (see text). 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.

The inhibition of specific ABC transporters from *C. albicans* and *C. glabrata* was assessed using defined strains of *S. cerevisiae* in which the various pumps from Candida spp. are overexpressed under the control of a common promoter: YEM139 (PDR5 deleted; FLU MIC 0.5 μg/mL), YEM172 (CDR1 over-expressed; FLU MIC 128 μg/mL), YEM171 (CDR2 over-expressed; FLU MIC 8 μg/mL), Y170 (CgCDR1 over-expressed; FLU MIC 128 mug/mL) and Y218 (CgCDR2 over-

expressed; FLU MIC 2 μg/mL). An inoculum of 10^4 cells of each strain was incubated at 30 °C in YPD in the presence of varying concentrations of inhibitor and a single concentration of FLU corresponding to the geometric mean of the MIC of the test strain and the MIC of the parent pump-deleted strain, for example, YEM172 was incubated with 8 μg/ml FLU, which is the geometric mean of 128 (MIC of YEM 172) and 0.5 (MIC of YEM139). MICs were determined as 80% inhibition of growth after 48 h.

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