

## Evaluation of Novel 2,4-Diaminoquinazoline Inhibitors of *Candida albicans* DHFR

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### ABSTRACT

**Background:** Systemic fungal infections are responsible for substantial morbidity and mortality worldwide. Existing therapies are associated with significant toxicity, consequently there is a need for better drugs. The essential enzyme dihydrofolate reductase (DHFR) has successfully been targeted for many therapeutic areas including antibacterial, antiparasitic and oncology. In contrast, exploitation of this target has proven relatively unsuccessful as an antifungal. As part of a structure based drug design (SBDD) antibacterial program, several hundred compounds based on a 2,4-diaminoquinazoline core were synthesized. Here we evaluate the antifungal potential of this series.

**Methods:** Human and *C. albicans* DHFR proteins were generated using standard recombinant techniques. Enzyme activity was determined using a spectrophotometric assay following NADPH oxidation coupled to reduction of substrate dihydrofolate.  $K_i$  values were calculated using the Morrison tight-binding equation. *C. albicans* and *C. glabrata* MIC values were determined according to CLSI guidelines.

**Results:** SAR indicates that this series contains compounds that are both highly potent ( $K_i < 1$  nM) and selective *C. albicans* DHFR inhibitors (ratio Human/*C. albicans* >3000). Furthermore, *in vitro* MIC testing indicated that several members of the series demonstrated promising antifungal activities.

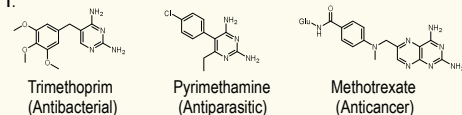
Cpd #	$K_i$ (nM)		$K_i$ Ratio Human/ <i>C. albicans</i>	MIC ( $\mu$ g/ml)	
	<i>C. albicans</i>	Human		<i>C. albicans</i> 24h 48h	<i>C. glabrata</i> 24h 48h
1	3.4	11	3.2	32 32	- -
2	45	702	16	32 32	- -
3	0.19	321	1690	1 >64	1 1
4	0.16	526	3288	1 >64	<0.5 2

**Conclusions:** Screening of a novel series of 2,4-diaminoquinazolines originally designed as antibacterials, revealed highly potent, selective inhibitors with antifungal activity. These leads are poised for specific optimization of antifungal properties.

### BACKGROUND

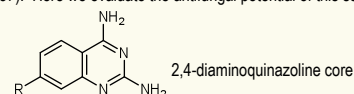
Systemic fungal infections are responsible for substantial morbidity and mortality worldwide. The prevalence of these infections continues to rise, in part due to the increased number of immune-compromised patients<sup>1,2</sup>. Existing antifungal therapies show limited spectrum and are often associated with significant toxicity, consequently there is a need for better drugs. The ubiquitous cofactor tetrahydrofolate ( $H_2F$ ) is essential for the synthesis of a variety of cellular metabolites including thymidine, purines as well as some amino acids<sup>2</sup>. Inhibitors of the folate pathway have been used clinically for infectious diseases (Trimethoprim and Pyrimethamine) and oncology (Methotrexate) (Figure 1). Within this pathway, the enzyme dihydrofolate reductase (DHFR) has been extensively studied and is arguably the most successfully exploited component<sup>2</sup>. Chemotherapeutic intervention at DHFR has proven relatively unsuccessful as an antifungal.

Figure 1.



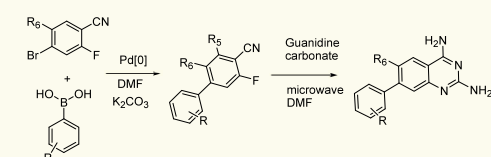
### BACKGROUND cont.

As part of a SBDD antibacterial effort targeting DHFR, several hundred compounds based on a 2,4-diaminoquinazoline core were synthesized. Properties, potencies at the enzyme and cellular levels, as well as selectivity relative to the human enzyme were optimized for bacterial infections (see posters F1-834, F1-835, F1-836, F1-837). Here we evaluate the antifungal potential of this series.



### METHODS

**Synthesis:** Compounds were prepared by using a Suzuki coupling to form the biaryl-nitrile. Treatment of the ortho-fluorobenzonitrile with guanidine yielded the desired quinazoline.



**Protein production:** The genes encoding human and *C. albicans* DHFRs were amplified and cloned into the pET30 expression vector (Novagen). The resulting constructs, containing C-terminal 6XHis tags, were expressed in *E. coli* BL21-AI (Invitrogen), and the resulting proteins purified with Nickel-chelating resin.

**Enzyme assays:** DHFR activity was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm resulting from the oxidation of NADPH coupled to reduction of substrate dihydrofolate ( $H_2F$ ). Enzymes (0.4-2 nM active site concentrations) were incubated with test compound in the presence of NADPH (150  $\mu$ M) for 10 minutes prior to initiation of reaction by addition of  $H_2F$  (100  $\mu$ M). Assay buffer consists of 100 mM Tris-Cl pH 7.2, 50 mM sodium chloride, 1 mM EDTA.

$K_m$  values for  $H_2F$  were determined at fixed NADPH (150  $\mu$ M) and varying  $H_2F$ . Inhibition modalities were confirmed to be competitive with respect to  $H_2F$  using standard procedures. Inhibition constants ( $K_i$ ) were determined using the Morrison tight-binding equation to account for ligand depletion. All analysis was carried out using Graphpad Prism 4.0.

**Microbiology:** Minimum inhibitory concentrations (MIC) values were determined using CLSI broth microdilution methods. Assays were conducted in RPMI media plus glutamine, lacking sodium bicarbonate +HEPES -phenol red. Compound stocks were prepared in 100% DMSO at 10 mg/ml, then serially diluted in two-fold steps, for final compound concentrations of 0.5-64  $\mu$ g/ml with a final DMSO concentration of 2% v/v. MICs were determined by OD<sub>600</sub> at 48h and by eye at 24h and 48h. Strains used were *C. albicans* ATCC 36082, *C. glabrata* ATCC 2001.

### RESULTS

- Kinetic parameters of *C. albicans* and human DHFRs were determined and found to be similar to published values.
- Inhibition modalities of lead compounds were examined against the fungal enzyme. Typical data for compound 1 are shown in Figure 2. This compound is competitive with respect to substrate  $H_2F$  and uncompetitive with respect to the cofactor NADPH (Hanes Woolf analysis shown).

Table 1: Inhibition of *C. albicans* and human DHFR

Cmpd #	Structure	$K_i$ (nM)		$K_i$ Ratio Human / <i>C. albicans</i>
		<i>C. albicans</i> DHFR	Human DHFR	
1		3.4	11	3.2
2		45	702	16
3		0.19	321	1690
4		0.16	526	3288
5		4.4	138	31
6		0.8	12.2	15
7		6.9	18	3
8		0.8	47	59
9		2.7	255	94
10		6.2	145	23
11		4.8	55	11.5
12		0.5	44	88

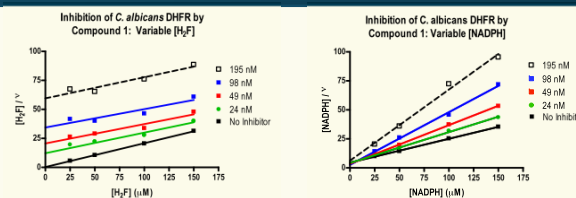


Figure 2: Inhibition of *C. albicans* DHFR

- Simple substitutions on the 7-position of the diaminoquinazoline core resulted in highly potent inhibitors.
- Addition of a methyl group to the 6-position of the diaminoquinazoline core showed substantial increases in potency, compounds 2 and 5. Potency increased 10-fold for the fungal enzyme but was associated with increased potency against the human enzyme.
- Additions of heteroatoms in the 7-substituted ring was tolerated, compounds 5 and 9. Potency increases 2-fold against the fungal enzyme and also decreases 2-fold against the human enzyme. This indicates scope for further optimization to both increase potency and selectivity.
- Addition of a methoxy-group at the 5-position of the diaminoquinazoline core increased potency but decreased selectivity, compounds 2 and 7.
- Addition of larger side chains on the 7-position substitution increases antifungal activity substantially, compounds 5, 6 and 8. Activity against the human enzyme can clearly be modulated from this vector, eg, addition of a third methoxy group, compound 8, decreases potency against the human enzyme 4-fold while having no detrimental effect on the fungal activity. This is further evidenced by compound 3, the only bicyclic 7-position group shown, which is extremely potent against the fungal enzyme and has a selectivity ratio of 1690.
- A series of nitrile-containing compounds were shown to have varying potencies and selectivities against the enzymes. Compound 11 shows similar potency to 5 against the fungal enzyme but is less selective. Addition of a methylene spacer, compound 12, increased antifungal activity 10-fold without substantially altering the human potency.
- Addition of a dimethylamine para- to the 7-position (compounds 4 and 11) results in a 30-fold increase in antifungal activity while also decreasing the human potency by almost 10-fold. Compound 4 has a  $K_i$  against *C. albicans* DHFR of 0.16 nM and a selectivity ratio of over 3000.

### RESULTS cont.

Cmpd #	MIC ( $\mu$ g/ml)			
	<i>C. albicans</i>		<i>C. glabrata</i>	
1	32	32	-	-
2	32	32	-	-
3	1	>64	1	1
4	1	>64	<0.5	2

Table 2: *C. albicans* *in vitro* MIC testing. In most cases, no antifungal activity was seen, despite excellent  $K_i$  values. This is similar to reports in the literature. Several compounds however, do display activity against both *C. albicans* and *C. glabrata*. Of particular note are the highly potent and selective compounds 3 and 4.

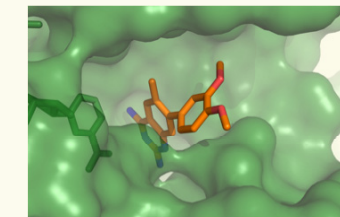


Figure 3: Compound 6 (orange) bound to *C. albicans* DHFR was modeled using the coordinates of a pyrroloquinazoline-bound *C. albicans* DHFR (PDB ID 1M7A) and compound 6 bound to *S. aureus* DHFR (unpublished coordinates). NADP(H) cofactor shown in green.

### SUMMARY

- A novel series of 2,4-diaminoquinazolines-based DHFR inhibitors, originally designed as antibacterials, were evaluated against *C. albicans*.
- Potent inhibitors of the *C. albicans* DHFR were found, with  $K_i$  values reaching sub-nM levels.
- Modification of the 7-position of the diaminoquinazoline core reveals clear SAR resulting in increased potency towards the fungal enzyme while also decreasing potency against the human counterpart.
- Molecular modeling suggests routes to further increase antifungal potency and selectivity.
- While MIC analysis indicated that most compounds were inactive (MIC >64  $\mu$ g/ml) despite good potencies against DHFR, several exhibited promising cellular activity.
- Compound 4 has excellent  $K_i$  potency (0.16 nM) and selectivity relative to the human enzyme (>3000-fold) and also shows encouraging cellular activity, in particular against *C. glabrata*, MIC <0.5 and 2  $\mu$ g/ml after 24 and 48 hours, respectively.
- This promising series is poised for specific optimization to fully exploit its antifungal potential.

### LITERATURE CITED

- Liu, J.; Bolstad, D.B.; Smith, A.E.; Priestley, N.D.; Wright, D.L.; Anderson, A.C. *Chem. Biol.* **2008**, *15*, 990
- Kompis, I.; Islam, K.; Then, R. *Chem. Rev.* **2005**, *105*, 593