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In Vitro Biological Activity and Structural Analysis of 2,4-Diamino-5-(2'-arylpropargyl)pyrimidine Inhibitors of *Candida albicans*

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Abstract

In order to develop new antifungal agents effective against two species of *Candida*, we have designed a series of dihydrofolate reductase (DHFR) inhibitors. Here, we explore the structure-activity relationships of these inhibitors toward *Candida albicans* DHFR by evaluating enzyme inhibition, antifungal activity and toxicity to mammalian cells. Analysis of docked complexes of the enzyme and inhibitors yields the structural basis of relative potency. The meta-biphenyl series of this class exhibits the greatest enzyme inhibition, selectivity and antifungal activity.

Keywords

antifolate; dihydrofolate reductase; *Candida albicans*; molecular modeling

INTRODUCTION

Since the early 1990s there has been an acute increase in the incidence of systemic fungal infections¹ along with associated high rates of morbidity and mortality^{2,3}. This is due, in part, to an increased number of immunocompromised patients who are susceptible to these infections. Although many different fungal organisms can cause systemic infections, those caused by *Candida* are the most prevalent². Furthermore, several studies have shown *Candida albicans* as the most abundant fungal species in these patients^{3–5}. Where the prevalence of *C. albicans* was 76 % between 1980 and 1990³, more recent studies show this prevalence has dropped to 45 % and that of other *Candida* species, most notably *Candida glabrata*, now account for 24 % of systemic *Candida* infections⁴. Despite this trend, *Candida albicans* continues to dominate as the leading causative agent of systemic fungal infections.

Limited previous efforts at Glaxo were directed at identifying antifungal agents that target the essential enzyme, dihydrofolate reductase (DHFR), from *Candida albicans* (CaDHFR). This

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program appears to have been discontinued, despite some initial success. Two classes of molecules were developed. Eight members of a 2,4-diamino-5-arylthiopyrimidine class exhibited nanomolar enzyme inhibition and greater than 100-fold selectivity⁶ and ten compounds exhibited antifungal activity. The second class of compounds was based on a 7,8-dialkylpyrroloquinazoline scaffold⁷. Members of this class were potent enzyme inhibitors but were nonselective against human DHFR. Researchers at Glaxo also determined crystal structures of CaDHFR bound to members of both classes of inhibitors^{8,9}.

Previously, we validated dihydrofolate reductase (DHFR) as an effective antifungal target^{10, 11}. A series of novel propargyl-linked compounds that target DHFR in *Candida glabrata* (CgDHFR) exhibit nanomolar enzyme inhibition and levels of activity against the organism equivalent to those observed with other clinically used therapeutics^{10,11}. Selectivity over human DHFR is greater than 1000-fold. Nicely illustrated by crystallographic evidence, inhibition is largely due to positioning of the inhibitor at optimal van der Waals distances in key hydrophobic regions of the substrate binding site. Owing to its similarity to *Candida glabrata*, a comparable path to inhibition for *Candida albicans* could be followed.

Here we present and analyze the detailed structure-activity relationships (SAR) between *C. albicans* DHFR and the propargyl-linked inhibitors. Screening an initial group of 33 inhibitors reveals that the *meta*-biphenyl subclass of these inhibitors have an IC₅₀ value for enzyme inhibition as low as 17 nM and exhibit fungal cell growth inhibition at concentrations of 23 µg/mL. In order to elucidate the structural basis of the SAR, we performed docking studies using a crystal structure of the enzyme. The models of the CaDHFR:inhibitor complexes were validated by a strong correlation between the docking scores and enzyme inhibition values. Examination of three pockets in the enzyme surrounding the docked inhibitors reveals a path toward second generation inhibitors designed for increased potency and selectivity. Additionally, a structural comparison of CaDHFR and CgDHFR opens the possibility of designing an inhibitor that is potent against both fungal enzymes yet maintains selectivity against the human enzyme.

RESULTS

Enzyme and Cell Growth Inhibition Assays with Propargyl Inhibitors

In order to evaluate the efficacy of the propargyl compounds as inhibitors of the CaDHFR enzyme and *C. albicans* growth, we performed in vitro enzyme inhibition and antifungal assays. Inhibition of CaDHFR was used to determine potency and comparison with inhibition of human DHFR (hDHFR) was used to determine selectivity for the pathogenic enzyme. Likewise, *C. albicans* growth inhibition was used to determine antifungal activity and inhibition of MCF-10 cells, a human breast carcinoma cell line, was used to evaluate overt human cell toxicity.

Thirty-five propargyl compounds were evaluated for potency and selectivity (Table 1); enzyme inhibition values for trimethoprim are also included for comparison. In all cases except **R-20** and **S-20**, compounds were screened as racemic mixtures when a chiral center was present. Several trends regarding potency emerged from this screen. In general, compounds with the simple trimethoxyphenyl scaffold (scaffold A) performed poorly as enzyme inhibitors and antifungal agents. When the simple scaffold was elaborated by making substitutions at the propargyl position (*gem*-dimethyl: **10**, ethyl: **9**) or an ethyl substitution at the C6 position of the pyrimidine (**11**, **12**), enzyme inhibition increased but antifungal activity remained low. Modifying this scaffold to include a dimethoxyphenyl ring (scaffold B) also failed to yield potent inhibitors at either the enzyme or cellular levels. However, when the scaffold was expanded to include a biphenyl series (scaffolds C and D), increased enzyme inhibition or antifungal activity was observed. The *meta*-biphenyl series (scaffold C) shows the greatest degree of potency with IC₅₀ values of 0.017 µM for compound **28** and antifungal activity of

24 $\mu\text{g/mL}$. Since this series shows the highest potency, individual enantiomers of the parent compound in this series (**R-20** and **S-20**) were evaluated. Interestingly, these enantiomers inhibit CaDHFR to the same degree, suggesting that the pocket can tolerate the methyl in either configuration. The inhibition of CaDHFR by a compound with a *gem*-dimethyl propargyl substitution (**10**) further validates this hypothesis. The two most active *meta*-biphenyl compounds in the enzyme assay were also the two most active in the whole cell assay. The *para*-biphenyl compounds (scaffold D) exhibited potent enzyme inhibition but low antifungal activity.

Selectivity for the pathogenic enzyme is highest (~100-fold) for the *para*-biphenyl compounds belonging to scaffold D. However, since these compounds do not exhibit appreciable antifungal activity, this class of compounds is of generally lower interest. Several of the *meta*-biphenyl compounds exhibit compelling selectivity with at least 30-fold greater potency for the pathogenic enzyme. These compounds also begin to explore a class with a promising trend toward cellular selectivity.

Receptor Modeling and Validation

In order to elucidate the structural determinants of potency and selectivity as well as to design future inhibitors, we created an accurate docking model for the propargyl-linked inhibitors. A model of CaDHFR derived from crystallographic data (PDB ID: 1AOE) extending to 1.6 Å resolution for a ternary complex containing a bound molecule of NADPH and an antifolate inhibitor was chosen as the docking receptor. The model was prepared according to the protocol outlined in Materials and Methods. Since DHFR has a highly conserved ligand binding orientation (Figure 1), distances between the hydrogen atoms of N1 and N2 were constrained to be no more than 2.75 Å from the oxygen atoms of Glu 32 to maintain the integrity of the binding core during the molecular dynamics simulation. Additionally, the hydrogen of the hydroxyl group in Thr 133 was constrained to be within 4.2 Å of the hydrogen (b) on N2 (Figure 1). Given that receptor flexibility is essential in obtaining accurate docking results, residues within a 3.5 Å shell of atoms around the active site were designated as flexible.

Docking experiments were analyzed using a neighbor binning technique (see Materials and Methods for a full description of the binning procedure). Ligands were ranked according to their IC_{50} values and divided into bins of approximately equal size (see Supplemental Material). After docking experiments were completed, ligands were also ranked according to docking score. If both metrics placed the ligand into the same bin or an adjacent bin, that ligand received a score of one. If this condition was not met then the ligand was assigned a score of zero. The predictive power of the model was assessed by evaluating the number of times the model placed ligands into the correct bin or its neighboring bin expressed as a percentage.

Sixty-seven ligands, representing the separate enantiomers of the racemic compounds in Table 1, were docked and ranked. Docking to a model of the receptor with minimal active site flexibility (as described in Materials and Methods) yielded poor accuracy (<50 % of the ligands were placed in the correct or a neighboring bin). Accuracy was increased, especially for ligands exhibiting poor potency for CaDHFR, by allowing additional residues to be flexible during the MD simulation, including residues in the loop that flanks the active site (Trp 59, Glu 60, Gln 64, and Lys 65) and Arg 34 and Lys 37 near the entrance to the active site (see Fig. 3A). Using the flexible receptor and the neighbor binning technique, an accuracy of 72 % was attained. This value for accuracy becomes meaningful when compared with random ligand placement (17 % binning).

With accurate models of the complexes of CaDHFR and the inhibitors, it is possible to investigate the structural basis of the SAR observed during the enzyme inhibition experiments. Specifically, we examined models of compound series that maintain the same scaffold and

differ by substitution at one of three positions: the propargyl position, the C6 position of the pyrimidine ring and the position of the biphenyl group.

Analysis of Propargyl Linker Position

The series **5–10** maintain the trimethoxyphenyl scaffold and explore varying substitutions at the propargyl position (Table 1). Increases in both enzyme inhibition and docking scores correlate with the addition of increasingly hydrophobic groups at the propargyl position. The correlation is likely due to increased van der Waals interactions with a hydrophobic pocket that is created by Ile 62, Leu 69, Ile 112, and Thr 58 found near the propargyl position (see Figure 2A). Gly 113 observed adjacent to the propargyl position accommodates the larger substitutions at this position. The additional two series comprising compounds **1–4** or **14, 17–19**, respectively, verify the correlation between potency and hydrophobicity at the propargyl linker position.

Interestingly, the *gem*-dimethyl substituted compound (**10**) is slightly more potent than the equally hydrophobic ethyl substituted compound (**9**). Docking models suggest that this potency difference is correlated to the number of potential hydrophobic contacts each compound can make. The ethyl substituted compound and one of the methyl groups of the *gem*-dimethyl substituted compound contact Thr 58. However, the second methyl of the *gem*-dimethyl substituted compound makes additional contacts above what the singly substituted ethyl allows, enabling favorable contacts with Ile 62, Leu 69 and Ile 112.

Analysis of the C6 position

Several compound series show that increasing the alkyl chain length at the C6 position of the diaminopyrimidine ring correlates with potency. Results from compounds **3, 7**, and **11** show that each additional methyl group increases potency 5- to 10-fold. Compound series **4, 8**, and **12** and series **13–16** show similar increases in potency with increasing alkyl chain length (see Table 1). This increase in potency is further explained and supported by docking models that show a correlation between increased alkyl chain length at the C6 position and increased docking score. From docking models it is evident that this trend is the result of increased contacts with a hydrophobic pocket formed by Met 25, Ile 33, and Phe 36 around the C6 position (see Figure 2B).

Analysis of the Biphenyl Position

Compounds with scaffolds A and B (Table 1) with one aryl ring attached to the propargyl position may be viewed as first-generation compounds based upon a similarity to trimethoprim. Models of CaDHFR show that the space occupied by this aryl ring is a 10–11 Å wide hydrophobic pocket created by Leu 69, Phe 66, Ile 62, and Phe 36 (see Figure 2C). Structural evaluation shows that these singly substituted aryl rings are not filling the entire pocket. The biphenyl derivatives with a phenyl ring extending from the 5' position of the proximal aryl ring were designed to better fill this pocket. Compounds **21–23**, exploring *ortho* distal ring substitutions all have similar potency. Compounds **24, 27, 29**, and **28**, exploring *para* and *meta* substitutions, show increases in docking scores and decreases in IC₅₀ values over the *ortho*-aryl substitutions. Models show that the preference for the *para* and *meta* substitutions arise because of additional contacts with Leu 69.

As noted previously, the *para*-biphenyl compounds (scaffold D) are some of the most potent enzyme inhibitors. Models of complexes with compounds **31–33** show additional hydrophobic contacts relative to complexes with the unsubstituted compound **30**, with residues in the pocket created by Phe 36, Ile 62, Phe 66 and Leu 69. On the basis of both docking scores and IC₅₀ values, compound **31** is the most potent of this series.

Selectivity over hDHFR

Interestingly, the majority of the inhibitors exhibit similar potency against hDHFR while exhibiting differing potency against CaDHFR. As a result, the varying inhibition of CaDHFR is responsible for the varying levels of selectivity. In order to achieve much higher levels of selectivity over the human enzyme, it should be possible to exploit structural differences between the enzymes. In the active site, three of the five residues differences are homologous (see Table 2) but may still provide opportunities to achieve selectivity. The other two residue differences represent significant changes in properties: Gln 35 and Asn 64 in human DHFR to Lys 37 and Phe 66, respectively in CaDHFR. These residue differences are found near the outer edge of the biphenyl pocket (see Figure 3). The models of the complexes show that the propargyl compounds do not extend to this part of the pocket; therefore these differences are not currently being exploited.

Some increased selectivity is evident with derivatives based on scaffolds C and D. When substitutions increasing the bulk of molecule are made on the 5'-aryl ring, increases in selectivity are observed. As shown in Figure 3A, the location of a loop region (residues 58–66) that flanks the biphenyl pocket may explain this trend. This loop adopts a more closed structure in human DHFR versus CaDHFR. Thus, increased bulk on the distal ring is tolerated in CaDHFR but is less well tolerated in the human DHFR model (see Figure 3B). However, it will be necessary to consider the flexibility of the loop region and its ability to accommodate larger biphenyl substituents in future inhibitor design based on these criteria.

Comparison to *Candida glabrata*

CaDHFR and CgDHFR share 85 % sequence homology. In the ligand binding site all residue differences are homologous (see Table 2). Therefore it is not surprising that the ligand binding trends observed in CaDHFR also hold true for CgDHFR. Compounds that increase potency for CaDHFR by the addition of hydrophobic groups at the C6 position of the diaminopyrimidine ring, propargyl linker position, and in the biphenyl pocket also increase potency for CgDHFR.

Although the same potency trends are observed for CaDHFR and CgDHFR, all of the propargyl compounds show greater enzyme inhibition for CgDHFR over CaDHFR, most likely due to better fit to the active site. The models show that the flexible loop described previously is closer to the active site in CgDHFR (see Figure 3), thus creating a pocket that is narrower in CgDHFR than CaDHFR (see Figure 3B). Inhibitors **27** and **28** form optimal van der Waal contacts with hydrophobic residues Phe 36, Ile 62, Phe 66, and Leu 69 lining the CgDHFR biphenyl pocket. The increased volume of the pocket in CaDHFR weakens these same hydrophobic contacts.

DISCUSSION

Systemic fungal diseases represent an increasing threat. Infections caused by *Candida albicans* continue to be most prevalent, although resistant species of *Candida*, particularly *C. glabrata*, now cause approximately 24 % of *Candida* infections. In this paper we have explored a class of antifolate compounds that show promising inhibition of the enzyme, dihydrofolate reductase (DHFR) from *C. albicans*. Several key findings have been revealed through in vitro evaluation and *in silico* experiments. Examination of Table 1 shows that compounds using scaffold C exhibit the highest potency while also showing target selectivity and antifungal activity. The best compounds, **27** and **28**, exhibit nanomolar potency and fifty-fold selectivity over human DHFR while also showing a promising two-fold selectivity at the cellular level. While it is evident that increased potency and selectivity is needed, the models reveal a strategy for design elements, outlined below, that are predicted to achieve those levels.

Interpretation of the in vitro data is strongly supported by the modeling studies. Three structural trends correlate potency and docking scores. First, within the propargyl linker pocket of scaffolds A–D, respectively, it was found that increasing hydrophobicity led to increased potency. This pocket is 5–6 Å deep (measured from the propargyl carbon to the α carbon of Gly 113) and therefore could accommodate much larger substitutes than were explored here, potentially leading to further increases in potency. Secondly, within the C6 pocket of the diaminopyrimidine ring increasing the alkyl chain length increased potency. However, models show that the volume of this pocket is largely filled by small alkyl substitutions, such as ethyl and *n*-propyl, and therefore that a repulsive effect may be observed if longer alkyl substitutions were created. Third, the space occupied by the biphenyl ring system of scaffolds C and D also showed that increasing hydrophobicity leads to increased potency. This is the largest pocket of the three (10–11 Å) and also yielded the largest gains in potency. The addition of the distal phenyl ring on scaffold C and D better fills the large volume of this large pocket than the proximal phenyl ring alone (scaffolds A and B). Structural analysis predicts that if methyl or possibly larger groups were added to positions R₂, R₃, R₄, R₅, and R₆ of the distal ring of scaffold C, this would lead to further hydrophobic interactions leading to additional increases in potency.

The compounds fit comfortably within the guidelines for good drug leads¹²: they have molecular weights between 284 and 442, clogD values between 1.7 and 4.8 (calculated for the most hydrophobic active inhibitors), a reasonable number of hydrogen bond donors and acceptors and they do not possess a large number of rotatable bonds. One issue that arose, not unexpectedly, during this study is that antifungal activity is not well correlated with enzyme inhibition. We observed the obvious case: compounds must be potent enzyme inhibitors in order to exhibit strong antifungal activity, yet not all potent enzyme inhibitors yielded corresponding antifungal activity. In order to understand this better, we have planned experiments to measure cell permeability, protein binding and solubility.

Dual Inhibitor design

Compounds that target more than one fungal species effectively are invaluable because of widespread species specific resistance to current therapeutics. Resistance necessitates species isolation and identification prior to treating the infection, wasting valuable treatment time. Here we have the opportunity to design a novel inhibitor that targets both CaDHFR and CgDHFR. This can be accomplished by capitalizing on the overwhelming similarities of CaDHFR and CgDHFR in the folate binding site while exploiting the differences between fungal and human DHFR. In vitro results show that the meta-biphenyl series of propargyl compounds is the most potent and selective for both *Candida* species and thus provides an excellent initial lead. Based upon evidence presented in this work, the basis of this potency and selectivity is the difference in size of the biphenyl pocket between human, *Candida glabrata*, and *Candida albicans* DHFR.

EXPERIMENTAL

Cloning

Candida albicans genomic DNA was obtained from the American Type Culture Collection (ATCC). The gene encoding for CaDHFR was obtained by PCR amplification followed by insertion into vector pET41 and the resulting plasmids were verified by sequencing. *Escherichia coli* BL21(DE3) cells were transformed and large scale protein expression was induced with isopropyl β -D-thiogalactoside. After several hours of growth, cells were collected by centrifugation and lysed using BugBuster (Novagen). Crude lysate was purified using a nickel affinity column. CaDHFR was eluted over a gradient using a buffer with 20 % glycerol, 0.3 M NaCl, 250 mM imidazole, 5 mM DTT, and 0.1 mM EDTA. The pure CaDHFR protein was frozen and stored at –80 °C until use.

Inhibitors

The synthesis and complete characterization of compounds **1–33** has been described previously^{10,11,13–15}.

Biological Assays

Inhibition assays were performed by determining the rate of NADPH consumption in the presence of the compound under investigation. This was accomplished by following the change in absorbance at 340 nm for 1 minute. Reactions were performed in the presence of 20 mM TES pH 7.0, 50 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM EDTA and 1 mg/mL bovine serum albumin. Saturating concentrations of cofactor (100 μ M NADPH) and substrate (100 μ M dihydrofolate) were used with a limiting concentration of enzyme. All assays were conducted in triplicate at 25 °C and 50 % inhibition concentration (IC₅₀) values and their standard deviations were calculated.

Antifungal Assays

C. albicans was stored as a suspension in 50 % glycerol at –78 °C. For susceptibility testing, a streak of stock culture was made on Sabouraud Dextrose agar (SDA) and grown at 30 °C for 48 h. One pure colony of the test organism was recovered from the plate, suspended in appropriate media and grown in a 5 mL shake flask culture. A sample of the shake flask culture was diluted to 1×10^5 cells/mL in media and added to 96-well test plates (100 μ L per well) containing test compounds dispensed in DMSO (1 μ L). Amphotericin and ketoconazole were used as controls. After an incubation period determined from the strain specific doubling time, Alamar Blue (10 μ L) was added and incubation was continued; each well was scored for dye reduction¹⁶. The MIC value was taken as the lowest concentration of test compound that inhibits growth such that less than 1 % reduction of the blue resazurin (λ_{max} 570 nm) component of the Alamar Blue to the pink resorufin (λ_{max} 600 nm) was observed^{10,11}.

Human cell toxicity assays

Adherent cell lines were maintained in Eagle's Minimal Essential Media with 2 mM glutamine and Earle's Balanced Salt Solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 % fetal calf serum. Fetal calf serum used in these assays was lot matched throughout. All cultures were maintained under a humidified 5 % CO₂ atmosphere at 37 °C, had media refreshed twice weekly and were subcultured by trypsinization and resuspension at a ratio of 1:5 each week. Toxicity assays were conducted between passages 10 – 20. Target compound toxicity was measured by incubating the test compound with the cells for four hours, washing the cells and finally treating the cells with Alamar Blue. After 12 – 24 hours the fluorescence of the reduced dye was measured. Fluorescence intensity as a function of test compound concentration was fit to the Fermi equation¹⁷ to estimate IC₅₀ values.

Modeling

All inhibitors were drawn using software within Sybyl (version 8.0, Tripos, Inc.). For compounds with a chiral center, individual enantiomers were drawn for docking. The diaminopyrimidine core was protonated at N1 to allow for the conserved interactions between the diaminopyrimidine ring and Glu 32 to be maintained during the docking process (Figure 1). Charges were computed and added according to Gasteiger-Marsili parameters. Each ligand was minimized using the Tripos force field integrated within Sybyl. Minimization was allowed to proceed in an iterative process until the energy between consecutive cycles was zero.

A model of CaDHFR, determined using diffraction data to 1.6 Å resolution, was obtained from the Protein Data Bank (PDB ID:1AOE⁹) and used as the docking receptor. The receptor was

prepared by using the Biopolymer application accompanying Sybyl to add hydrogens and assign charges and atom types according to AMBER99 ([http://ambermd.org]).

To simulate protein flexibility, the prepared receptor was subjected to a molecular dynamics (MD) simulation. All atoms within 3.5 Å of the ligand were allowed to move during the MD while the ligand and NADPH were fixed. In order to preserve hydrogen bonds known to be essential for ligand binding (see Figure 1), each oxygen atom of the carboxyl group of Glu 32 was constrained to maintain a distance between 1.6–2.75 Å of the hydrogen atoms of N1 and N2 on the diaminopyrimidine ring of the co-crystallized ligand. The MD simulation was allowed to proceed according to a NTV ensemble (i.e. canonical ensemble; constant number of particles, temperature, and volume) at 300 K for 30,000 fs with snapshots taken every 500 fs from 20,000 to 30,000 fs, resulting in an ensemble set containing 21 members. The first 19,999 fs were not used in order to allow the MD algorithm to converge prior to sampling. Each ensemble member was brought to a local minimum using AMBER99 parameters. Formal charges were also assigned.

Ligand docking was implemented according to Surflex-Dock¹⁸ and executed by Sybyl. Surflex-Dock requires three-dimensional ligands that have been energy minimized, a model of the receptor complete with hydrogens and void of any ligand in the active site, and a protomol created by flooding the active site with hydrogen bond donors, acceptors and steric probes. An internal scoring function keeps probes that represent interactions with the highest affinity, thus approximating a negative image of the protein's active site. Surflex-Dock's paradigm is as follows: ligand fragments are generated according to preset parameters, a lead ligand fragment is aligned with protomol probes, additional ligand fragments are placed within the protomol and combined until the ligand is complete, and the highest scoring poses are retained.

The docking scores produced by Sybyl are related to binding affinities by the formula –log₁₀(K_d) and include terms for hydrophobic, polar, and repulsive interactions as well as entropic effects and solvation. Top-scoring poses were assessed for adherence to the geometry shown in Figure 1. It was assumed that top-scoring poses that show the conserved geometry contributed equivalently to the docking score and were averaged across the ensemble.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Conserved geometry for compounds with a 2,4-diaminopyrimidine ring bound to DHFR.

Figure 2. Residues surrounding the a) propargyl, b) C6 pyrimidine and c) biphenyl groups of the DHFR inhibitors. CaDHFR is rendered as a surface and key residues are labeled.

Figure 3.

The biphenyl pocket: A) CaDHFR (cyan), CgDHFR (pink) and human DHFR (green) are superimposed. Labels are shown for CaDHFR residues; the corresponding residues for CgDHFR and human DHFR are shown in Table 2. B) Surface mesh diagram of the biphenyl pocket with CaDHFR (blue), CgDHFR (green) and human DHFR (red).

TABLE 1

In vitro evaluation of the propargyl inhibitors

ID	Scaffold	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	CaDHR IC ₅₀ (μM)	Human DHR IC ₅₀ (μM)	SI ^d	Ca MIC (μg/mL) (μM)	Human IC ₅₀ (μM) ^b
Scaffold A												
Scaffold B												
Scaffold C												
Scaffold D												
TMP	-	-	-	-	-	-	-	42 ± 11	198 ± 0.003	4.7	- ^c	-
1	A	H	OH	-	-	-	-	11.9 ± 2.7	14.3 ± 0.24	1.2	Inact	510
2	A	H	OMe	-	-	-	-	2.5 ± 0.64	1.16 ± 0.005	0.5	Inact	440
3	A	H	H	-	-	-	-	0.82 ± 0.09	1.46 ± 0.02	1.8	Inact	Inact
4	A	H	Me	-	-	-	-	0.18 ± 0	1.46 ± 0.01	8.1	Inact	Inact
5	A	Me	OH	-	-	-	-	0.98 ± 0.09	5.71 ± 0.05	5.8	Inact	1000
6	A	Me	OMe	-	-	-	-	0.32 ± 0.06	1.22 ± 0.007	3.8	Inact	490
7	A	Me	H	-	-	-	-	0.18 ± 0.05	0.40 ± 0.06	2.2	Inact	250

Scaffold C

Scaffold A

Scaffold D

Scaffold B

ID	Scaffold	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	CaDHR IC ₅₀ (μM)	Human DHFR IC ₅₀ (μM)	SI ^a	Ca MIC (μg/mL) (μM)	Human IC ₅₀ (μM) ^b
8	A	Me	Me	-	-	-	-	0.11 ± 0.04	1.38 ± 0.02	13	172(500)	125
9	A	Me	Et	-	-	-	-	0.045 ± 0.009	0.20 ± 0.01	4.4	357(1000)	175
10	A	Me	<i>gem</i> -Me	-	-	-	-	0.033 ± 0.01	0.29 ± 0.08	8.8	357(1000)	185
11	A	Et	H	-	-	-	-	0.018 ± 0.001	0.057 ± 0.002	3.2	343(1000)	34
12	A	Et	Me	-	-	-	-	0.020 ± 0.002	0.088 ± 0.007	4.4	-	-
13	B	H	H	-	-	-	-	1.4 ± 0.4	3.2 ± 0.05	2.3	285(1000)	107
14	B	Me	H	-	-	-	-	0.41 ± 0.06	1.3 ± 0.003	3.2	449(1500)	125
15	B	Et	H	-	-	-	-	0.10 ± 0.007	1.28 ± 0.02	13	78(250)	63
16	B	<i>n</i> -Pr	H	-	-	-	-	0.045 ± 0.009	1.18 ± 0.01	26	-	-
17	B	Me	OH	-	-	-	-	0.73 ± 0.36	1.2 ± 0.20	1.6	-	-

Scaffold C

Scaffold A

Scaffold D

Scaffold B

ID	Scaffold	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	CaDHR IC ₅₀ (μM)	Human DHR IC ₅₀ (μM)	SI ^a	Ca MIC (μg/mL) (μM)	Human IC ₅₀ (μM) ^b
18	B	Me	OMe	-	-	-	-	0.55 ± 0.09	0.060 ± 0.001	0.1	Inact	-
19	B	Me	Me	-	-	-	-	0.048 ± 0.04	0.40 ± 0.1	8.3	157(500)	163
rac-20	C	Me	H	H	H	H	H	0.055 ± 0.02	1.7 ± 0.01	31	90(250)	90
R-20	C	Me	H	H	H	H	H	0.033 ± 0.006	1.36 ± 0.026	41	-	-
S-20	C	Me	H	H	H	H	H	0.037 ± 0.004	1.38 ± 0.026	37	-	-
21	C	Me	Me	H	H	H	H	0.045 ± 0.009	1.36 ± 0.05	30	373(1000)	91
22	C	Me	Me	H	H	H	Me	0.045 ± 0.009	1.25 ± 0.006	28	387(1000)	88
23	C	Me	i-Pr	H	H	H	i-Pr	0.038 ± 0.001	7.2 ± 0.15	189	Inact	45
24	C	Me	H	H	OMe	H	H	0.03 ± 0.003	0.18 ± 0.006	6.0	-	102
25	C	OH	H	H	H	H	H	0.091 ± 0.04	2.3 ± 0.04	25	Inact	58

Scaffold C

Scaffold A

Scaffold D

Scaffold B

ID	Scaffold	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	CaDHFR IC ₅₀ (μM)	Human DHFR IC ₅₀ (μM)	SI ^a	Ca MIC (μg/mL) (μM)	Human IC ₅₀ (μM) ^b
26	C	H	Me	H	H	H	Me	0.055 ± 0.02	1.58 ± 0.009	29	93(250)	54
27	C	Me	H	H	Me	H	H	0.022 ± 0.001	1.41 ± 0.015	64	23(62)	54
28	C	Me	H	Me	H	Me	H	0.017 ± 0.002	0.75 ± 0.006	44	24(62)	47
29	C	Me	H	H	<i>i</i> -Bu	H	H	0.034 ± 0.009	0.21 ± 0.006	6.2	-	50
30	D	H	H	-	-	-	-	0.055 ± 0.009	1.42 ± 0.012	26	45(125)	80
31	D	Me	H	-	-	-	-	0.027 ± 0.009	2.77 ± 0.06	103	93(250)	76
32	D	Me	Me	-	-	-	-	0.032 ± 0.005	3.37 ± 0.015	105	Inact	117
33	D	<i>i</i> -Pr	<i>i</i> -Pr	-	-	-	-	0.041 ± 0.002	4.2 ± 0.1	102	Inact	37

^a SI = Selectivity Index = Human DHFR IC₅₀/ CaDHFR IC₅₀

^b Human IC₅₀ measurement of preliminary human cell (MCF-10) toxicity

^c not tested

TABLE 2

Active Site Residue Differences in CaDHFR, CgDHFR and Human DHFR

CaDHFR	CgDHFR	Human DHFR
Ile 33	Met 33	Phe 31
Met 25	Leu 25	Leu 22
Ile 112	Ile 121	Val 115
Lys 37	Arg 37	Gln 35
Phe 66	Phe 66	Asn 64