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High Throughput Screening Identifies Novel Inhibitors of *Escherichia coli* Dihydrofolate Reductase that are Competitive with Dihydrofolate

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Abstract—This communication describes the high-throughput screen of a diverse library of 50,000 small molecules against *Escherichia coli* dihydrofolate reductase to detect inhibitors. Sixty-two compounds were identified as having significant inhibitory activity against the enzyme. Secondary screening of these revealed twelve molecules that were competitive with dihydrofolate, nine of which have not been previously characterized as inhibitors of dihydrofolate reductase. These novel molecules ranged in potency (K_i) from 26 nM to 11 μM and may represent fresh starting points for new small molecule therapeutics directed against dihydrofolate reductase.

Dihydrofolate reductase (DHFR) is a well-characterized enzyme (EC 1.5.1.3) that catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). Tetrahydrofolate is an important cofactor for a number of one carbon transfer reactions and is essential for the biosynthesis of purines, pyrimidines and several amino acids.[1] One of the most significant consequences of inhibition of this enzyme is thymidylate deficiency leading to the disruption of DNA synthesis. Thus DHFR has long been recognized as a drug target for a wide range of diseases including cancer,[2] malaria[3] and bacterial infections.[4] Trimethoprim has found particular clinical utility as an inhibitor of DHFR that shows striking selectivity for the bacterial enzymes over that from the human host.[5] Clinical resistance to trimethoprim has, however, limited its use to all but a few therapeutic indications.[6] The aim of this work was to perform a high-throughput screen of *Escherichia coli* DHFR using a diverse, high-quality library of compounds in order to identify novel inhibitors of the bacterial enzyme.

A library of compounds (50,000) sourced from Maybridge (Cornwall, England) were screened against recombinant *E. coli* DHFR in a highly automated format. The gene (*folA*) encoding DHFR was PCR amplified from *E. coli* MG1655 chromosomal DNA with primers, $^{5'}$-C ATC TTA CAT ATG ATC AGT CTG ATT GCG GC-$^{3'}$ and $^{5'}$-CTA CTC GAG CCG CCG CTC CAG AAT CT-$^{3'}$, containing *Nde*I and *Xho*I restriction sites (underlined), respectively. The gene was cloned lacking a stop codon into *Nde*I and *Xho*I digested pET26b to form pET26b-folA, which incorporates a C-terminal polyhistidine-tag. Polyhistidine-tagged DHFR was purified to homogeneity as described previously.[7]

DHF reductase activity was assayed continuously in 96-well microplates by monitoring the decrease of NADPH at an absorbance of 340 nm.[8] Assays were carried out at 25 °C and performed in duplicate. Each 200 μL reaction mixture contained 40 μM NADPH, 30 μM DHF, 5 nM DHFR, 50 mM Tris (pH 7.5), 0.01% (w/v) Triton and 10 mM β-mercaptoethanol. Test compounds from the screening library were added to the reaction before initiation by enzyme and at a final concentration of 10 μM. High activity controls consisted of reaction

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mixtures with DMSO only and low activity controls contained 1.5 μM Trimethoprim. Automation for high throughput screening included assay reagent handling in 96-well format. Compound addition, assay monitoring and plate handling were performed using a Sagian-Beckman Coulter linear track with a Biomek FX liquid handler and SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA, USA) integrated into Core Assay System (Beckman Coulter, Mississauga, ON, Canada). ActivityBase (IDBS Inc., Emeryville, CA), SARgen (IDBS Inc., Emeryville, CA, USA) and Spotfire DecisionSite (Spotfire, Inc., Somerville, MA, USA) were used for data analysis.

The assay data were of high quality with Z and Z' statistical scores[9] of 0.66 and 0.73, indicative of good signal to noise and signal to background ratios in the compound and control wells, respectively. Figure 1 shows a plot of the screening replicates against one another and illustrates the quality of the entire screen, where absolute replicates would lie on a perfect diagonal. All data are reported as percent residual activity relative to the average of the high controls. Active molecules were identified as those showing less than 75% residual activity, a statistical cut-off three standard deviations below the high control mean. Using this threshold 62 compounds were found to be inhibitors of DHFR, giving a primary hit rate of 0.12% over the entire screen.

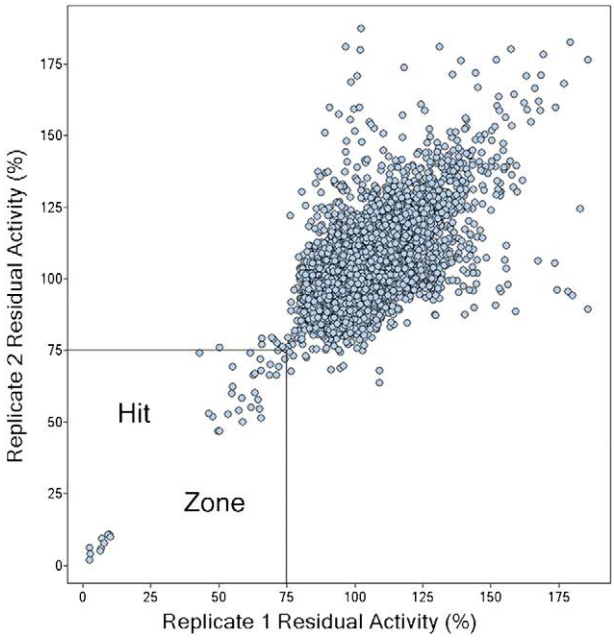


Figure 1. Replicate plot of the screen of 50,000 small molecules against *E. coli* DHFR. Compounds that perturbed *E. coli* DHFR activity to three standard deviations below the high control mean in both replicates (Hit Zone) were deemed to be active against DHFR, and were selected for secondary screening.

In secondary screening, IC_{50} determinations were performed for actives from primary screening. This potency analysis was done at two DHF concentrations, 30 μM and 100 μM, to identify compounds that were competitive with DHF. Figure 2 illustrates sample IC_{50} curves for trimethoprim, a known inhibitor competitive with DHF,[10] and the active compound **10**. The ratio of the two calculated IC_{50} values at the two concentrations of DHF was used to evaluate the competitiveness of each primary hit with respect to DHF. Using the equation $IC_{50} = K_i\,(1 + [S]/K_m)$,[11] where $K_{m,\ DHF} = 9.5$ μM (data not shown), the IC_{50} ratio of a true competitive inhibitor should equal 2.8 for determinations at 100 and 30 μM DHF. This secondary screen identified 12 inhibitors of DHFR, out of the 62 actives from primary screening, having IC_{50} ratios consistent with competitive behavior. Table 1 shows the structures of these 12 molecules along with their IC_{50} data and K_i values calculated from the two IC_{50} determinations. The identity of each of the 12 compounds was confirmed by positive ion electrospray mass spectrometry. Compounds **1**, **2**, **4**, **10** and **12** from Table 1 have IC_{50} values in the nanomolar range and those for the remainder of the compounds were in the micromolar range.

The 12 hits can be grouped into 3 broad structural classifications, 2,4-diaminoquinazolines (**1**–**5**), 2,4 diaminopyrimidines (**6**–**9**), and molecules without diaminoheterocyles (**10**–**12**). Of the 2,4-diaminoquinazolines identified compounds **3**–**5** represent novel inhibitors of DHFR while the 5-arylthioquinazolines (**1** and **2**) have

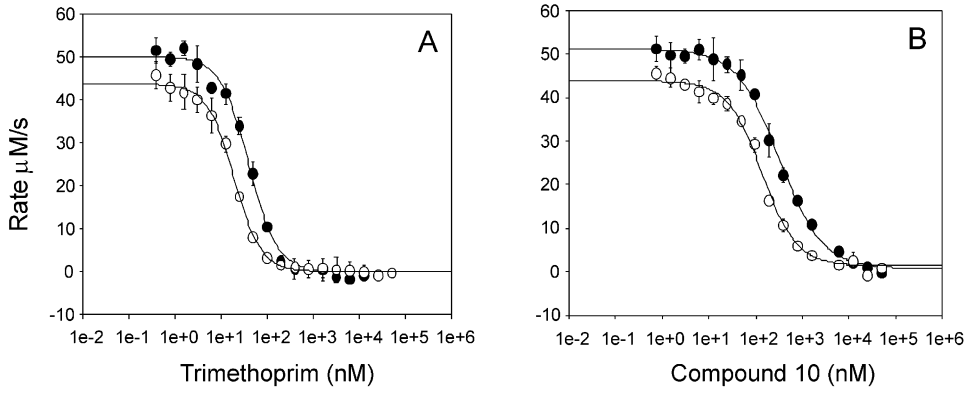
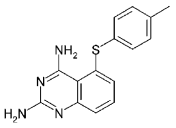
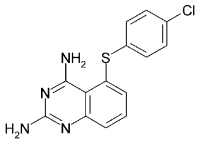
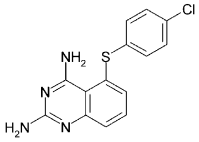
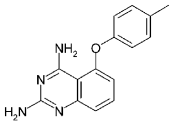
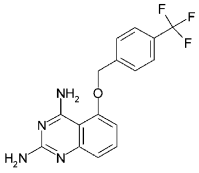
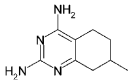
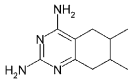
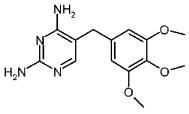
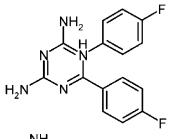
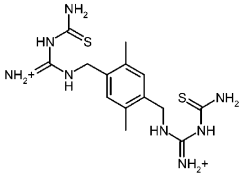
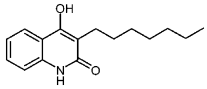
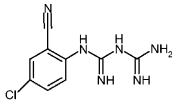


Figure 2. IC_{50} analysis for competitive inhibition. Data are shown for DHFR with a known DHF-competitive inhibitor, Trimethoprim, (panel A) and apparent competitive inhibitor **10** (panel B). Plots for IC_{50} determination are shown at 30 μM DHF (○) and 100 μM DHF (●). IC_{50} values were extracted from assay data using nonlinear regression analysis (SigmaPlot 8.0 software, SPSS Science, Chicago, IL) of the equation $v = a\,(1 - [I]/(IC_{50} + [I])) + c$, where v is the reaction rate, a is the amplitude of inhibition, [I] is the inhibitor concentration and c is residual activity at infinite inhibitor concentration.

Table 1. DHFR inhibitors found to be competitive with dihydrofolate

Compd	Structure	IC_{50} (nM) 30 μM DHF	IC_{50} (nM) 100 μM DHF	IR[a]	K_i[b] (nM)
1		310	820	2.6	73
2		320	510	1.6	61
3		400	1.0×10^3	2.6	93
4		190	420	2.3	41
5		660	1.1×10^3	1.7	130
6		1.1×10^4	2.4×10^4	2.3	2.3×10^3
7		790	2.1×10^3	2.6	190
8		18	37	2.1	3.8
9		1.1×10^4	1.6×10^4	1.5	2.0×10^3
10		109	302	2.8	26
11		4.8×10^4	1.2×10^5	2.5	1.1×10^4
12		320	620	1.9	65

[a]IC_{50} ratio, (IC_{50} in the presence of 100 μM DHF)/(IC_{50} in the presence of 30 μM DHF). Calculated value of IR for competitive inhibitors is 2.8.
[b]Inhibition constant K_i was calculated using the relationship $K_i = IC_{50}/(1 + [S]/K_m)$,[11] where [*S*] the substrate concentration (100 or 30 μM) and K_m was determined to be 9.5 μM (data not shown). The K_i indicated was the average of determinations at the two substrate concentrations.

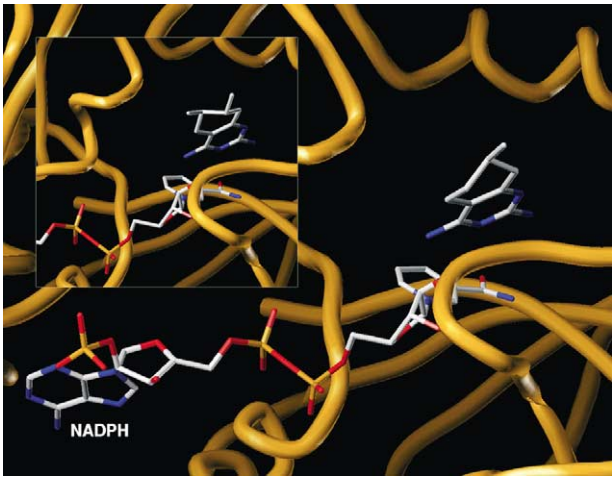


Figure 3. Model of DHFR binding by **6** and **7** (inset) into the *E. coli* DHFR active site, in the presence of NADPH (PDB code 1RX3[14]). Modeling is based upon the structure of **1** bound to *C. albicans* DHFR (PDB code 1IA1),[13] and was constructed using SYBYL 6.8 with the Biopolymer module (Tripos Inc., St Louis, MO, USA).

been previously identified as active against *Candida albicans* DHFR.[12] The structure of **1** bound to *C. albicans* DHFR has been published.[13] Among the 2,4-diaminopyrimidines identified was the antibiotic trimethoprim (**8**) and three molecules, **6**, **7** and **9**, not previously characterized as inhibitors of DHFR. Based on the structure of DHFR with **1** present in the active site of *Candida albicans* DHFR,[13] we have modeled **6** and **7** into the active site of *E. coli* DHFR. Each of these compounds nicely occupies the DHF binding pocket (Fig. 3) as expected based on the competitive behavior with respect to DHF. Of the remaining molecules, **10** and **12** are novel inhibitors of DHFR that have guanidine in common and are remarkably potent (K_i values of 26 and 65 nM, respectively). Compound **11** is a quinolinone not previously reported to be active against dihydrofolate reductase.

In conclusion, we have screened a 50,000 compound library for novel inhibitors of *E. coli* DHFR and performed secondary screening to identify actives that are competitive with the substrate DHF. Of 62 compounds that were active against DHFR, 12 showed kinetic behavior consistent with competitive inhibition with respect to DHF. Of these 12 molecules, 9 have not been previously reported as inhibitors of DHFR. Co-crystallization and other experiments aimed at more fully describing the activity of these molecules are currently in progress.

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