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Selective Antifolates for Chemically Labeling Proteins in Mammalian Cells

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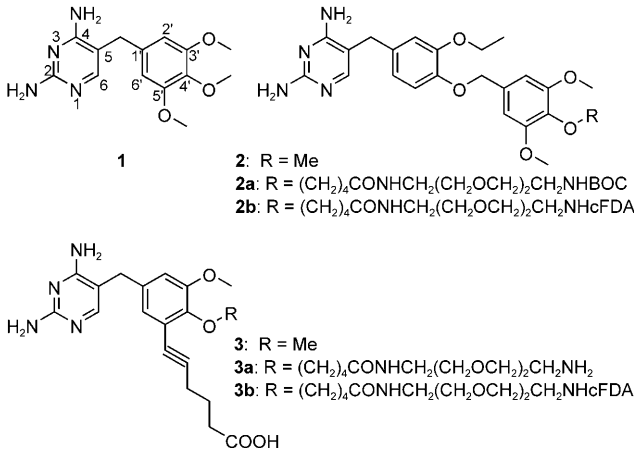
Pairs of small molecule ligands and receptor proteins that associate to the exclusion of all other biological components (that is, orthogonal interaction pairs) are powerful tools for analyzing, modulating, or otherwise perturbing protein function in living cells and organisms.[1] Trimethoprim (TMP, **1**, Scheme 1)



Scheme 1. Schematic representation of antifolates under study. The common numbering Scheme for 5-substituted benzyl pyrimidines is given for **1** (trimethoprim, TMP). Compounds **2b** and **3b** are heterodimeric conjugates of **2** and **3** to acetylated 5(6)-carboxy fluorescein (cFDA).

is a selective inhibitor of bacterial forms of dihydrofolate reductase (DHFR). TMP strongly inhibits *Escherichia coli* DHFR (eDHFR) (K_i = about 1 nM), yet weakly inhibits mammalian forms of the enzyme ($K_i > 1$ μM).[2] The orthogonality of the TMP–eDHFR interaction to eukaryotic cells has been exploited to develop a general method for labeling eDHFR fusion proteins in wild-type mammalian cells with cell-permeable TMP-fluorophore conjugates.[3,4] A heterodimeric conjugate of TMP with synthetic ligation factor (SLF) has been developed as a chemical inducer of dimerization (CID) and used to activate transcription in a yeast three-hybrid assay as well as modulate glycosylation in mammalian cells.[5,6]

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We sought to determine whether selective DHFR inhibitors (antifolates) other than TMP could be utilized for biotechnological applications in mammalian cells. DHFR has been the focus of intense drug discovery efforts for several decades,[7] and in recent years there has been considerable focus put on developing selective inhibitors against DHFRs from pathogenic organisms such as *Pneumocystis carinii*, *Plasmodium falciparum*, and *Staphylococcus aureus*.[8] Hundreds of TMP analogues (substituted 5-benzyl pyrimidines) have been prepared in efforts to analyze binding modes and to develop improved therapies against resistant organisms.[9–16] We chose to focus our efforts on compounds **2** and **3** (Scheme 1, Table 1) which exhibit approximately nanomolar inhibitory activities against *P. falciparum* DHFR (pfDHFR) and *P. carinii* DHFR (pcDHFR), while they exhibit minimal or no activity against mammalian DHFRs.[9,15,16]

Table 1. DHFR enzyme inhibition (K_i, [nM]) for selected antifolates (Scheme 1).[a]

Compound	1	2	3
rat liver DHFR	193000	No inhibition	540
E. coli	1.3	n.d.[b]	n.d.
P. falciparum	10.3	0.3	n.d.
P. falciparum (C59R, S108N)	242	2.2	n.d.
P. carinii	6700	n.d.	0.054

[a] Values taken from previously published data[2,9,15–16] [b] n.d.: not determined.

Conjugates of **2** and **3** to a hydrophobic, acetylated fluorescein were prepared (Scheme 1, **2b, 3b)**. The conjugation strategy was informed by previously reported structural analyses that revealed the binding modes of each compound to their respective targets. Yuthavong and co-workers used semiempirical methods to model the binding of a close analogue of **2** to the known structure of pfDHFR.[15] The 4′-trimethoxy-benzyl substituent was modeled to form a π–π stacking interaction with Phe116 of the wild-type pfDHFR. The *para*-methoxy is in van der Waals contact with Cys50 near the entrance to the binding pocket, and we surmised that alkyl linkage at this position would be the least disruptive to binding. The structure of pcDHFR in complex with **3** and NADPH was reported by Cody and co-workers.[17] The 5′-(5-carboxy-1-pentynyl side chain interacts with Arg75, and this contributes strongly to the high affinity. The 4′-methoxy group interacts with the hydrophobic side chains of Leu25 and Ile33 near the entrance to the binding pocket, and we chose to conjugate at this position.

We first sought to validate our conjugation strategy by directly characterizing the ability of the nonfluorescent analogues **2a** and **3a** to inhibit *Toxoplasma gondii* DHFR (tgDHFR), a protozoan DHFR used as a model for biochemical characteri-

zation of pfDHFR inhibitors[18] and pcDHFR. By using an assay based on measurement of the change in absorbance at 340 nm when dihydrofolate is reduced to tetrahydrofolate in the presence of NADPH, inhibition constants for each analogue were determined (Table 2).[12] Methotrexate (MTX) and TMP were included in the assay as controls. **2a** potently inhibited tgDHFR ($IC_{50}=0.032$ μM), while it poorly inhibited rat liver DHFR ($IC_{50}=6$ μM). **3a** also exhibited excellent potency and selectivity, and strongly inhibited pcDHFR ($IC_{50}=0.025$ μM), while it showed about 350-fold lower activity against mammalian DHFR. The inhibition data suggested that heterodimers of **2** and **3** linked at the indicated positions would retain effective potency against their putative targets, while maintaining sufficient selectivity for applications in mammalian cell lines.

Table 2. DHFR enzyme inhibition (IC_{50}, [μM]) of substituted inhibitor analogues.

Compound	**1**[a]	**2a**	**3a**
Rat liver DHFR	180	6	9.1
pcDHFR	12	1.373	0.025
tgDHFR	2.8	0.032	0.026

[a] Values taken from previously published data.[9]

The linker-substituted analogues, **2a** and **3a** were further characterized by determining their ability to inhibit *E. coli* growth. The minimum inhibitory concentrations (MIC) of TMP, **2a** and **3a** were about 0.2 μM, 20 μM and >100 μM, respectively. We were interested in knowing the affinity of **2a** for eDHFR relative to that of TMP. Compound **2** is about 30-fold and about 100-fold more potent than TMP against wild-type pfDHFR and the TMP-resistant double mutant (C59R, S108N), respectively (Table 1). Because an analogue of **2** is substantially less potent against eDHFR than TMP, it is possible that **2b** or other heterodimers of **2** could be used simultaneously with TMP for assays in mammalian cells. In the case of **3a**, we expected that it would strongly inhibit eDHFR, as a 5′-(5-carboxyalkyloxy)-TMP analogue was shown to be about 50-fold more potent against eDHFR than TMP itself.[10] The fact that **3a** does not inhibit *E. coli* growth up to the concentrations tested suggests the molecule cannot cross the cell membrane. This is not entirely surprising as the carboxyl group of **3a** is likely deprotonated, and localized charges typically lower the membrane permeability of small molecules.[3]

In order to be useful as a research tool for chemical biology applications, orthogonal ligand–receptor pairs must meet, at minimum, three criteria: 1) The protein acceptor must be soluble and expressible in mammalian cells; 2) the ligand and its conjugates must be cell permeable; and 3) the ligand must diffuse freely within cells and not partition to particular organelles or subcellular domains. We used epifluorescence microscopy to determine the cell permeability and subcellular distribution of **2b** and its selective binding to a soluble, TMP-resistant mutant (K27E, C59R, S108N) of the DHFR domain of *P. falciparum* DHFR-thymidylate synthetase.[19] An expression vector that targeted pfDHFR to the nucleus was prepared. Targeting was achieved by encoding pfDHFR soluble domain with a N-terminal fusion of three copies of the canonical simian virus 40 large T-antigen nuclear localization sequence (DPKKKRKV).[4] We then transiently transfected NIH 3T3 fibroblast cells with the vector. Approximately 24 h after transfection, the cells were incubated with low (500 nM) concentrations of **2b** and imaged microscopically (Figure 1).

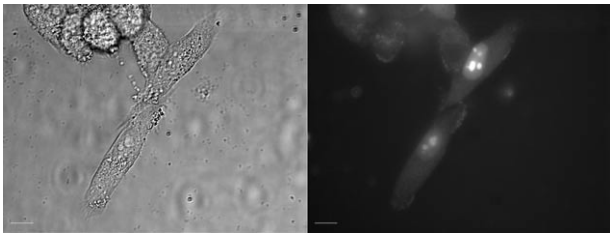

Figure 1. Selective chemical labeling of subcellularly targeted pfDHFR in living mammalian cells. Bright field (left) and fluorescence (right) micrographs show adherent NIH3T3 fibroblast cells transiently expressing nucleus-localized pfDHFR soluble domain (K27E, C59R, S108N). The cells were incubated in growth medium containing 500 nM **2b** for 15 min, washed two times with PBS, immersed in medium without compound and imaged.

Diffuse fluorescence was observed in all cells incubated with **2b**, and some of the cells exhibited distinct nuclear fluorescence with more brightly fluorescent nucleoli, characteristic of the nucleus-targeting sequence.[4] The diffuse fluorescence indicates that **2b** readily enters cells, where the fluorescein moiety is hydrolyzed by intracellular esterases; this yields the fluorescent fluorescein dianion. We attribute nuclear staining to the specific binding of **2b** to nucleus-targeted pfDHFR soluble domain. We performed analogous imaging experiments with **3b** and cells transfected with a vector encoding pcDHFR fused to the N-terminal nucleus localization sequence. However, we did not observe any intracellular fluorescence or nuclear staining with **3b**; this provides further evidence that analogues of **3** cannot passively diffuse into cells due to the presence of the 5′-(5-carboxy-1-pentynyl) moiety.

Substituted analogues of the established antifolates **2** and **3** retain similar potency and selectivity of the parent compounds when assessed in an inhibition assay. Compound **2a**, a heterodimeric conjugate of **2** to a hydrophobic, acetylated fluorescein passively diffused into mammalian cells and selectively labeled a recombinantly expressed fusion of the soluble domain of pfDHFR. These results demonstrate that the considerable efforts devoted to finding selective inhibitors of pathogenic DHFRs can be leveraged to identify and develop new tools for chemical biology. Further studies are underway to comprehensively characterize the interaction between substituted analogues of **2** and soluble mutants of pfDHFR, and to further exploit this interaction for chemically labeling proteins in living mammalian cells.

Experimental Section

The complete details of antifolate syntheses and characterization, plasmid vector construction, cell culture conditions and microscopy protocols are reported in the Supporting Information.

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[1] A. Bishop, O. Buzko, S. Heyeck-Dumas, I. Jung, B. Kraybill, Y. Liu, K. Shah, S. Ulrich, L. Witucki, F. Yang, C. Zhang, K. M. Shokat, *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 577–606.
[2] D. P. Baccanari, S. Daluge, R. W. King, *Biochemistry* **1982**, *21*, 5068–5075.
[3] N. T. Calloway, M. Choob, A. Sanz, M. P. Sheetz, L. W. Miller, V. W. Cornish, *ChemBioChem* **2007**, *8*, 767–774.
[4] L. W. Miller, Y. Cai, M. P. Sheetz, V. W. Cornish, *Nat. Methods* **2005**, *2*, 255–257.
[5] J. L. Czlapinski, M. W. Schelle, L. W. Miller, S. T. Laughlin, J. J. Kohler, V. W. Cornish, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 13186–13187.
[6] S. S. Gallagher, L. W. Miller, V. W. Cornish, *Anal. Biochem.* **2007**, *363*, 160–162.
[7] I. M. Kompis, K. Islam, R. L. Then, *Chem. Rev.* **2005**, *105*, 593–620.
[8] D. C. Chan, A. C. Anderson, *Curr. Med. Chem.* **2006**, *13*, 377–398.
[9] R. A. Forsch, S. F. Queener, A. Rosowsky, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1811–1815.
[10] L. F. Kuyper, B. Roth, D. P. Baccanari, R. Ferone, C. R. Beddell, J. N. Champness, D. K. Stammers, J. G. Dann, F. E. Norrington, D. J. Baker, *J. Med. Chem.* **1985**, *28*, 303–311.
[11] L. F. Kuyper, B. Roth, D. P. Baccanari, R. Ferone, C. R. Beddell, J. N. Champness, D. K. Stammers, J. G. Dann, F. E. Norrington, D. J. Baker, P. J. Goodford, *J. Med. Chem.* **1982**, *25*, 1120–1122.
[12] A. Rosowsky, V. Cody, N. Galitsky, H. Fu, A. T. Papoulis, S. F. Queener, *J. Med. Chem.* **1999**, *42*, 4853–4860.
[13] A. Rosowsky, R. A. Forsch, S. F. Queener, *J. Med. Chem.* **2002**, *45*, 233–241.
[14] A. Rosowsky, R. A. Forsch, S. F. Queener, *J. Med. Chem.* **2003**, *46*, 1726–1736.
[15] C. Sirichaiwat, C. Intaraudom, S. Kamchonwongpaisan, J. Vanichtanankul, Y. Thebtaranonth, Y. Yuthavong, *J. Med. Chem.* **2004**, *47*, 345–354.
[16] B. Tarnchompoo, C. Sirichaiwat, W. Phupong, C. Intaraudom, W. Sirawaraporn, S. Kamchonwongpaisan, J. Vanichtanankul, Y. Thebtaranonth, Y. Yuthavong, *J. Med. Chem.* **2002**, *45*, 1244–1252.
[17] V. Cody, J. Pace, K. Chisum, A. Rosowsky, *Proteins Struct. Funct. Bioinf.* **2006**, *65*, 959–969.
[18] M. G. Reynolds, D. S. Roos, *J. Biol. Chem.* **1998**, *273*, 3461–3469.
[19] D. Japrung, S. Chusacultanachai, J. Yuvaniyama, P. Wilairat, Y. Yuthavong, *Protein Eng. Des. Sel.* **2005**, *18*, 457–464.

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