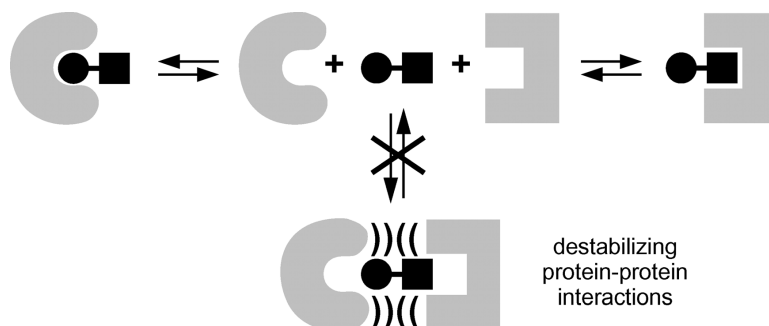## Article

A Bifunctional Molecule That Displays Context-Dependent Cellular Activity

Patrick D. Braun, Katherine T. Barglow, Yun-Ming Lin, Thomas Akompong,
Roger Briesewitz, Gregory T. Ray, Kasturi Haldar, and Thomas J. Wandless

destabilizing
protein-protein
interactions

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 4 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

A Bifunctional Molecule That Displays Context-Dependent Cellular Activity

Patrick D. Braun,[†] Katherine T. Barglow,[†] Yun-Ming Lin,[†] Thomas Akompong,[‡] Roger Briesewitz,[§] Gregory T. Ray,[†] Kasturi Haldar,[‡] and Thomas J. Wandless*,[†]

Contribution from the Department of Chemistry and the Howard Hughes Medical Institute Stanford University, Stanford, California 94305, and the Departments of Pathology and Microbiology & Immunology, Northwestern University Medical School, Chicago, Illinois 60611

Received March 15, 2003; E-mail: wandless@stanford.edu

Abstract: The cell-permeable dihydrofolate reductase inhibitor methotrexate was covalently linked to a ligand for the protein FKBP to create a bifunctional molecule called MTXSLF. The covalent tether between the two ligands was designed to be prohibitively short, so that unfavorable protein−protein interactions between DHFR and FKBP preclude formation of a trimeric complex. In vitro and in vivo experiments demonstrate that MTXSLF is an effective inhibitor of human DHFR, but that efficacy is decreased in the presence of human FKBP due to the high concentration of FKBP and its tight affinity for MTXSLF. MTXSLF also inhibits *Plasmodium falciparum* DHFR in vitro, but a low concentration of the weaker binding *Plasmodium* FKBP has no effect on the inhibitory potency of MTXSLF in vivo. These studies illustrate a potentially general strategy for modulating the biological activity of synthetic molecules that depends on the ligand-binding properties of a nontarget protein.

Introduction

The synthesis of inhibitory molecules that bind selectively to target proteins is a longstanding goal for the development of new therapeutic agents.[1] In recent years, the focus of this activity has significantly expanded to include the use of cell-permeable inhibitors as probes of fundamental cellular processes.[2−5] For either application, pharmaceutical development or chemical biology, the binding selectivity profile for the compound of interest largely determines its utility. Many target proteins are members of larger families (e.g., kinases, proteases), and related nontarget proteins often compete with the desired target protein for binding to inhibitory compounds, thus eroding the specificity of the desired interaction. Inhibitors that are unable to discriminate between related proteins are of limited value for many fundamental and therapeutic applications, and methods that provide predictive power for the design and synthesis of selective inhibitors are still acutely needed.

One approach to elicit a selective cellular response relies on significant genomic differences between organisms to provide specificity. For example, β-lactam antibiotics kill bacteria because they inhibit the bacterial transpeptidase enzymes, which

have no mammalian homologues.[6] Therefore, the presence (in bacteria) or absence (in human cells) of the target protein determines biological activity. However, it is particularly difficult to target specific eukaryotic cell subpopulations (e.g., cancer cells, certain parasites, and fungi) in an environment of related nontarget cells due to similarities in biochemical pathways. We have developed an alternative strategy that uses induced protein−protein interactions to control the specificity of a binding interaction and, as a result, the selectivity of a biological response.[7] This approach may prove useful for cell-selective targeting of toxins or other drugs.

Our strategy involves the design and synthesis of molecules that are capable of binding to two different proteins (Figure 1A). These bifunctional molecules are composed of a ligand for one protein connected by a covalent tether to a ligand for a second protein. When the tether between the two ligands is sufficiently long, both proteins can simultaneously bind to the bifunctional molecule to form a trimeric complex.[7,8] In situations where a very short linker is used, the two proteins are forced into close proximity, and unfavorable protein−protein interactions (e.g., steric collisions, electrostatic repulsions) destabilize the trimeric complex relative to the two possible dimeric complexes.[9] If the destabilizing protein−protein interactions are sufficiently severe, then the bifunctional molecule will be able to bind to only one of its two possible protein partners at any given time. The distribution of the bifunctional molecule

† Department of Chemistry, Stanford University.
‡ Northwestern University Medical School.
§ HHMI, Stanford University.

(1) Cohen, P. *Curr. Opin. Chem. Biol.* **1999**, *3*, 459−465.
(2) Amara, J. F.; Clackson, T.; Rivera, V. M.; Guo, T.; Keenan, T.; Natesan, S.; Pollock, R.; Yang, W.; Courage, N. L.; Holt, D. A.; Gilman, M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10618−10623.
(3) Bierer, B. E.; Somers, P. K.; Wandless, T. J.; Burakoff, S. J.; Schreiber, S. L. *Science* **1990**, *250*, 556−559.
(4) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019−1024.
(5) VanDuyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. *Science* **1991**, *252*, 839−842.
(6) Frere, J. M. *Mol. Microbiol.* **1995**, *16*, 385−395.
(7) Briesewitz, R.; Ray, G. T.; Wandless, T. J.; Crabtree, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1953−1958.
(8) Rosen, M. K.; Amos, C. D.; Wandless, T. J. *J. Am. Chem. Soc.* **2000**, *122*, 11979−11982.
(9) Scheinerman, F. B.; Norel, R.; Honig, B. *Curr. Opin. Struct. Biol.* **2000**, *10*, 153−159.
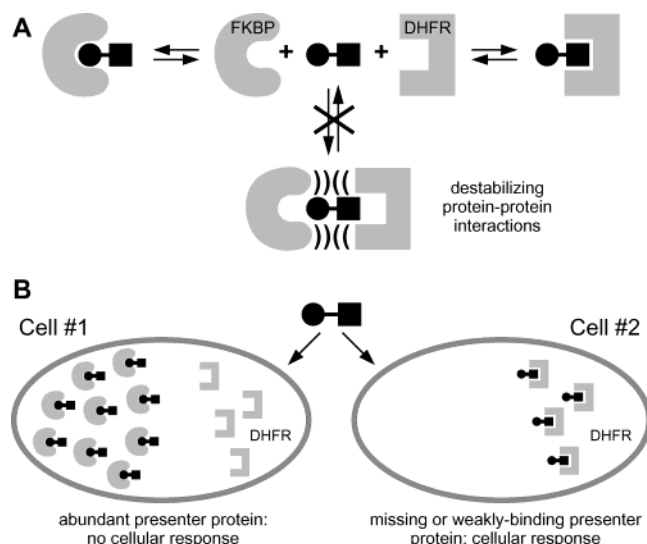
Figure 1. Schematic representation of the presenter protein strategy. (A) The bifunctional molecule, MTXSLF, can bind to either the FKBP presenter protein (gray semicircle) or to the DHFR target protein (gray rectangle) but not to both proteins simultaneously due to destabilizing protein−protein interactions. (B) Schematic representation of MTXSLF's activity in different cells. Bifunctional molecules entering cells that possess an abundant, high-affinity FKBP presenter protein will partition preferentially to bind to FKBP, leaving DHFR uninhibited. Upon entering cells that either lack the FKBP presenter protein entirely or possess an FKBP in low abundance or that binds weakly to FKBP ligands, the MTXSLF bifunctional molecule will selectively partition to inhibit DHFR and elicit a cytotoxic response.

between the two proteins is a function of the concentration of each protein as well as the molecule's affinity for each protein. We reasoned that this behavior could be used to control the binding preferences and subsequent cellular activity of bifunctional molecules (Figure 1B). Independently, a theoretical treatment of a similar strategy has been outlined.[10] We set out to test our hypothesis by identifying protein−ligand combinations with the appropriate characteristics.

One of the two proteins is defined as the target protein, and the binding of a ligand to the target protein is designed to elicit a specific desired cellular response (e.g., cytotoxicity, transcription, enzyme inhibition). The second protein is defined as the presenter protein, which is an intracellular protein that binds tightly to cell-permeable ligands. A key characteristic of the presenter protein is that binding to its cognate ligand is biologically silent. At any one time, a fraction of the total cellular complement of presenter protein may be occupied by the bifunctional molecule, and this binding event should not elicit a cellular response. Rather, it is the binding of the bifunctional molecule to the target protein that causes the cellular response (Figure 1B). These bifunctional molecules would thus be expected to display biological activity that depends on the presence or absence of the presenter protein.

Experimental Section

1-(3,3-Dimethyl-2-oxopentanoyl)piperidine-2-carboxylic Acid 1-{3-[4-*tert*-Butoxycarbonyl-4-(9H-fluoren-9-ylmethoxycarbonylamino)-butyrylamino]phenyl}-3-(3,4-dimethoxyphenyl)propyl Ester (3). Samples of Fmoc-Glu-α-ᵗBu (48 mg, 0.11 mmol), DMAP (14 mg, 0.12 mmol), HOAt (16 mg, 0.12 mmol), and the Synthetic Ligand for FKBP (SLF, compound **2**, 40 mg, 0.8 mmol) were dissolved in 2 mL of dry CH_2Cl_2 under argon, and triethylamine (10 μL, 0.16 mmol) was added.

(10) Varshavsky, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2094−2099.

The reaction mixture was stirred to homogeneity (10 min) at which time EDC (23 mg, 0.12 mmol) was added. After being stirred for 2 h, the reaction was concentrated by evaporation, and the residue was dissolved in CH_2Cl_2 and washed with 1% aqueous citric acid (2 × 10 mL), 5% aqueous $NaHCO_3$ (2 × 10 mL), and saturated aqueous NaCl (1 × 10 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated, and the product was purified by silica gel chromatography (1:1 hexane:EtOAc) to yield compound **3** (60 mg, 87%) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.89 (t, $J = 7.4$ Hz, 3H), 1.11 (d, $J = 3.6$ Hz, 1H), 1.22 (s, 3H), 1.23 (s, 3H), 1.47 (s, 9H), 1.54−1.66 (m, 3H), 1.71 (q, $J = 7.4$ Hz, 2H), 1.94−2.1 (m, 3H), 2.20 (m, 1H), 2.25−2.40 (m, 2H), 2.44 (t, $J = 6.7$ Hz, 2H), 2.55 (m, 2H), 3.07 (dt, $J = 10$ Hz, 1H), 3.33 (d, $J = 15$ Hz, 1H), 3.83 (s, 3H), 3.84 (s, 3H), 4.19 (m, 1H), 4.36 (m, 1H), 4.36 (d, $J = 7$ Hz, 1H), 4.43 (m, 1H), 5.34 (d, $J = 5.1$ Hz, 1H), 5.81 (m, 1H), 6.60 (m, 2H), 6.75 (m, 1H), 7.01 (d, $J = 7.7$ Hz, 1H), 7.28 (m, 3H), 7.38 (m, 2H), 7.57 (m, 3H), 7.70 (d, $J = 8.1$ Hz, 1H), 7.75 (d, $J = 7.5$ Hz, 2H), 8.53 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 8.74, 14.17, 20.90, 21.03, 23.26, 23.31, 24.94, 26.12, 27.95, 29.48, 31.05, 32.47, 33.85, 38.28, 44.22, 46.76, 47.08, 51.12, 53.92, 55.79, 55.76, 55.87, 60.38, 67.12, 76.41, 82.77, 94.72, 111.19, 111.64, 117.15, 119.18, 119.96, 119.99, 120.11, 121.86, 125.00, 125.01, 127.05, 127.73, 129.18, 133.47, 140.79, 141.26, 143.61, 143.72, 147.21, 148.78, 156.64, 166.90, 169.43, 170.44, 170.94. ESI-MS ($C_{54}H_{65}N_3O_{11}$ + Na^+): calcd m/z 954.45, found m/z 954.30.

1-(3,3-Dimethyl-2-oxopentanoyl)piperidine-2-carboxylic Acid 1-[3-(4-*tert*-Butoxycarbonyl-4-{4-[(2,4-diaminopteridin-6-ylmethyl)-methylamino]benzoylamino}butyrylamino)phenyl]-3-(3,4-dimethoxyphenyl)propyl Ester (MTXSLF, 1). Compound **3** (60 mg, 0.063 mmol) was dissolved in 2 mL of anhydrous DMF and 0.5 mL of piperidine and stirred at room temperature for 1 h. DMF and piperidine were removed under high vacuum to yield deprotected **3** as a light yellow solid (45 mg) that was carried on without further purification. 4-[(2,4-Diaminopteridin-6-ylmethyl)methylamino]benzoic acid (compound **4**, 28 mg, 0.074 mmol) was dried overnight under high vacuum and then placed in an oven-dried, argon-filled flask and dissolved in 2 mL of anhydrous DMF. Diethylcyanophosphonate (38 μL, 0.25 mmol) and triethylamine (43 μL, 0.30 mmol) were added, and the solution was stirred in the dark for 3 h. Deprotected **3** (45 mg, 0.064 mmol) was dissolved in 1 mL of anhydrous DMF and added via syringe to the reaction solution. After 20 h the solution was concentrated under high vacuum and redissolved in 10 mL of $CHCl_3$ and 10 mL of 1% aqueous NH_3 and stirred for 1 h. The organic layer was removed, and the aqueous layer washed with CH_2Cl_2 (2 × 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified twice by flash chromatography over silica (10:1 $CHCl_3$:MeOH then 25:1 $CHCl_3$:MeOH) to yield the desired MTXSLF (compound **1**, 40 mg, 63%). ^{1}H NMR (500 MHz, CD_3OD) δ 0.845 (t, $J = 7.5$ Hz, 3H), 1.05 (m, 1H), 1.19 (m, 5H), 1.65 (m, 5H), 1.46 (s, 9H), 2.02 (m, 1H), 2.16 (m, 2H), 2.29 (m, 2H), 2.53 (m, 4H), 3.16 (m, 3H), 3.22 (m, 2H), 3.30 (m, 3H), 3.35 (bs, 1H), 3.38 (bs, 1H), 3.77 (s, 3H), 3.78 (m, 3H), 4.48 (q, $J = 4.5$ Hz, 1H), 4.75 (s, 2H), 5.20 (bd, $J = 5$ Hz, 1H), 5.67 (m, 1H), 6.68 (m, 1H), 6.75 (m, 3H), 6.82 (d, $J = 10.0$ z, 1H), 7.02 (d, $J = 7.5$ Hz, 1H), 7.22 (t, $J = 8.0$ Hz, 1H), 7.35 (m, 1H), 7.68 (m, 3H), 8.51 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD) δ 9.11, 22.10, 23.59, 23.89, 25.90, 27.37, 27.83, 28.27, 32.22, 33.57, 34.4, 39.24, 39.57, 45.78, 47.68, 52.80, 54.89, 56.42, 56.51, 76.18, 76.67, 78.09, 82.92, 84.12, 90.69, 112.58, 113.17, 113.52, 119.08, 120.63, 121.69, 122.36, 123.29, 130.00, 130.14, 135.19, 140.26, 142.23, 148.80, 149.01, 150.24, 153.40, 155.89, 164.32, 164.71, 169.07, 170.24, 170.97, 192.90, 173.48. High-res MALDI-FTMS ($C_{54}H_{68}N_{10}O_{10}$ + Na^+): calcd m/z 1039.5012, found m/z 1039.4977.

Inhibition of DHFR Enzymatic Activity. Human FKBP12 was expressed and purified as described previously.[7] Human DHFR was expressed in BL21(DE3) cells, purified over a methotrexate-column, and dialyzed against the assay buffer (50 mM Tris, 1 mg/mL bovine

serum albumin, pH 7.0).[11] *Plasmodium* DHFR in pET17b and *Plasmodium* thymidylate synthase tagged with His6 in pET24a were coexpressed in BL21(DE3) pLysS, purified over a Ni^{2+}-column, and dialyzed against the assay buffer.

DHFR activity was measured by incubating the enzyme (25 nM) in assay buffer with NADPH (25 μM) and various concentrations of MTXSLF in the presence or absence of the FKBP presenter protein. The reaction was initiated by adding freshly synthesized dihydrofolate (to 24 μM), and DHFR activity was quantitated spectrophotometrically at 340 nm. Initial velocities were measured and relative reaction rates were determined (observed velocity/uninhibited velocity, V/V_0).

Identification and Synthesis of PfFKBP. The FKBP12 amino acid sequence was used to query the *Plasmodium falciparum* sequence database (PlasmoDB). The single gene identified by the search (called PfFKBP) was synthesized using overlapping oligonucleotides (Interactiva Biotechnology, Ulm, Germany) and subcloned into a pET22b expression plasmid (Novagen, Madison, WI). The synthetic gene was sequenced in both directions to ensure accuracy of the assembly process. Codon preferences of *P. falciparum* are significantly different than those of *Escherichia coli*, so the PfFKBP gene was synthesized using *E. coli* codon preferences to optimize protein expression. PfFKBP-His6 protein was expressed in BL21(DE3) cells, purified by Ni^{2+}-column, and dialyzed against assay buffer. Pure protein (300 μg) was used to immunize rabbits, and antisera crossreactive against PfFKBP were obtained.

Characterization of PfFKBP. *P. falciparum* parasites and infected human erythrocytes were tested by Western blot for the presence of PfFKBP. Parasites were separated from infected host erythrocytes by incubating with 0.01% saponin for 10 min. Parasite and erythrocyte lysates were fractionated into membrane-associated and cytosolic fractions, and 20 μg of total protein was resolved on a 12% PAGE gel, transferred to nitrocellulose, and probed with α-PfFKBP antisera. The affinity of fluorescein-SLF for PfFKBP was measured using fluorescence polarization.[12] The fluorescein-SLF tracer (2 nM) was incubated in buffer (50 mM KH_2PO_4, 150 mM NaCl, 0.1 mg/mL bovine γ-globulin, pH 7.8) along with various concentrations (2.0 nM to 5.0 μM) of purified recombinant PfFKBP. The concentration of bound tracer can be calculated by comparing the intensity of emitted fluorescence both parallel and perpendicular to polarized incident light. Data were transferred to Kaleidagraph (Synergy Software, Reading, PA) and fitted to obtain the dissociation constant. Equation: $F_o - (F_o - F_\infty)(K_d + [\text{tracer}]_{\text{total}} + f) - ((K_d + [\text{tracer}]_{\text{total}} + f)^2 - (4[\text{tracer}]_{\text{total}}f))^{0.5}/(2[\text{tracer}]_{\text{total}})$, where F_o is the polarization of free fluorescent tracer, F_∞ is the polarization of infinitely bound tracer, $[\text{tracer}]_{\text{total}}$ is the total tracer concentration, and f is the measured polarization signal at each protein concentration.

Activity of Synthetic DHFR Inhibitors in *P. falciparum* Culture. The anti-parasite activity of MTXSLF was assessed by measuring hypoxanthine incorporation into parasite DNA.[13] Parasite suspensions at 1–2% parasitemia and 1% hematocrit were dispensed into 96-well plates. Various concentrations of the test compound were added to the culture medium, and the cells were incubated for 4 h. [³H]-Hypoxanthine (0.5 μCi/well) was then added and the sample incubated overnight under standard culture conditions. The cells were harvested onto a glass fiber filter, liquid scintillation cocktail was added, and the sample was counted to determine incorporated [³H]-hypoxanthine.

Activity of Synthetic DHFR Inhibitors in Human Tissue Culture. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the cytotoxicity of MTXSLF in the MES-

SA cell line (uterine tumor).[14] Cells were plated in 96-well microtiter plates (100 μL at 80 000 cells/mL). After 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, the cells were exposed to various concentrations of MTXSLF and incubated for 72 h. MTT reagent (20 μL of 5 mg/mL in PBS buffer) was added to each well. After a 3 h incubation, the medium was aspirated and 0.1 N HCl in 2-propanol was added to solubilize the formazan salts and thoroughly mixed. Absorbances were measured using a multiwell spectrophotometer (Molecular Devices) at 570 nm. The data were plotted in Excel to determine the IC_{50}.

Results

Dihydrofolate reductase (DHFR) was chosen as the target protein due to its essential function in most organisms.[15] DHFR catalyzes the synthesis of tetrahydrofolate, which is a necessary cofactor for nucleic acid biosynthesis. DHFR sequences are highly conserved among prokaryotes and eukaryotes, and as a result, many DHFR inhibitors display limited selectivity between enzymes from different organisms.[16] High-affinity DHFR ligands have been clinically used as antibiotics; however the conservation of DHFR sequences in different cell types often leads to a narrow therapeutic window that can limit the utility of these drugs. To compensate for this lack of selectivity, our approach takes advantage of a second cellular protein (the presenter protein) to engineer selectivity.

The human FK506-binding protein, hFKBP, is well suited to serve as a presenter protein. hFKBP is an abundant intracellular protein (4–5 μM) that is widely expressed in human cells and tissues.[2–4] The hFKBP–FK506 complex inhibits calcineurin and the process of T cell activation.[17–21] Neither FK506 nor hFKBP alone binds to calcineurin, but the hFKBP–FK506 complex binds to calcineurin with high affinity. hFKBP also binds tightly to dozens of different synthetic ligands that, unlike FK506, lack the ability to bind to calcineurin, and the synthetic ligand of FKBP (SLF) is one such ligand. As a result, cell-permeable ligands such as SLF bind tightly to hFKBP but do not elicit a significant cellular response. Similarly, a slight synthetic modification to the C21 allyl group of FK506 abolishes its ability to bind to calcineurin, although the resulting compound (FK506-M) retains high affinity for hFKBP.[4]

Methotrexate (MTX) was chosen as the ligand for DHFR. It is a tight binder ($K_d <$ 1 nM)[22] to and potent inhibitor of DHFR and is used therapeutically to treat cancer. It is well documented that the γ carboxylic acid can be functionalized with minimal effect on efficacy, so this functional group was chosen as the site of derivitization.[23] To obtain unfavorable protein–protein

(11) Kaufman, B. T. *Methods Enzymol.* **1974**, *34*, 272–281.
(12) Clackson, T.; Yang, W.; Rozamuns, L. W.; Hatada, M.; Amara, J. F.; Rollins, C. T.; Stevenson, L. F.; Magari, S. R.; Wood, S. A.; Courage, N. L.; Lu, X, D.; Cerasoli, F.; Gilman, M.; Holt, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10437–10442.
(13) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710–718.
(14) Mosmann, T. J. *J. Immunol. Methods* **1983**, *65*, 55–63.
(15) Bertino, J. R.; Hillcoat, B. L. *Adv. Enzyme Regul.* **1968**, *6*, 335–349.
(16) Kuyper, L. F.; Baccanari, D. P.; Jones, M. L.; Hunter, R. N.; Tansik, R. L.; Joyner, S. S.; Boytos, C. M.; Rudolph, S. K.; Knick, V.; Wilson, H. R.; Caddell, J. M.; Friedman, H. S.; Comley, J. C. W.; Stables, J. N. *J. Med. Chem.* **1996**, *39*, 892–903.
(17) Liu, J.; Farmer, J. D.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807–815.
(18) Vogel, K. W.; Briesewitz, R.; Wandless, T. J.; Crabtree, G. R. In *Advances in Protein Chemistry*; Scolnick, E. M., Ed.; Academic Press: San Diego, 2001; Vol. 56, pp 253–291.
(19) Siekierka, J. J.; Hung, S. H.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755–757.
(20) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758–760.
(21) Liu, J.; Albers, M. W.; Wandless, T. J.; Luan, S.; Alberg, D. G.; Belshaw, P. J.; Cohen, P.; Mackintosh, C.; Klee, C. B.; Schreiber, S. L. *Biochemistry* **1992**, *31*, 3896–3901.
(22) Chunduru, S. K.; Cody, V.; Luft, J. R.; Pangborn, W.; Appleman, J. R.; Blakley, R. L. *J. Biol. Chem.* **1994**, *269*, 9547–9555.
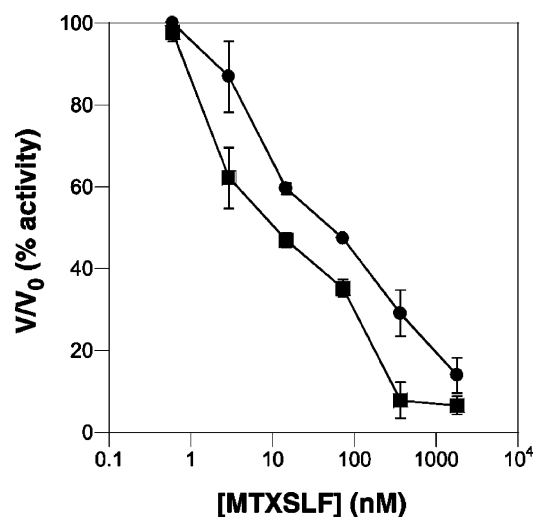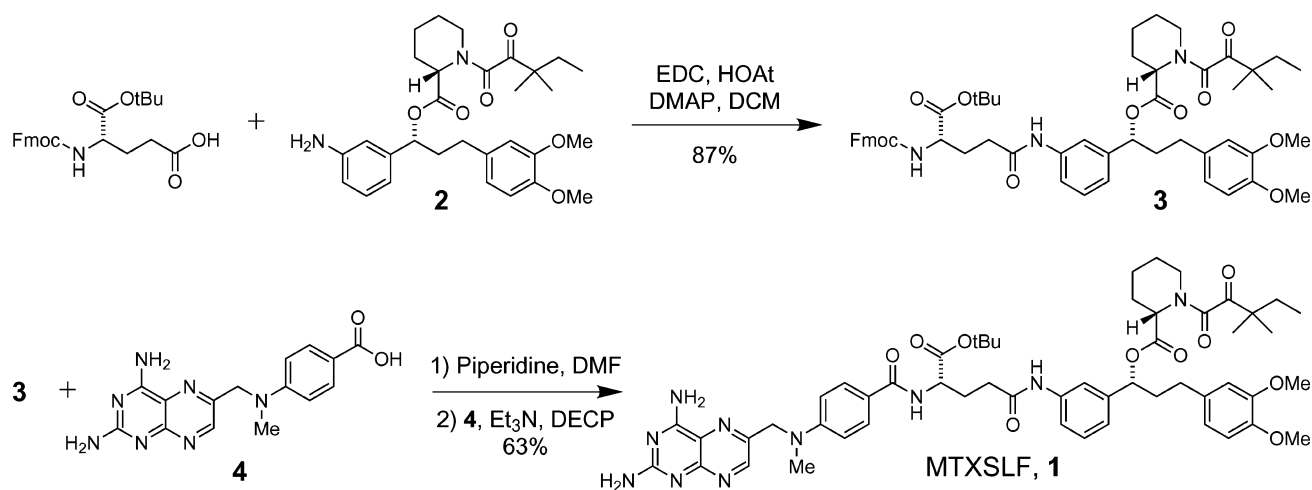(23) Galivan, J. *Mol. Pharmacol.* **1980**, *17*, 105–110.

Scheme 1

Figure 2. The ability of MTXSLF to inhibit purified hDHFR is attenuated by hFKBP. The symbols represent 25 nM hDHFR alone (squares) and hDHFR plus 5 μM hFKBP (circles). All points shown are the averages of duplicate experiments, and error bars show the measured experimental range.
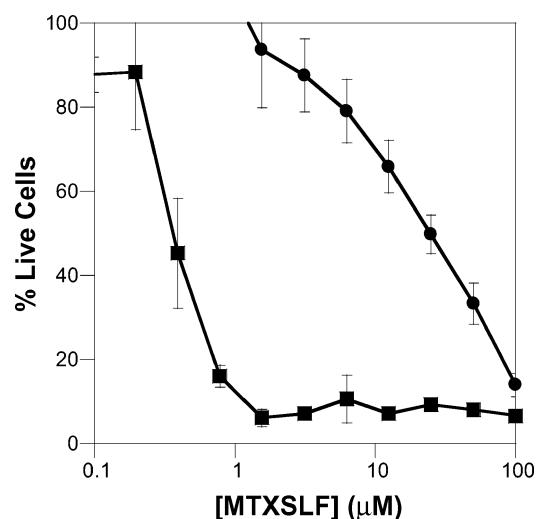

Figure 3. The cytotoxicity of MTXSLF in cells is diminished by hFKBP. MES-SA cells, a human uterine cell line, were treated with MTXSLF in the absence (circles) or presence (squares) of 5 μM FK506-M and cell proliferation was measured with the MTT reagent. Addition of FK506-M should competitively saturate endogenous hFKBP, reducing the ability of hFKBP to attenuate MTXSLF activity. Each point is an average of eight experiments, and error bars show one standard deviation.

interactions, we desired the minimum separation between MTX and SLF. Therefore, SLF, **2**,[24] was coupled to protected glutamate *tert*-butyl ester and then PABA-methyl-pteridine, **4**, to create bifunctional molecule **1** possessing the shortest possible linker between the two protein ligands (Scheme 1).

MTXSLF was first tested as an inhibitor of DHFR in a cell-free system using purified proteins. Human FKBP and human DHFR were expressed in *E. coli* and purified to homogeneity.[7,11] A standard assay of DHFR catalysis was used to quantitate the inhibitory effects of MTXSLF in the presence and absence of hFKBP.[25] For this assay, the concentrations of hDHFR and hFKBP were chosen to closely match those measured in human cells. The bifunctional molecule MTXSLF is a potent inhibitor of hDHFR activity with a measured IC$_{50}$ of 12 nM (Figure 2). When hFKBP (5 μM) is present in the assay solution, the potency of MTXSLF is reduced 5-fold (IC$_{50}$ = 60 nM) (Figure 2). The addition of FK506-M as a monomeric ligand to saturate the hFKBP presenter protein restores inhibitory activity and

provides curves that are indistinguishable from assays lacking hFKBP (data not shown).

To further test the selective detoxification hypothesis, we tested MTXSLF in a cell-based system that is sensitive to DHFR inhibitors. The cytotoxic activity of MTXSLF was assessed using MES-SA cells (human uterine) and the MTT reagent.[14,26] MTXSLF alone displays poor cytotoxic activity with a measured IC$_{50}$ of 25 μM (Figure 3). To determine if endogenous hFKBP is responsible for reducing the activity of the bifunctional molecule, MTXLSF was also tested in the presence of FK506-M, which was used to saturate the hFKBP presenter protein. Addition of 5 μM FK506-M improves the cytotoxic activity of MTXSLF (IC$_{50}$ = 0.37 μM) relative to cells treated with MTXSLF alone (Figure 3). Encouraged by this apparent 68-fold attenuation by hFKBP, we sought to test MTXSLF's activity in a different cellular context.

P. falciparum is a single-cell, eukaryotic parasite that causes

(24) Keenan, T.; Yaeger, D. R.; Courage, N. L.; Rollins, C. T.; Pavone, M. E.; Rivera, V. M.; Yang, W.; Guo, T.; Amara, T. F.; Clackson, T.; Gilman, M.; Holt, D. A. *Bioorg. Med. Chem.* **1998**, *6*, 1309−1335.

(25) Baccanari, D. P.; Tansik, R. L.; Joyner, S. S.; Fling, M. E.; Smith, P. L.; Freisheim, J. H. *J. Biol. Chem.* **1989**, *264*, 1100−1107.

(26) Harker, W. G.; Mackintosh, F. R.; Sikic, B. I. *Cancer Res.* **1983**, *43*, 4943−4950.

```
hFKBP12-GVQVETISPGDG--RTFPKRGQTCVVHYTGMLED-GKKFDSSRDRNKPFKFMLGKQEVIRGWEE
        GV    +   GD     PK+G   VHY G LE  GK FDSS DRN PFKF L + EVI+GW+
MTTEQEFEKVELTADGGVIKTILKKGDEGEENIPKKGNEVTVHYVGKLESTGKVFDSSFDRNVPFKFHLEQGEVIKGWDI


GVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELL
 V+ M    ++   + I    Y YG  G     IP ++ L+F++ELL
CVSSMRKNEKCLVRIESMYGYGDEGCGESIPGNSVLLFEIELLSFREAKKSIYDYTDEEKVQSAFDIKEEGNEFFKKNEI
NEAIVKYKEALDFFIHTEEWDDQILLDKKKNIEISCNLNLATCYNKNKDYPKAIDHASKVLKIDKNNVKALYKLGVANMY
FGFLEEAKENLYKAASLNPNNLDIRNSYELCVNKLKEARKKDKLTFGGMFDKGPLYEEKKNSAN
```

Figure 4. The protein sequence of the *P. falciparum* FKBP protein (PfFKBP) is aligned below the human FKBP12 sequence, which is underlined for clarity. Throughout the region of homology, PfFKBP is 43% identical to human FKBP, and the residues of human FKBP that directly contact FK506 in the FKBP−FK506 complex[5] are highlighted in boldface type.
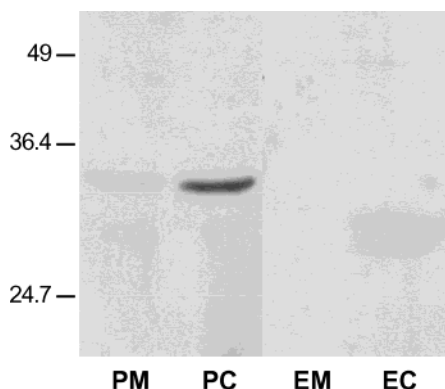

Figure 5. Parasites and infected human erythrocytes were analyzed by Western blot for the presence of PfFKBP. Parasite membrane (PM) and cytosolic (PC) fractions are shown next to erythrocyte membrane (EM) and cytosolic (EC) fractions. The α-PfFKBP antibodies do not react with human FKBP12 or FKBP52 or with other His6-tagged proteins (data not shown).

the most virulent of human malarias.[27] Folate mimics similar to MTX are currently used as antimalarials due to their ability to inhibit malarial DHFR (PfDHFR).[28] However, the conservation of DHFR sequences throughout eukaryotes often leads to a narrow therapeutic window that can limit the utility of these drugs. Though PfDHFR has been well characterized, FKBP had not been identified in malaria parasites.[29] The *P. falciparum* genome has been sequenced, so we queried the PlasmoDB database with the protein sequence of hFKBP.[30] One putative open reading frame encoding a protein of 304 amino acids was identified as a result of this search (Figure 4). The sequence of the 35-kDa *P. falciparum* FKBP protein is 43% identical to hFKBP throughout the region of overlap, and of the 11 amino acids that directly contact FK506 in the hFKBP−FK506 complex, 10 are identical in the parasite protein.[5] If this gene is expressed in parasites, one might expect the protein product to bind to hFKBP ligands such as FK506 or SLF.

The codon-optimized candidate PfFKBP35 gene (hereafter called PfFKBP) encoding the His6-tagged protein was synthesized, expressed in *E. coli*, and purified to homogeneity. Purified protein was used to immunize rabbits, and polyclonal antisera against PfFKBP were generated. Western blot analysis of cell lysates using these antibodies shows that a single major band of ∼35 kDa is detected in the cytosolic fraction of parasite-infected human erythrocytes (Figure 5). No protein is observed in either cytosolic or membrane fractions of uninfected human erythrocytes. Quantitative Western blot analysis using multiple concentrations of parasite lysates and recombinant PfFKBP were
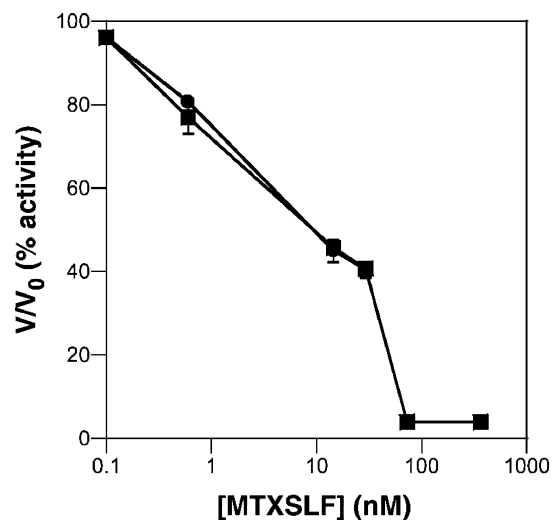
(27) Practical Chemotherapy of Malaria; WHO Technol. Rep. Ser. No. 805, 1990.
(28) Olliaro, P. L.; Yuthavong, Y. *Pharmacol. Ther.* **1999**, *81*, 91−110.
(29) Berriman, M.; Fairlamb, A. H. *Biochem. J.* **1998**, *334*, 437−445.
(30) The Plasmodium Genome Consortium. *Nucleic Acids Res.* **2001**, *29*, 66−69. http://plasmodb.org.

Figure 6. The ability of MTXSLF to inhibit purified PfDHFR is not attenuated by PfFKBP. The symbols represent 25 nM PfDHFR alone (squares) and PfDHFR plus 100 nM PfFKBP (circles). All points shown are the averages of duplicate experiments, and error bars show the measured experimental range.
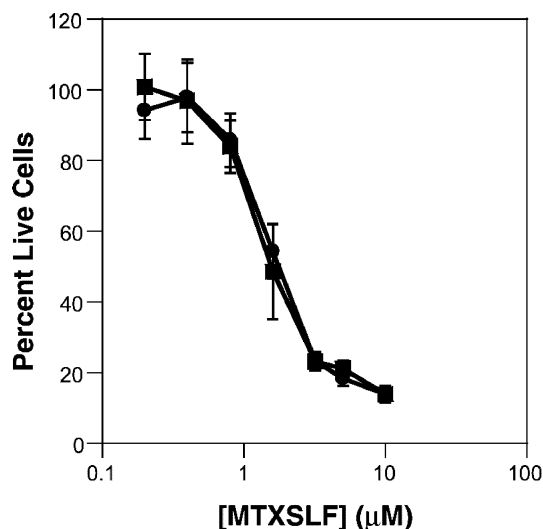

Figure 7. PfFKBP has no effect on the cytotoxicity of MTXSLF toward *P. falciparum* parasites. The anti-parasite activities were assessed by hypoxanthine incorporation into parasite DNA.[13] Symbols represent MTX-SLF (circles) and MTXSLF with 1 μM FK506-M (squares). Each point shown is the average of three independent experiments, and error bars show one standard deviation.

compared to obtain an estimated PfFKBP concentration of 50−100 nM within parasites. Using fluorescence polarization,[12] we determined that recombinant PfFKBP binds to fluorescein-labeled SLF with a dissociation constant that is 14-fold weaker than the corresponding interaction between fluorescein-labeled SLF and hFKBP (K_d values of 43 and 3 nM, respectively, data not shown).

P. falciparum DHFR (PfDHFR) was expressed in *E. coli* and purified to homogeneity.[31] MTXSLF shows significant inhibition of PfDHFR (IC$_{50}$ = 13 nM) (Figure 6). When a biologically relevant concentration of PfFKBP is added (100 nM), there is no observable change in the inhibitory potency of MTXSLF (IC$_{50}$ = 13 nM) (Figure 6). We then tested MTXSLF using a cell-based assay involving human erythrocytes infected with *P. falciparum*.[13] The parasites utilize hypoxanthine for DNA biosynthesis, so incorporation of tritiated hypoxanthine provides a quantitative measure of parasite viability. Treatment of infected erythrocytes with MTXSLF causes parasite death observed as a considerable reduction in hypoxanthine incorporation with a measured IC$_{50}$ of 1.5 μM (Figure 7). To determine if the endogenous PfFKBP is reducing the potency of the DHFR inhibitor, MTXSLF was tested in the presence of excess FK506-M (1 μM). Saturating FKBP with FK506-M does not affect the potency of MTXSLF (Figure 7). As a negative control, treatment with FK506-M alone does not cause significant antimalarial activity (data not shown).

Discussion

In certain cases, cellular responses that depend on the presence or absence of the target protein can be quite specific. However, this approach is most effective when two organisms or cell types possess significantly different genomic or biochemical profiles (e.g., β-lactam antibiotics in bacteria and humans). But consider two different cell types that possess equivalent amounts of an identical or closely related target protein (Figure 1B). A specific cellular response is often desired in one cell type without perturbing nontargeted bystander cells. Potent inhibitors of one target protein may be available, but because the target proteins are effectively identical, traditional medicinal chemistry may be unable to provide selective inhibitors. The presenter protein strategy described above demonstrates that the biological activity of bifunctional molecules can be engineered to depend on the ligand-binding properties of a second protein that is not the target protein.

The bifunctional molecule MTXSLF inhibits the in vitro activities of human DHFR and *P. falciparum* DHFR to similar extents (12 and 13 nM, respectively) for a therapeutic index of 0.92. By taking advantage of genomic differences in a nontarget protein, the therapeutic index can be increased. In the cell-free assay of DHFR activity, addition of hFKBP to hDHFR and PfFKBP to PfDHFR increases MTXSLF's therapeutic index to 4.7. Part of the increase is attributable to the difference in affinity of SLF for the FKBP proteins. SLF does bind to both proteins but its affinity for hFKBP is 14-fold tighter than for PfFKBP. Furthermore, the concentration of hFKBP in human cells is higher than the concentration of PfFKBP in malaria parasites (5 μM and 100 nM respectively). An even greater effect on the therapeutic index is seen in cellular experiments. In human MES-SA cells, hFKBP detoxifies MTXSLF almost 70-fold whereas no detoxification is observed in malaria cells.

The preference for a bifunctional molecule to bind to either the presenter protein or the target protein is determined by its affinity for each protein as well as the concentrations of all three molecules. With a knowledge of these five variables, one may predictably engineer the binding characteristics, and thus the activity of a given bifunctional molecule. Even though two cell types express similar presenter proteins, biophysical differences between the two proteins (e.g., protein concentration, affinity for ligand, state of posttranslational modification, subcellular localization) can be utilized to modulate the activity of the bifunctional molecule (Figure 1B). This general approach may be useful for designing compounds that display selective biological activity in situations ranging from two very different cell types to the other extreme embodied by two nearly identical cell types.

The experiments in this paper describe an intermediate example involving two eukaryotic organisms (human and malaria) that both possess an FKBP presenter protein. As predicted, the distinct properties of each FKBP protein differentially modulate the cytotoxic activity of MTXSLF. Ultimately, the presenter protein strategy may be useful for eliciting selective cellular responses within populations of nearly identical cells (e.g., normal and cancer cells) that differ in the expression of a small number of proteins or perhaps even a single protein.

The potential utility of this approach is not limited to cellular responses derived from loss-of-function binding events such as enzyme inhibition. Selective regulation of gain-of-function cellular responses is also possible. Any molecule that elicits a cellular response by binding to a protein or other macromolecular target (e.g., hormones, transcriptional regulators) may potentially be engineered to be more selective through the identification of an appropriate presenter protein. This presenter protein strategy should increasingly benefit from the rapidly expanding body of genomic and proteomic information. The genomes of many different organisms including several pathogens have been sequenced, and annotated databases are easily accessed. The current availability of these data, coupled with the increasing volume of similar information that will become available in the future, create opportunities to develop new strategies to predictably modulate the binding selectivity of ligands for proteins.

(31) Sirawaraporn, W.; Prapunwattana, P.; Sirawaraporn, R.; Yuthavong, Y.; Santi, D. V. *J. Biol. Chem.* **1993**, *268*, 21637−21644.