Supplementary Methods

Materials. Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. The dNTPs used in the Polymerase Chain Reaction (PCR) were purchased from Amersham Biosciences. Oligonucleotides were purchased from Invitrogen. The DMEM, DMEM/F12 and fetal calf serum media were purchased from Gibco. Fugene 6 transfection reagent was purchased from Roche. Fluorescein cadaverine and BODIPY Texas Red-cadaverine were obtained from Molecular Probes. Except as otherwise noted, all chemicals were purchased from Aldrich or Sigma. The pMONDHFR plasmid was obtained from Professor Jim Hu. The pECFP-N1-lyn plasmid was obtained from Professor Tobias Meyer. The pECFP-NUC plasmid was obtained from Professor Kai Johnsson. The pSIREN RetroQ plasmid was purchased from BDBiosciences.

Construction of plasmids. Restriction digests were carried out as recommended by New England Biolabs. All standard molecular biology techniques were carried out essentially as described.1,2

Construction of plasma membrane-localized eDHFR vector (plasmid pLM1208). The plasmid PECFP-N1-lyn was a modified form of the vector pECFP-N1 (Clontech) with a 43 bp sequence encoding a Kozak translation sequence (CGCCACCATGG) and the amino acids MGCIKSKGKDS inserted between the EcoR1 and BamH1 sites of the multiple cloning site. The gene encoding dihydrofolate reductase (DHFR) from E. coli was subcloned from plasmid pMONDHFR to pECFP-N1-lyn to generate pLM1208. A 577 bp AgeI to NotI fragment encoding DHFR with an N-terminal Kozak sequence, a valine in the second position and a (Gly-Ser-Gly)2 linker as well as a C-terminal His6 tag was prepared by PCR from pMONDHFR using the primers 5’-GCA TAC GTC ACC GGT CGC CAC CAT GGT GGG TTC TGG TGG TTC TGG TAT CAG TCT GAT TGC GGC-3’ (AgeI, coding strand) and 5’-GCA TAC GTC GCG GCC GCT TTA GTG GTG GTG GTG GTG GTG CCG CCG CTC CAG AAT C-3’ (NotI, non-coding strand). This fragment was inserted between the AgeI site and the NotI site in pECFP-N1-lyn to give to pLM1208. Upon transfection into mammalian cells, pLM1208
expressed the protein fusion MGCIKSKGD-RADPPVATMVSGSG-[DHFR]-HHHHHH.

Construction of nucleus-localized eDHFR vector (plasmid pLM1302). The plasmid pLM1302 was prepared by replacing the gene for CFP in pECFP-NUC (Clontech) with DNA encoding eDHFR. A 521 bp Nhel to XhoI fragment encoding DHFR with an N-terminal Kozak sequence, a valine in the second position was prepared by PCR from pMONDHFR using the primers 5’-GCA TAC GTC GCT AGC GCT ACC GGT CGC CAC CAT GGT GAT CTC GAT TGC GGC-3’ (Nhel, coding strand) and 5’-GCA TAC GTC GCT ATG TGA GTC CGG ACC GCC GCT CCA GAA TC-3’ (XhoI, non-coding strand). This fragment was inserted between the Nhel site and the XhoI site in pECFP-NUC to give pLM1302. Upon transfection into mammalian cells, pLM1302 expressed the protein fusion eDHFR-SGLRSRA-(DPKKKRKV)3-GSTGS.

Construction of pSIREN RetroQ vector expressing puromycin N-acetyl transferase-eDHFR (plasmid pLM1304). Two complementary oligonucleotides encoding an siRNA sequence targeting the β-actin gene were annealed: coding strand, 5’-GAT CCG TGA AGA TCA AGA TCA TTG CTT CAA GAG AGC AAT GAT CTT GAT CTT CAC TTT TTT TCT AGA G-3’; non-coding strand, 5’-GCA CTT CTA GTT CTA GTA ACG AAG TTC TCT CGT TAC TAG AAG TGA AAA AAA GAT CTC TTA A-3’. The double-stranded DNA, containing 5’ BamHI and 3’ EcoRI overhangs was ligated between the respective restriction sites of the linearized pSIREN RetroQ vector. The gene encoding eDHFR was inserted C-terminally to the gene encoding puromycin N-acetyl transferase. A 550 bp DraIII to XhoI fragment encoding DHFR with an N-terminal (Gly-Ser-Gly)2 linker as well as a C-terminal His6 tag was prepared by PCR from pMONDHFR using the primers 5’-GCA TAC GTC GCT CAC CTG GTG CAT GAC CCG CAA GCC CGG TGC CGG CTC TGG CGG CTC TGG CAT CAG TCT GAT TGC GGC-3’ (DraIII, coding strand) and 5’-GCA TAC GTC CTC GAG TTA GTG GTG GTG GTG GTG CCG CCG CTC CAG AAT C-3’ (XhoI, non-coding strand). This fragment was inserted between the DraIII site and the XhoI site in
pSIREN RetroQ to give to pLM1304. Upon transfection into mammalian cells, pLM1304 expressed the protein fusion Puro\textsuperscript{R}-GSGGSG-eDHFR-HHHHHH.

**Synthesis of TMP-BODIPY Texas Red (TMP-BTR) and TMP-fluorescein (TMP-FI).** Trimethoprim (TMP) was converted to a 4'-substituted phenol derivative by preferential cleavage of the 4'-methoxy group.\textsuperscript{3} 25 g of TMP was dissolved in 300 mL 48% HBr pre-heated to ca. 100°C. Reaction was stirred for ca. 20 min. and then quenched by slow addition of 60 mL 50% NaOH. Reaction was allowed to cool to room temperature, and placed at 4°C overnight, allowing crystals to form. Crystals were filtered and washed with ice-cold water. Crystals were dissolved in ca. 125 mL boiling water, and the solution was neutralized with NH\textsubscript{4}OH, leading to recrystalization. Crystals were filtered and dried under vacuum, yielding the desired phenol at ca. 60% yield.

Preparation of 4'-methoxy substituted carboxylic acid of TMP was prepared essentially as described in Kuyper, et al.\textsuperscript{4} To a solution of the 4'-phenol derivative of TMP (300 mM) in DMSO was added 1.1 equiv. of t-BuOK with stirring under Ar atmosphere. The solution was stirred at room temperature for 0.25-0.5 hr. To the solution was added 1.1 equiv. of ethyl 5-bromo valerate, and the solution was stirred at room temperature. The reaction was monitored by TLC on silica gel with CH\textsubscript{2}Cl\textsubscript{2}:CH\textsubscript{3}OH (4:1). When complete (within 2 h), the solvent was removed under vacuum, and the residual brown oil was subjected to column chromatography on silica gel, elution with 10% CH\textsubscript{3}OH in CH\textsubscript{2}Cl\textsubscript{2}. Fractions containing the desired product were pooled and concentrated to dryness with an overall yield of ca. 30% of the 4'-substituted ester. To a solution of the ester (200 mM) in CH\textsubscript{3}OH was added 3.0 equiv. of 1.0 N NaOH. The solution was stirred at room temperature for ca. 1 h, and neutralized with 3.0 equiv. of 1.0 N HCl. This led to precipitation of the hydrolyzed 4'-alkylated TMP derivative, which was then filtered, washed with ice-cold water and dried under vacuum (70% yield).

The 4'-substituted carboxylic acid derivative of TMP was coupled to either BODIPY Texas Red cadaverine or fluorescein cadaverine in a single step. TMP (0.01-
0.02 mmol) and PyBop (0.03-0.04 mmol) were dissolved in 0.5 mL dimethyl formamide under Ar atmosphere. 0.5 mL of a 0.02 M solution of the desired fluorophore in dimethyl formamide was added to the reactants, and the solution was stirred at room temperature for 0.25 h. Ca. 20 L (ca. 8 equiv wrt the fluorophore) of diisopropyl ethyl amine was added, and the reaction mixture was stirred under Ar atmosphere at room temperature overnight (ca. 16 h). The TMP-BODIPY Texas Red product was subjected to column chromatography on silica gel, elution with 10% CH$_3$OH in CH$_2$Cl$_2$. Fractions containing the desired product were pooled and concentrated to dryness (yield 30%). The product was confirmed by $^1$H NMR (CH$_3$OH-d$_4$): δ 8.05 (d, 1H, ArH), 7.98 (d, 2H, ArH), 7.60 (d, 1H, ArH), 7.46 (s, 1H, ArH), 7.37 (s, 1H, ArCH=), 7.21 (d, 1H, ArH), 7.19 (d, 1H, ArH), 7.13, (m, 1H, ArH), 7.05 (d, 2H, ArH), 6.87 (d, 1H, ArH), 6.78 (d, 1H, ArH), 6.50 (s, 2H, ArH), 4.60 (s, 2H, CH$_2$), 3.87 (m, 2H, CH$_2$), 3.78 (m, 2H, CH$_2$), 3.74 (s, 6H, CH$_3$), 3.65 (m, 2H, CH$_2$), 3.60 (s, 2H, CH$_2$), 2.2 (m, 2H, CH$_2$), 1.75 (m, 4H, CH$_2$), 1.65 (m, 2H, CH$_2$), 1.55 (m, 4H, CH$_2$). Low-resolution MS (C$_{49}$H$_{56}$BF$_2$N$_2$O$_6$S): calculated m/z 866.4, found m/z 867.4. The TMP-Fluorescein product was subjected to column chromatography on silica gel, elution with 20% CH$_3$OH in CH$_2$Cl$_2$. Fractions containing the desired product were pooled and concentrated to dryness. The resulting product mixture was purified by HPLC (50% acetonitrile in H$_2$O with 25 mM NH$_4$Oac) on a Waters Delta 600 with a GraceVydac Protein and Peptide C18 (cat. No. 218TP54). Fractions containing only the desired compound were pooled, rotovapped to remove acetonitrile, and lyophilized to yield the desired TMP-FI (1.3 mg, 13%). Low-resolution MS (C$_{44}$H$_{47}$N$_2$O$_9$S): calculated m/z 850.0, found m/z 850.7.

**Fluorescence Polarization Binding Assay.**

**Protein Purification.** The eDHFR was purified from the E. coli strain Tuner BL21-(DE3) carrying the eDHFR-His$_6$ expression vector pAED4-eDHFR. Briefly, 1 mL of an LB overnight culture was used to inoculate 50 mL of LB. Both cultures contained 1000 g/mL ampicillin to select for the expression vector. The 50 mL culture was grown at 37 °C, shaking at 300 rpm, to an OD600 of 0.6, at which time expression of the protein was induced by the addition of IPTG to a final concentration of 0.1 mM. After growth for an additional 4 h, the cells were harvested by centrifugation. The cell pellet underwent one freeze-thaw cycle (from -80 °C to room temperature) before being lysed by 1 mL of Bug Buster protein extraction reagent on ice for 30 min. The protein was then purified under
standard nondenaturing conditions using the Ni-NTA spin kit according to the manufacturer’s protocol (Qiagen).

**Binding Affinity Assay.** The affinity of TMP-fluorescein and for eDHFR was determined by fluorescence polarization. TMP-Fluorescein at a final concentration 10 nM, was incubated with purified eDHFR with a 100-fold excess of NADPH at varying concentrations ranging from 0.1 nM to 500 nM in MTEN buffer (50 mM morphlinoethane sulfonic acid (MES), 25 mM Tris-HCl, 25 mM ethanolamine, 100 mM NaCl, pH 7.2). By comparing the intensity of emitted fluorescence both parallel and perpendicular to incident light (measured on a Perkin Elmer Victor2 FP analyzer), it is possible to compute the amount of bound tracer. Using Kaleidagraph (Synergy Software, PA), the data were fit to the following equation in order to obtain the dissociation constant:

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where \(F_0\) is the polarization of the tracer with no receptor, \(F_{100}\) is the maximum polarization with an infinite amount of receptor, \([F]T\) is the total amount of tracer used, and \([P]T\) is the total amount of receptor used. A measured \(K_D\) of 32 ± 3 nM was obtained (Supplementary Figure 1).

**Cell growth, transfection and probing with small molecule.** In preparation for transfection, Chinese Hamster Ovary (CHO) cells or mouse embryonic fibroblast (MEF) cells were seeded at 10^5 cells per well into a 6-well plate. Cells were grown in DMEM/F12 medium containing 5% Fetal Calf Serum and supplemented with 30 M thymidine, HEPES, L-glutamine and penicillin/streptomycin (CHO cells) or DMEM medium containing 10% Fetal Calf Serum, HEPES, L-glutamine and penicillin/streptomycin (MEF cells) at 37 °C and 5% CO₂. After ca. 18 hrs., adherent cells (ca. 80% confluent) were transfected with 2 g of the desired plasmid DNA using Fugene 6 transfection reagent according to manufacturers instructions. Ca. 24 hrs. after transfection, cells were trypsinized and reseeded onto either 22 mm² coverslips in 6-well plates (50,000 cells/well) or into chambered microscopy slides. Indicator-free DMEM
containing the desired concentration was added to the adherent cells, and the cells were either imaged without washing, or first washed 3X with phosphate buffered saline, re-immersed in indicator-free medium, and then imaged.

**Microscopy.** Confocal fluorescent microscopy of adherent live cells was performed using an Olympus 1X81 scanning laser confocal microscope equipped with a 60X Plan Fluor oil immersion objective. Laser excitation at 458 nm, 488 nM or 568 nm was used to excite CFP, GFP or TMP-Fl, and TMP-BTR fluorescence respectively. DIC and fluorescent images were captured using Fluoview FV500 software and rendered using NIH Image J and Adobe Photoshop 5.5.

**Dissociation Kinetics.** The signal/noise ratio of a single MEF cell expressing puromycin n-acetyl transferase-eDHFR and labeled with TMP-fluorescein was monitored over a period of 2.5 hrs. to determine the rate of dissociation of TMP-fluorescein from eDHFR in vivo (Supplementary Figure 3). The measured signal/noise ratio vs. time was found to decline at a rate of ca. 13% per hour.

**References.**