Chemically induced dimerization of dihydrofolate reductase by a homobifunctional dimer of methotrexate

Stephan J Kopytek[1], Robert F Standaert[2]*, John CD Dyer[1] and James C Hu[1,3]*

Background: Chemically induced dimerization (CID) can be used to manipulate cellular regulatory pathways from signal transduction to transcription, and to create model systems for study of the specific interactions between proteins and small-molecule chemical ligands. However, few CID systems are currently available. The properties of, and interactions between, *Escherichia coli* dihydrofolate reductase (DHFR) and the ligand methotrexate (MTX) meet many of the desired criteria for the development of a new CID system.

Results: *Bis*MTX, a homobifunctional version of MTX, was synthesized and tested for its ability to induce dimerization of DHFR. Gel-filtration analysis of purified DHFR confirmed that, *in vitro,* the protein was a monomer in the absence of dimerizer drug; in the presence of *bis*MTX, a complex of twice the monomeric molecular weight was observed. Furthermore, the off-rate was found to be 0.0002 s^{-1}, ~100 times slower than that reported for DHFR–MTX . Interestingly, the addition of excess *bis*MTX did not result in formation of the binary complex (1 protein:1 dimerizer) over the ternary complex (2 proteins:1 dimerizer), which suggests cooperative binding interactions (affinity modulation) between the two DHFR molecules in the *bis*MTX:$DHFR_2$ ternary complex.

Conclusions: The combination of DHFR and *bis*MTX provides a new CID system with properties that could be useful for applications *in vivo*. Formation of the *bis*MTX:$DHFR_2$ ternary complex *in vitro* is promoted over a wide range of dimerizer concentrations, consistent with the idea that formation of the ternary complex recruits energetically favorable interactions between the DHFR monomers in the complex.

Addresses: [1]Department of Biochemistry and Biophysics, [2]Department of Chemistry, and [3]Center for Macromolecular Design, Texas A&M University, College Station, TX 77843-2128, USA.

Correspondence: James C Hu
E-mail: jimhu@tamu.edu

*These authors contributed equally to this work.

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Introduction

Dimerization (or oligomerization) of proteins or protein domains can be mediated by low molecular weight organic compounds termed chemical inducers of dimerization or 'dimerizers' [1–6]. Several dimerizer systems have been developed based on both natural products and completely synthetic molecules [2,7–11] (Table 1). One of the original chemically induced dimerization (CID) systems is based on the immunosuppressive drug FK506 and the immunophilin FK506-binding-protein 12 (FKBP). Spencer *et al.* [2] synthesized FK1012, a homobifunctional version of FK506, and used this semisynthetic dimerizer to regulate signal transduction. By fusion of ζ, the intracellular signaling domain of the T-cell receptor, to a protein with drug-binding activity (in this case FKBP), signal transduction was rendered absolutely dependent on the bivalent drug [2]. This demonstrated the use of CID as a method to regulate signal transduction.

Regulation of eukaryotic transcription has also been shown by use of several different chemical dimerizers and fusion protein pairs. Because eukaryotic transcription factors are modular in nature, the DNA-binding and activation domains can be expressed as separate proteins, and, as long as they are brought in close proximity to one another, will reconstitute sequence-specific transcriptional activation [12]. Dimerizer-dependent transcriptional regulation has been demonstrated using various different dimerizers and transcription-factor domains fused to FKBP. Using the

Table 1

Chemically induced dimerization systems.

Target protein(s)*		Dimerizer ligand	References
FKBP	FKBP	FK1012	[2,7]
FKBP	CNA	FK506	[7]
FKBP	CyP	FK506–CsA	[8]
FKBP	FRB	Rapamycin	[7,9]
GyrB	GyrB	Coumermycin	[10]
DHFR	DHFR	*Bis*MTX	This study

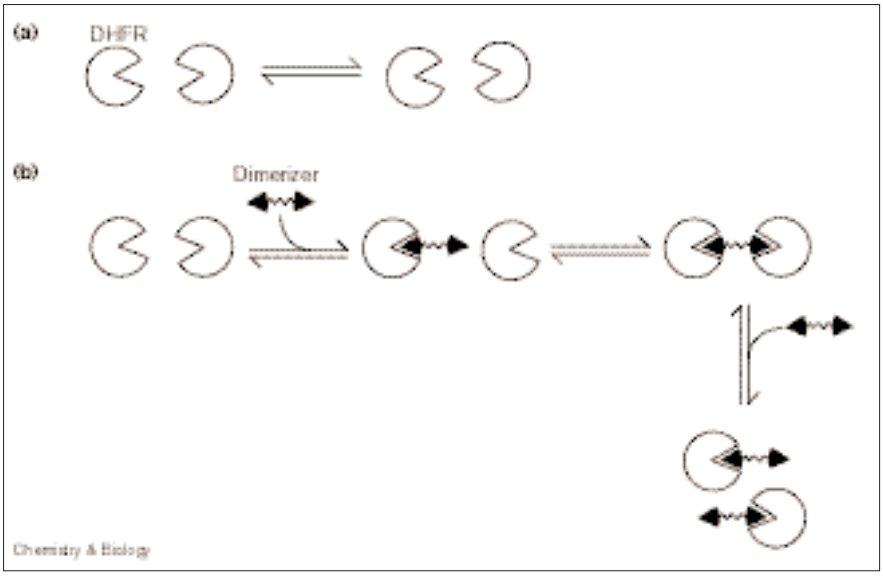
*Only the fragment of the fusion protein(s) that binds the dimerizer ligand is listed. FKBP, FK506-binding-protein 12; CNA, calcineurin A; CsA, cyclosporin A; CyP, cyclophilin; FRB, FKBP-rapamycin binding domain of FKBP-rapamycin-associated protein; GyrB, B subunit of bacterial DNA gyrase.

semisynthetic homobifunctional dimerizer FK1012, Ho *et al.* [7] showed dimerizer-dependent activation of transcription in cells expressing the yeast GAL4 DNA-binding domain and the herpes simplex virus (HSV) VP16 activation domain, each fused to multiple copies of FKBP [12]. Belshaw *et al.* [8] showed that the semisynthetic heterobifunctional dimerizer FK506-cyclosporin A could be used to regulate transcription using the fusion protein FKBP–GAL4 DNA-binding domain and cyclophilin–VP16 activation domain. The naturally occurring rapamycin is also an effective dimerizer. Rivera *et al.* [9] demonstrated rapamycin-dependent regulation of transcription by use of transcription factors fused to FKBP and FRB (FKBP-rapamycin binding domain of FKBP-rapamycin-associated protein). The DNA-binding domain was based on ZFHD1 [13], the activation domain on the carboxy-terminal region of the NF-κB p65 protein [14]. Activation of the Raf-1 serine/threonine kinase cascade by coumermycin-induced dimerization was demonstrated by Farrar *et al.* [10]. The natural homobifunctional dimerizer can bring about the dimerization of proteins fused to the amino-terminal subdomain of the B subunit of bacterial DNA gyrase, the binding target of coumermycin. In all cases, the use of dimerizers allows these activities to be promoted at will and in a dose-dependent and reversible way, suggesting various experimental and therapeutic applications [1]. For the researcher, use of dimerizers provides a powerful means with which to study the function of a gene by allowing the consequences of its regulated expression to be analyzed both *in vitro* and *in vivo* [11]. For the clinician, the ability to exert pharmacologic control over the expression of a therapeutic gene, in the context of gene therapy, would allow the timing of delivery and the dosage of gene product to be optimized [11].

Because there are currently few protein dimerizer systems (Table 1), additional protein–ligand pairs would be useful. An ideal CID pair would include a well-characterized protein, a ligand that is chemically malleable but metabolically stable, and a specific, well-characterized interaction between the two. Dihydrofolate reductase (DHFR) and methotrexate (MTX) have these characteristics. DHFR catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, which is then used directly or converted to other reduced folates. DHFRs have been characterized from various sources ranging from bacteria to humans. Most forms, including the *Escherichia coli* and mammalian enzymes, function as monomers [15,16]. Many crystallographic and nuclear magnetic resonance (NMR) structures are available, with DHFR from numerous sources bound to a wide variety of ligands. Because DHFR is involved in thymidine biosynthesis, it has been used extensively as a drug target for the treatment of various forms of cancer, rheumatic diseases and bacterial infections [17]. MTX is a folate analog that binds DHFR very tightly and the K_d for the interaction with *E. coli* DHFR is ~0.6 nM [18]. Since its synthesis in the late 1940s, MTX has been thoroughly characterized and many analogs synthesized [19–22]. The three-dimensional structure of the binary *E. coli* DHFR–MTX complex has been determined to 1.7 Å resolution [23,24].

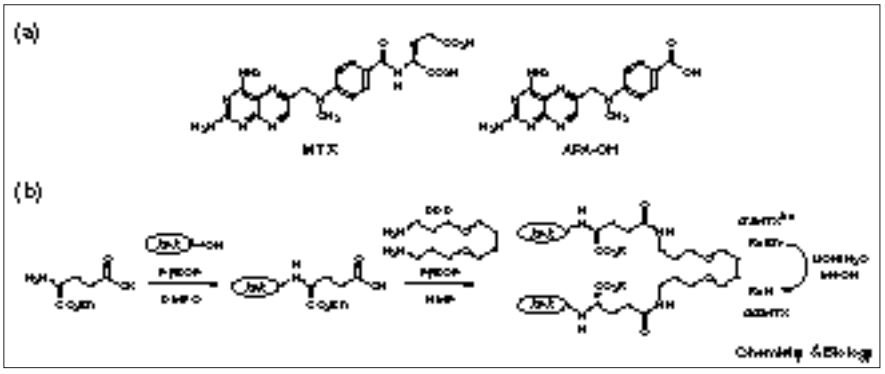
Here, we describe the synthesis of a homobifunctional version of MTX (*bis*MTX) and show that it can induce dimerization of *E. coli* DHFR (Figure 1). Further, the ternary complex formed between *bis*MTX and DHFR is shown to be more stable than the binary complex between MTX and DHFR. This suggests a dimerizer-induced affinity modulation [25], in which an increase in

Figure 1



Model of *bis*MTX-inducible DHFR dimerization. **(a)** In the absence of dimerizer, the proteins (*E. coli* DHFR) remain monomeric. **(b)** In the presence of dimerizer (*bis*MTX), the molecules of DHFR can bind the ligand to form binary and ternary complexes.

Figure 2



Synthesis and molecular structure of *bis*MTX. **(a)** Structure of MTX and APA-OH. **(b)** Synthesis of *bis*MTX. APA-OH, 4-amino-4-deoxy-N^{10}-methylpteroic acid; Bn, benzyl; *bis*MTX, γ,γ′-DDD-linked methotrexate dimer; *bis*MTX^{Bn}, γ,γ′-DDD-linked methotrexate dimer di-α-benzyl ester; DDD, 4,9-dioxa-1,12-dodecanediamine; DMSO, dimethyl sulfoxide; MeOH, methanol; MTX, methotrexate; NMP, *N*-methylpyrrolidinone; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate.

the affinity of the binding event is observed owing to the generation of additional protein–protein interactions outside of the ligand-binding sites.

Results and discussion

Dimerizer synthesis and bioavailability

The central issue in design of a dimerizer is the availability of a site on the ligand that can be derivatized without decreasing affinity for the target. In the case of MTX, the glutamate-γ-carboxyl (Figure 2a) meets this criterion readily, because wide structural variety is tolerated in amide derivatives of this group [19,26–29]. A second important element is the linker; although many factors, some quite subtle, can be important [30], here a simple analysis provided a satisfactory result. Commercial methotrexate agarose (Sigma) uses an eight-atom spacer; we reasoned that a chain approximately twice as long would assure adequate separation, and that oxygenation would decrease hydrophobicity and increase water solubility. DDD (4,9-dioxa-1,12-dodecanediamine; 14 atoms) is a commercially available diamine meeting both requirements.

Synthesis of the dimerizer required three steps from commercial materials (Figure 2b). MTX, protected as its α-benzyl ester (MTX^{Bn}), was prepared by coupling of 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA–OH) to L-glutamic acid α-benzyl ester by use of a slight variation of the procedure of Nagy *et al.* [31]. The α-protected MTX^{Bn} was then doubly coupled to DDD with benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and deprotected by alkaline hydrolysis to afford *bis*MTX in ~50% overall yield. All intermediates and the final product were purified by reverse-phase (C_{18}) high-performance liquid chromatography (HPLC) and characterized by NMR (^{1}H and ^{13}C) and fast atom bombardment mass spectrometry (FAB–MS).

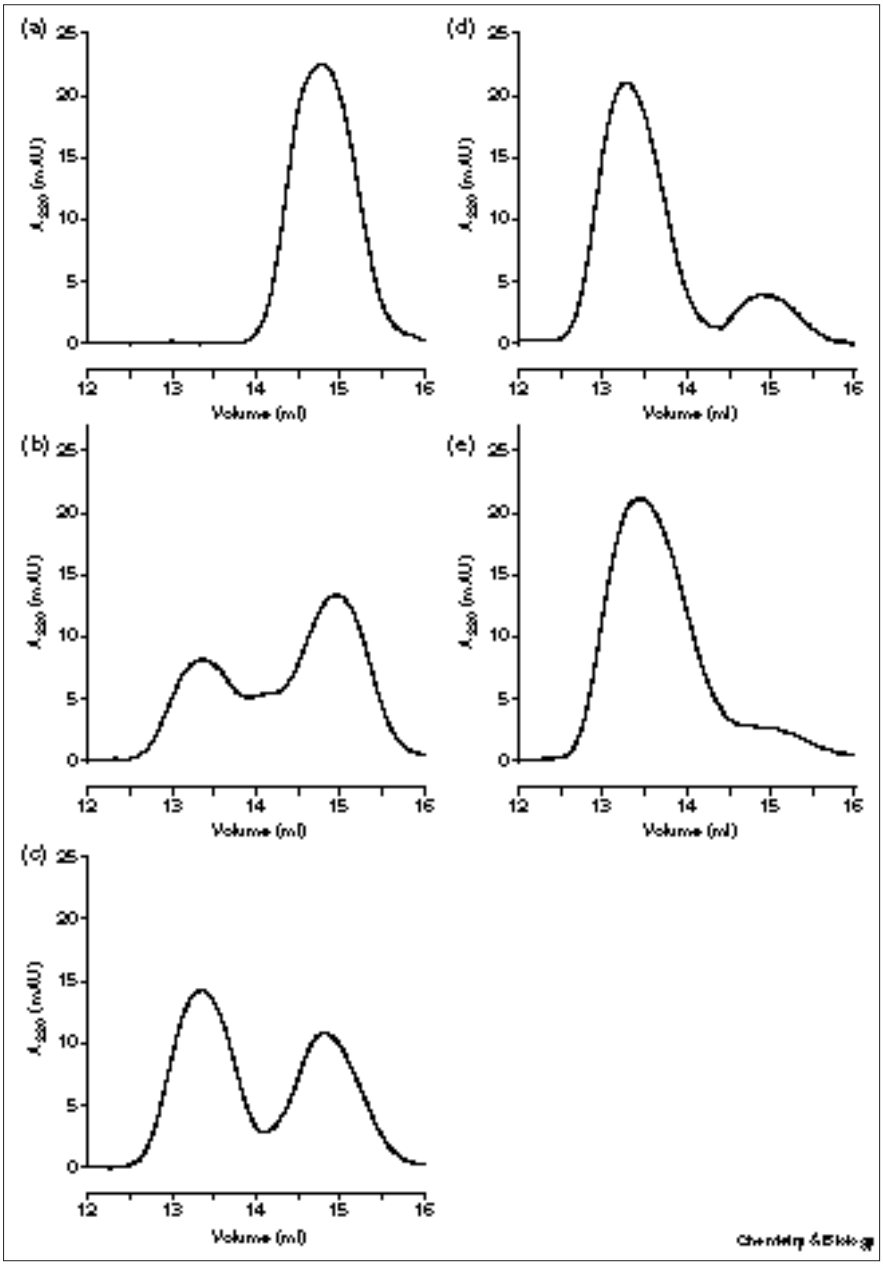
*Bis*MTX inhibited the growth of *E. coli tolC*, a strain that is sensitive to growth inhibition by methotrexate (S.J.K. *et al.*, unpublished observations), indicating that the molecule can cross biological membranes and bind *E. coli* DHFR *in vivo*.

Dimerized complex detected by gel filtration

To determine whether dimerization of purified DHFR molecules could be induced by the presence of *bis*MTX *in vitro*, we prepared a series of samples containing either protein only (Figure 3a) or protein plus increasing concentrations of dimerizer drug (Figure 3b–e). The mixtures were allowed to reach equilibrium by incubation at 25°C for at least 12 hours, and they were then fractionated on a Superdex 75 gel filtration column. In the absence of drug, a single peak was observed corresponding to a molecular weight of 17 kDa (Figure 3a), which is consistent with this peak being monomeric DHFR (17.68 kDa [16]). In the presence of dimerizer, a second peak corresponding to a molecular weight of 34–35 kDa was observed (Figure 3b–e). The higher molecular weight peak corresponds to the expected size of a ternary complex consisting of two molecules of DHFR tethered together by one molecule of *bis*MTX. Furthermore, the generation of the ternary peak coincided with a decrease in the intensity of the monomer peak, which supports the interpretation that this new peak was the product of a successful chemically induced dimerization event. As the concentration of drug was increased, the amount of ternary complex increased and the amount of monomer decreased (Figure 3a–e).

In a noncooperative binding model in which each end of the dimerizer binds independently to the protein, addition of an increasing amount of dimerizer should increase the fraction of protein in the ternary complex up to the stoichiometric dimerizer to protein ratio of 1 to 2. Excess drug would drive equilibrium back toward the binary complex. The fraction of protein expected to be in the ternary complex can be calculated on the basis of a noncooperative binding model from the published value for the binding of MTX to *E. coli* DHFR (590 pM) [18]) as an approximation for the binding of the *bis*MTX to DHFR (solid lines in Figure 4). As the *bis*MTX:DHFR ratio is

Figure 3



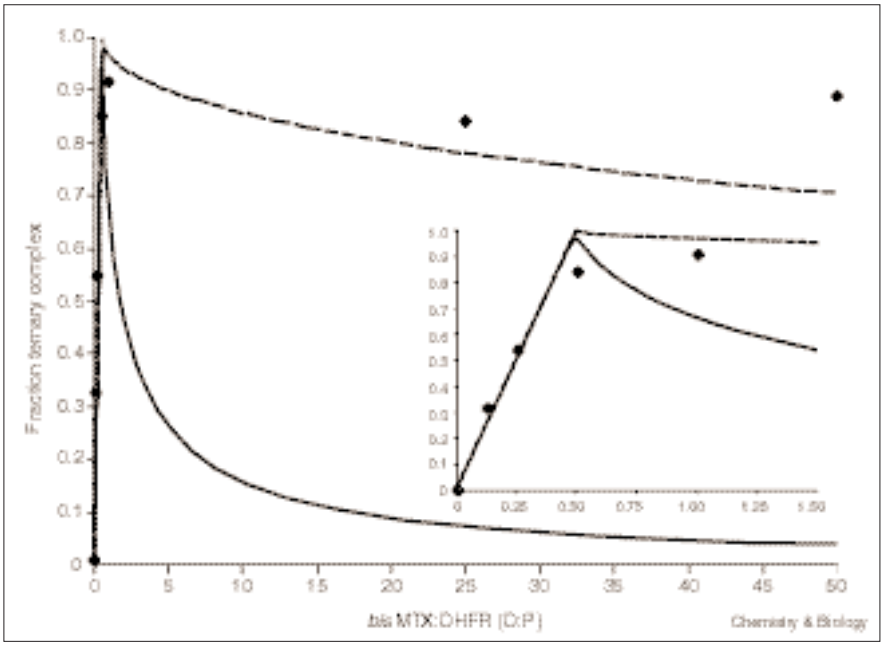
Gel-filtration analysis showing *bis*MTX-dependent dimerization of DHFR. Samples consisting of purified DHFR (1 μM final concentration) with increasing concentrations of *bis*MTX in final concentrations of **(a)** 0 μM, **(b)** 0.125 μM, **(c)** 0.25 μM, **(d)** 0.50 μM and **(e)** 50 μM were injected onto a Superdex 75 gel filtration column and chromatographed with the ÄKTA™ *purifier* system. Protein elution profiles (monitored at 280 nm) are shown. At 0 μM *bis*MTX (a), the fraction of total protein in the ternary complex is 0%. The fraction of total protein in the ternary complex was calculated to be (b) 32.5%, (c) 54.7%, (d) 85%, and (e) 88.8%. The observed molecular sizes were determined by comparison with a set of molecular standards: aprotinin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), and blue dextran (2 MDa).

increased, the fraction of protein in the ternary complex would increase to a maximum at a *bis*MTX:DHFR ratio of 0.5. As the *bis*MTX:DHFR ratio increased further, the fraction of protein in the ternary complex would decrease. The shape of this curve is fairly insensitive to the value used for the binding constant (not shown) as long as the dissociation constant is below the total concentration of protein used in the modeling (1 μM).

At *bis*MTX:DHFR ratios below 0.5, the experimental data fit the noncooperative binding model well. However, at concentrations of dimerizer equal to or greater than the concentration of the protein, virtually all of the protein remained in the ternary form. This finding is not consistent with a simple noncooperative binding model in which, as the concentration of dimerizer exceeds half the concentration of protein, the proportion of ternary complexes at equilibrium would be expected to decrease (and the proportion of binary complexes to increase). At a *bis*MTX:DHFR ratio of 50, only 4% of the protein would be predicted to be in ternary complexes, with the rest of the DHFR molecules bound to dimerizer in a 1:1 ratio. In fact, we found that virtually all of the protein was in the ternary complex at this ratio of drug to protein (Figures 3e and 4).

Figure 4

The fraction of protein in the ternary complex is shown as a function of the ratio of dimerizer (*bis*MTX) to protein (DHFR). Solid circles indicate data derived from integration of the ternary complex peaks shown in Figure 3. The solid line shows the calculated curve for a noncooperative binding model where $K_a = 1.69 \times 10^9$ and $K_{coop} = 1$. The dashed line shows the calculated curve for a cooperative binding model where $K_a = 1.69 \times 10^9$ and $K_{coop} = 200$. The inset focuses on the fraction ternary complex at low drug to protein ratios. See the Materials and methods section for binding model calculations.



Our data fit better with a model in which formation of the ternary *bis*MTX:$DHFR_2$ complex is cooperative, (i.e. DHFR has a higher affinity for the binary complex than for the free drug; dashed lines in Figure 4). Note that the predictions of the cooperative and noncooperative models are indistinguishable at low *bis*MTX:DHFR ratios (Figure 4, inset). As the cooperativity constant increases, the ternary complexes disappear more slowly with increasing concentrations of the drug (data not shown).

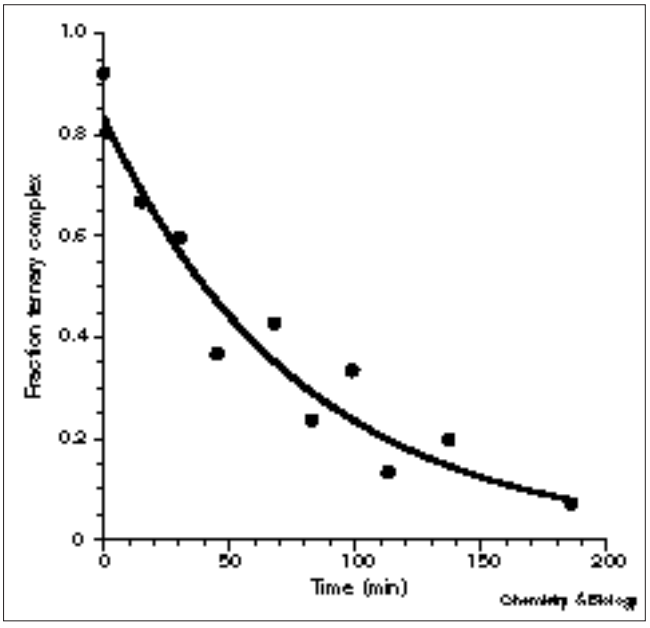
Cooperative binding could occur if formation of the ternary complex involves additional interactions outside the ligand binding sites on the DHFR molecules. This situation would be analogous to the 'affinity modulation' observed by Briesewitz *et al*. [25]; binding of the heterobifunctional ligand FKpYEEI (consisting of an SH2-binding peptide linked to FK506) to the Fyn SH2 domain was increased in the presence of FKBP52 but not FKBP12. The specificity for FKBP52 suggested that the increase in affinity for the Fyn SH2 domain was due to favorable protein–protein interactions involving the surface of FKBP52.

Additional protein–protein contacts in the ternary complex should stabilize it by decreasing the dissociation rate. To address this possibility, the off-rate of the ligand was investigated by a competition assay using MTX. By gel filtration, *bis*MTX:$DHFR_2$ ternary complexes were purified and mixed with 800-fold molar excess of MTX over protein. At timed intervals from mixing, aliquots of the mixture were fractionated by gel filtration and the amount of ternary complex remaining measured. A plot of fraction ternary complex versus time fitted a first-order exponential decay process and yielded a calculated k_{off} of 0.0002 s^{-1} (Figure 5). This off-rate is ~100 times slower than the reported value of 0.0190 s^{-1} for DHFR–MTX [18]. In addition, the half-life of the complexes is longer than the elution time of the ternary complex from the gel filtration column; thus the column should provide a good estimate of the species present in the mixture.

The *E. coli* DHFR–MTX binary complex crystallizes with two molecules per unit cell and it is interesting to note that the MTX-binding sites of the two DHFR molecules face each other [23,24]. Although this was not part of our design of *bis*MTX, binding of the dimerizer could potentially achieve affinity modulation by recruiting the interactions that form crystal contacts between monomers. The additional stability could also result from favorable contacts between *bis*MTX and DHFR that are not present in the complex between MTX and DHFR. For example, contacts with the flexible DDD linker might be entropically unfavorable in the binary complex. However, once the conformation of the linker has been constrained in the binary complex, structurally similar contacts in the ternary complex might be favorable.

These two possibilities are obviously not mutually exclusive; the extra stability, which corresponds to only a few kilocalories of binding energy, could be due to a combination of protein–protein and protein–drug interactions. However the phenomenon arises, the stability of

Figure 5



Dissociation of the ternary complex. Ternary complexes were formed by incubation of DHFR (25 μM) and *bis*MTX (12.5 μM) at 25.5°C for 12 h and purified by gel filtration. The purified complexes were combined with an 800-fold molar excess of MTX (10 mM; time 0). At timed intervals, aliquots (1000 μl) of the ternary complex/MTX sample were fractionated by gel filtration as in the experiments shown in Figure 3. The amount of ternary complex present was calculated by integration of the area under the peak corresponding to the ternary complex. The ternary complex as a fraction of total DHFR was plotted versus time and the data fitted to the first-order exponential equation $T = T_0 e^{-kt}$, where T is the fraction of DHFR in the ternary complex, T_0 is the fraction of DHFR in the ternary complex at time zero, and *k* is a first-order rate constant. The half-life and apparent off-rate were calculated to be 55 min and 0.0002 s^{-1}, respectively.

the ternary complex in the presence of excess ligand should be useful for the application of this CID system to the creation of new molecular switches *in vivo*, where maintaining a precise ratio of drug to protein is confounded by bioavailability.

Our results led us to examine the literature on other CID systems to see whether this serendipitous affinity modulation is widespread. *In vivo*, reversibility of ternary complex formation has been observed at high concentrations of dimerizer [10]. However, in many cases the measured response does not decrease at higher concentrations of the dimerizer [2,7,9]. Although this finding could be interpreted as showing cooperativity, several factors confound the interpretation of *in vivo* data including nonlinearity of the assays, buffering of the free dimerizer concentration by endogenous proteins, and uncertainty about the intracellular concentrations of both the drug and the target protein.

Significance

We synthesized a novel bifunctional compound, *bis*MTX (γ,γ′-4,9-dioxa-1,12-dodecanediamine-linked methotrexate dimer) to drive the chemically induced dimerization (CID) of dihydrofolate reductase (DHFR). The ability of this dimerizer compound to drive the assembly of monomeric DHFR into the *bis*MTX:$DHFR_2$ ternary complex was shown *in vitro* by use of the *Escherichia coli* DHFR. The homobifunctional ligand binds more tightly to its protein target than would be expected from a non-cooperative binding model, possibly by forming novel protein–protein interactions between the DHFR molecules in the ternary complex. The combination of *bis*MTX and DHFR can be used as an additional CID system in the design of artificial gene regulatory circuitry. The insensitivity of ternary complexes to drug concentration should be useful for applications of this CID system *in vivo*, where a plateau in the dose-response curve may be beneficial.

Materials and methods

Materials

APA–OH hemihydrochloride dihydrate was purchased from Sigma; PyBop, 1-hydroxybenzotriazole (HOBT), *N*-methylpyrrolidinone (NMP), *N,N*-diisoproylethylamine (DIEA), and L-glutamic acid α-benzyl ester (H-Glu-OBn) were purchased from Advanced ChemTech; DDD was purchased from Aldrich.

General methods

Reverse-phase HPLC was performed with binary gradients formed (except where indicated) from solvent A (water + 0.1% v/v trifluoroacetic acid; TFA) and solvent B (acetonitrile + 0.08% v/v TFA) and use of absorbance detection at the indicated wavelengths. Analytical HPLC was performed with a Waters Symmetry C_{18} column (4.6 × 100 mm) at a flow rate of 1 ml/min, and preparative HPLC was performed with a Waters Delta-Pak C_{18} radial compression column (100 Å pore size, 25 × 100 mm) at a flow of 10 ml/min. Except for the final step, reaction yields are uncorrected for residual solvent, moisture and TFA. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75.4 MHz) spectra were recorded in CD_3OD (deuterated methanol) and referenced to solvent peaks (HCD_2OD = 3.30 ppm or CD_3OD = 49.0 ppm).

MTX α-benzyl ester (MTX^{Bn})

A slight variation of the procedure of Nagy *et al.* [31] for the coupling of APA–OH was used. APA–OH hemihydrochloride dihydrate (102 mg, 29 μmol) and DIEA (76 μl, 43 μmol) were dissolved in 870 μl dimethyl sulfoxide (DMSO). PyBOP [32] (166 mg, 32 μmol) was added, and the reaction was stirred at room temperature for 30 min. To this solution was added 870 μl of the mixture obtained by combining H-Glu-OBn (151 mg, 64 μmol) with finely pulverized K_2CO_3 (44 mg, 32 μmol) in 1.74 ml DMSO and sonicating for several minutes at 50°C. The reaction mixture was agitated at 50°C until a clear, yellow solution was obtained (about 5 min) and then stirred at room temperature for 3 h, at which time it was diluted with 1 M aqueous TFA (1 ml), acetonitrile (2 ml), and water (to a volume of 10 ml). The product was purified by preparative HPLC (detection by A_{400}) with a two-segment linear gradient (min/%B): 0/0, 5/25, 30/35. Lyophilization of the product peak afforded 140 mg (89%) of MTX^{Bn}. FAB–MS (thioglycerol/NaI), *m/z* calculated for $C_{27}H_{28}N_8O_5$, 544.570; found, 545 ($[M+H]^+$). ^{1}H-NMR: δ 2.00–2.15 (m, 1H), 2.18–2.31 (m, 1H), 2.42 (t, 2H, J = 7.2 Hz), 3.24 (s, 3H), 4.53 (dd, 1H, J = 5.4, 9.3 Hz), 4.92 (s, 2H), 5.15 (s, 2H), 6.81 (d, 2H, J = 9.3 Hz), 7.22–7.36 (m, 5H), 7.72 (d, 2H, J = 9.0 Hz), 8.60 (s, 1H). ^{13}C-NMR: δ 27.38, 31.36, 39.72, 54.03, 56.57, 67.92, 112.68, 122.63, 123.30,

129.10, 129.22, 129.50, 130.28, 37.22, 147.07, 150.35, 152.94, 153.56, 157.93, 164.86, 170.29, 173.46, 176.52.

MTX dimer di-α-benzyl ester (bisMTX^{Bn})

Control of stoichiometry in the coupling is important, but the presence of residual moisture and acid in the MTX^{Bn} led to uncertainty in its quantification by mass. Therefore, the optimum stoichiometry was determined empirically. Thus, MTX^{Bn} (67 mg, 123 μmol nominal) was dissolved in 600 μl NMP, to which was added 147 μl (74 μmol, 0.6 equivalents) HOBT (0.5 M in NMP) followed by PyBOP (71 mg, 136 μmol, 1.1 equivalents). After 20 min, the diamine (11 μl, 51 μmol, 0.42 equivalents) was added with mixing, and the reaction was allowed to stand for 2 h. Analytical HPLC (A_{330}, 0–50% B over 10 min) of the reaction showed a 3:5 area ratio of monoacylated product to the later-eluting diacylated product, indicating the need for 25% more MTX^{Bn}. Accordingly, an additional 17 mg (31 μmol) of MTX^{Bn} was dissolved in 75 μl NMP, to which was added 37 μl (18 μmol) HOBT (0.5 M in NMP), 11 μl (63 μmol) DIEA, and 20 mg PyBOP. After 20 min, the bolus was added to the main reaction, and the mixture was allowed to stand for 3 h, at which time reaction was complete as judged by HPLC. The reaction was quenched by the addition of DDD (2 μl) followed after 30 min by 1 M TFA (100 μl), and then diluted with water (10 ml) resulting in precipitation of the product. After addition of just enough 20% acetonitrile in 0.1% aqueous TFA to redissolve the product (~150 ml), the product was purified by preparative HPLC (detection by A_{400}) with a two-step gradient (min/%B): 0/0, 2/30, 42/50. Lyophilization afforded 89 mg (115% yield) of the product. The excess mass included some tripyrrolidino phosphine oxide, a byproduct of the PyBOP coupling. Because this compound is inert, it was not removed before the next reaction; for the analytical sample, it was removed by chromatographing the product on silica gel with 5:1 chloroform/methanol as the eluent. FAB-MS (thioglycerol/NaI): *m/z* calculated for $C_{64}H_{76}N_{18}O_{10}$, 1256.599; found, 1279 ([M+Na]$^+$).

^{1}H-NMR: δ 1.47–1.55 (m, 4H), 1.66 (p, 4H, J = 6.4 Hz), 2.01–2.16 (m, 2H), 2.17–2.30 (m, 2H), 2.33 (br t, 4H, J = 6.6 Hz), 3.19 (dt, 4H, J = 2.6, 6.8 Hz), 3.26 (s, 6H), 3.30–3.39 (m, 8H), 4.57 (dd, 2H, J = 4.6, 9.4 Hz), 4.90 (s, 4H), 5.14 (s, 4H), 6.82 (d, 4H, J = 9.0 Hz), 7.24–7.75 (m, 10H), 7.74 (d, 4H, J = 9.0 Hz), 8.62 (s, 2H).

^{13}C-NMR: δ 27.45, 28.05, 30.54, 33.51, 38.13, 39.94, 54.44, 56.72, 68.04, 69.54, 71.86, 112.82, 122.71, 123.45, 129.23, 129.38, 129.68, 130.46, 137.39, 147.02, 150.50, 153.05, 153.79, 157.91, 165.00, 170.20, 173.59, 174.99.

BisMTX

A solution of 43 mg (34 μmol) *bis*MTX^{Bn} was dissolved in 0.5 ml of methanol and diluted with 0.5 ml of water. This solution was added dropwise to 4 ml of a well-stirred solution of LiOH (0.5 M) in 50% (v/v) methanol/water such that the solution remained homogeneous. After 3 h, the reaction was quenched with 5 ml 0.1 M aqueous TFA and diluted with 50 ml 20% acetonitrile in 0.1% aqueous TFA. The product was purified by preparative HPLC (detection by A_{400}) using a two-segment gradient as follows (min/%B): 0/0, 2/10, 32/40. The product was lyophilized and desalted by repeating the HPLC with 0.2% CH_3CO_2H (pH adjusted to 4.0 with NH_4OH) and CH_3CN, respectively, as the A and B mobile phase components. Lyophilization afforded 53 mg of a yellow solid, which was dissolved in 2 ml of water by the addition of 75 μl 1 M NaOH to give a homogeneous solution at pH 8. The concentration of this stock solution was determined spectrophotometrically by diluting a 1 μl aliquot in 1 ml 10 mM sodium-phosphate (pH 7.4); ε_{309} was taken as 47,400 $M^{-1}cm^{-1}$ (twice the value of ε_{304} = 23,700 reported for a MTX γ-amide at this pH [22]). The reaction yield based on spectrophotometry was 49%. To verify that no epimerization at glutamate H_α had occurred, an analogous reaction was done with LiOD in methanol-d_4/D_2O; no loss of the glutamate H_α signal (δ 4.53 ppm) was observed in the ^{1}H NMR spectrum of the product, confirming that no racemization occurred. FAB-MS (thioglycerol/NaI): *m/z* calculated for $C_{50}H_{64}N_{18}O_{10}$, 1076.505; found, 1078 (8%, [M+H]$^+$), 1100 (15%, [M+Na]$^+$), 1122 (8%, [M+2Na–H]$^+$), 1144 (16%, [M+3Na–2H]$^+$). ^{1}H-NMR: δ 1.45–1.55 (m, 4H), 1.67 (p, 4H, J = 6.5 Hz), 2.00–2.18 (m, 2H), 2.18–2.46 (m, 6H), 3.20 (dt, 4H, J = 2.5, 6.9 Hz), 3.24 (s, 6H), 3.28–3.45 (m, 8H), 4.53 (dd, 2H, J = 4.6, 9.1 Hz), 4.91 (s, 4H), 6.79 (d, 4H, J = 9.0 Hz), 7.72 (d, 4H, J = 9.0 Hz), 8.59 (s, 2H). ^{13}C-NMR: δ 27.45, 28.27, 30.37, 33.54, 37.96, 39.77, 53.98, 56.58, 69.38, 71.71, 112.62, 122.63, 123.32, 130.25, 147.00, 150.29, 152.82, 153.56, 157.84, 164.83, 169.89, 175.06, 175.44.

Expression and purification of E. coli *DHFR*

A plasmid encoding *E. coli* DHFR (pMONDHFR; gift of Carl Frieden) was transformed into the *E. coli* strain BL21DE3 to yield JH710. DHFR was expressed and purified essentially as previously described [16,33] except with the noted modifications. JH710 was streak-isolated from frozen stock on LB (Luria-Bertani) plates that contained ampicillin (200 μg/ml). An isolated colony was used to inoculate 10 ml LB broth that contained ampicillin (200 μg/ml). This seed culture was grown at 37°C in a roller drum to yield a saturated culture. The seed culture was used to inoculate 1 l LB broth in a 2.8 l Buchner flask, and then grown at 37°C with shaking until an A_{600} of about 0.5 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM to induce transcription of the recombinant DHFR gene from the T7 promoter. The induced culture was left to grow at 37°C with shaking for an additional 3 h at which time the cells were harvested. Cells were pelleted at 6500 g for 30 min at 4°C in a Beckman model JA-10 rotor and then resuspended in ice-cold buffer 1 (40 mM Tris-HCl,pH 8.0 at 4°C, 20 mM EDTA, 1 mM dithiothreitol). The cells were lysed using a French press cylinder at 10,000 psi for 30 s and the cellular debris was pelleted at 15,000 g for 30 min at 4°C in a Beckman JA-20 rotor. The pellet was then washed using 5 ml buffer 1 and centrifuged as above. The supernatants were combined and polyethylenimine was added to a final concentration of 0.25% v/v. The mixture was stirred at 4°C for 5 min, then centrifuged at 12,100 g for 30 min at 4°C using a Beckman JA-20 rotor. The supernatants were combined, and protein precipitated by the addition of ammonium sulfate to a final concentration of 40% w/v. The mixture was stirred at 4°C for at least 2 h (up to 72 h) and protein pelleted by centrifugation at 12,100 *g* for 15 min at 4°C using a Beckman JA-20 rotor. Pelleted protein was discarded, and the protein remaining in the supernatant was further purified by affinity chromatography.

The protein (~50 ml) was loaded onto a 25 ml MTX-agarose affinity column that had been equilibrated in LS buffer (40 mM potassium phosphate, pH 6.0 at 4°C, 200 mM KCl, 2 mM EDTA, 1 mM dithiothreitol), and run at 1 ml/min using the ÄKTA purifier system (Pharmacia). The column was washed using two column volumes of HS buffer (200 mM potassium phosphate, pH 6.0 at 4°C, 1 M KCl, 2 mM EDTA, 1 mM dithiothreitol). Retained protein was eluted from the column by the addition of elution buffer (200 mM potassium phosphate, pH 8.0 at 4°C, 1 M KCl, 2 mM EDTA, 1 mM dithiothreitol) that contained 2 mM folic acid. Eluted column fractions were monitored at 280 nm and analyzed for protein content by SDS–PAGE. The fractions containing DHFR were pooled, diluted 10-fold with P500 buffer (50 mM potassium phosphate, pH 7.0 at 4°C, 500 mM NaCl, 1 mM EDTA), then diafiltered using an Amicon (Beverley, MA) stirred cell equipped with a YM10 membrane. This diafiltration cycle was performed four times in total to remove any remaining folic acid (detection by A_{350}). Equal weights of activated charcoal and protein were combined and gently stirred for 10 min at 4°C. The supernatant was retrieved by centrifugation and then filtered through a 0.45 μm filter. This treatment was performed three times in total to remove any contaminating NADPH (detection by A_{340}). The protein preparation was then further purified by gel filtration with a Superdex 75 HR 10/30 FPLC column (Pharmacia) with a bed volume of 24 ml. The fractions containing DHFR were pooled, and glycerol was added to a final concentration of 25% v/v. A sample of the final protein preparation was analyzed by SDS–PAGE and found to be greater than 95% pure by Coomassie staining. The concentration of the purified protein preparation was determined using

the Beer–Lambert law (with ε_{280} calculated to be 33,710 M^{-1} cm^{-1} [34]); the final preparation was subaliquoted and stored at –80°C.

Gel filtration assay

Molecular sizes were determined by gel filtration with a Superdex 75 HR 10/30 FPLC column (Pharmacia) with a bed volume of 24 ml. Samples (1000 µl) containing purified DHFR (1 µM) along with increasing concentrations of *bis*MTX (see Figures 3 and 4 for final concentrations of dimerizer) were prepared with P500 buffer and allowed to reach equilibrium by incubation at 25.5°C for at least 12 h. Controls containing *bis*MTX only (up to 5 µM), MTX only (up to 40 µM), and DHFR and MTX at a 1:2 molar ratio were prepared in the same way. Samples were injected onto the column and chromatographed with the ÄKTA system (Pharmacia) at 0.5 ml/min at room temperature with the same buffer. The observed molecular sizes were determined by comparison with a set of molecular standards (Sigma) that consisted of aprotinin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), and blue dextran (2 MDa). The area under each curve was integrated and used to calculate the fraction of protein in the ternary complex. Column fractions were collected and further analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie staining.

Modeling complex formation

Theoretical binding isotherms were generated in a Microsoft Excel spreadsheet based on the equations:

$$[P] = \frac{-(1+K_a[D]) + \sqrt{1+K_a[D]^2 + (\ 8P_{tot}K_a^2K_{coop}[D])}}{4K_a^2K_{coop}[D]}$$

$$[PD] = K_a[P]\ [D]$$

$$[P_2D] = K_a^2K_{coop}P^2D$$

$$P_{tot} = [P] + [PD] + 2[P_2D]$$

$$D_{tot} = [D] + [PD] + [P_2D]$$

where [P], [PD] and $[P_2D]$ are the concentrations of the free DHFR protein, the binary complex, and the ternary complex, respectively; [D] and D_{tot} are the free and total concentrations of the dimerizer drug *bis*MTX, respectively; K_a is the intrinsic association constant for the binding of the dimerizer to the protein; and K_{coop} is the cooperativity constant associated with formation of the ternary complex. A fixed value of $P_{tot} = 1$ µM was used, [D] was varied, and all other species were calculated. The ratio *bis*MTX:DHFR is D_{tot}/P_{tot}, and the fraction of protein as ternary complex is $[P_2D]/P_{tot}$. For the noncooperative model, $K_{coop} = 1$.

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