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Dexamethasone−Methotrexate: An Efficient Chemical Inducer of Protein Dimerization *In Vivo*

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Cell-permeable small molecules that can control cellular processes *in vivo* are integral to basic research in biochemistry and cell biology. Isopropyl-β-D-thiogalactoside (IPTG), for example, has been used for decades to activate gene transcription by stabilizing a conformation of lac repressor with reduced operator affinity. A clever extension of this approach involves the use of dimeric ligands or "chemical inducers of dimerization" (CIDs) to manipulate protein−protein interactions *in vivo*.[1−6] The inspiration for CIDs were studies of the mechanism of action of the natural immunosuppressants FK506 and rapamycin.[7,8] It was found that the immunosuppressant activity of both compounds results from the fact that they dimerize two proteins that otherwise do not interact. To generalize this molecular mechanism, Spencer *et al*. showed in 1993 that two FK506 molecules tethered via their C_{21} allyl groups could oligomerize proteins fused to FKBP12. CIDs are particularly powerful because the same dimeric ligand can be used over and over again simply by fusing the proteins of interest to the receptors for the CID. The majority of CIDs described to date are dimers of FK506[1,3,4] or FK506-analogues,[5,6] although other ligands have been reported also.[2] Given the broad utility of CIDs, we sought to design an optimized CID that could be prepared readily from commercially available materials and that was based on different ligand−receptor pairs. Here, we report such a compound: a heterodimeric dexamethasone−methotrexate molecule that can dimerize proteins efficiently *in vivo* (Figure 1).

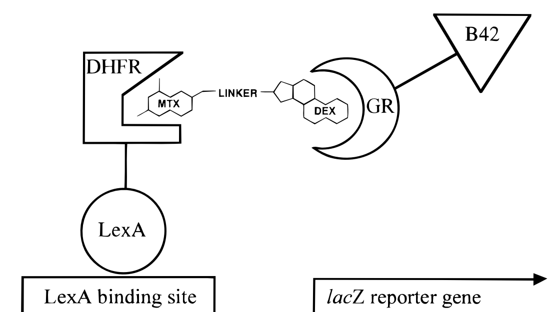

Figure 1. Yeast three-hybrid assay. A heterodimeric ligand (Dex-Mtx) bridges a DNA binding protein−receptor protein chimera (LexA−DHFR) and a transcription activation protein−receptor protein chimera (B42−GR), effectively reconstituting a transcriptional activator and stimulating transcription of a *lacZ* reporter gene. The levels of *lacZ* transcription serve as an indicator of the efficiency of Dex−Mtx-induced protein dimerization.

We chose to build a heterodimeric CID based on the well-characterized ligand−receptor pairs dexamethasone (Dex)−glucocorticoid receptor (GR) and methotrexate (Mtx)−dihydrofolate reductase (DHFR). Both Dex and Mtx present chemical functionality that can be modified readily without disrupting receptor binding.[12−15] Because the interactions of both Dex and Mtx with their respective receptors are well characterized,[9,10,14,15] future characterization and optimization of the Dex−Mtx system should also be facilitated. The rat glucocorticoid receptor (rGR) binds Dex with a K_D of 5 nM, and mutants of rGR with increased affinity for Dex have been isolated.[9] The steroid dexamethasone has been used extensively as a cell-permeable small molecule to regulate the activity and nuclear localization of GR fusion proteins in vivo.[10] Recently, a yeast "three-hybrid" system has been reported in which a GR-fusion and an FKBP12-fusion could be dimerized by the small molecule Dex-FK506.[11] Likewise, DHFR fusion proteins have been used for a variety of biochemical applications[16,17] due to Mtx's picomolar affinity for DHFR.[18] Both Dex and Mtx are commercially available, and Mtx can be synthesized readily from simple starting materials.

The retro-synthetic analysis of the Dex−Mtx heterodimer is shown in Scheme 1. The synthesis is based on previous syntheses of Dex and Mtx derivatives.[12,19−21] The synthesis is designed to allow the chemical linker between the two ligands to be varied readily. Both ligands were introduced as thiol derivatives to a di-halo linker. Following oxidative cleavage with periodate, Dex was derivatized with cystamine using standard peptide coupling reagents. The γ-carboxylate in Mtx was replaced with a thiol simply by replacing glutamate with homocysteine. Homocysteine, protected as the *tert*-butyl ester and disulfide, was coupled to 4-methylaminobenzoic acid. The resulting Dex and Mtx disulfide derivatives were reduced to their corresponding thiols using tri-*n*-butylphosphine, and the two thiols were coupled to a di-bromo linker in a one-pot reaction. The 2,4-diamino-6-bromomethyl pteridine was added after introduction of the dibromo linker to simplify purification of the intermediates, and the final step was cleavage of the *tert*-butyl ester. Thus, the Dex−Mtx heterodimer was prepared from two components in eight steps in 2% overall yield. The modular synthesis of the Dex−Mtx heterodimer will facilitate future studies to evaluate the influence of the linker on CID activity in different systems.

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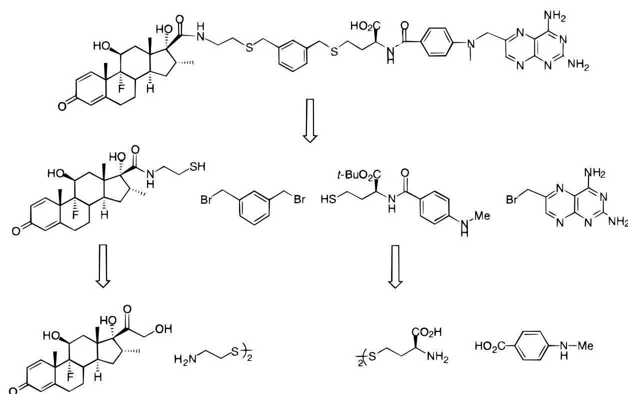
Table 1. Small-Molecule Induced Activation of β-Galactosidase Synthesis[a]

protein chimeras[c]	hydrolysis of ONPG[b] (μmol/min/mg total protein) no CID	0.01 μM Dex−Mtx	0.1 μM Dex−Mtx	1 μM Dex−Mtx	10 μM Dex−Mtx	10 μM Mtx 1 μM Dex−Mtx
LexA-DHFR, B42-GR	0.06 ± 0.01	0.1 ± 0.05	1.3 ± 0.1	10.9 ± 2.0	12.3 ± 2.0	0.30 ± 0.10
LexA, B42	0.25 ± 0.01	ND	ND	0.33 ± 0.04	ND	ND

[a] β-Galactosidase is encoded by the *lacZ* gene. Small-molecule-dependent protein dimerization was quantified in duplicate for cells grown in synthetic complete liquid media (containing 2% galactose and 2% raffinose, lacking uracil, histidine, and tryptophan) based on levels of β-galactosidase synthesis. The error estimates for the rates of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis correspond to the standard error between the two measurements. [b] β-Galactosidase synthesis levels were determined from the rate of hydrolysis of ONPG. [c] Plasmids encoding the protein chimeras were introduced into *S. cerevisiae* FY250 containing a *lacZ*-reporter gene under control of 4 tandem LexA operators.

Scheme 1. Retro-synthetic Analysis of Dex−Mtx



To demonstrate that Dex−Mtx can dimerize proteins *in vivo*, we employed the yeast "three-hybrid" system,[11,22,23] which consists of a dimeric small molecule, two protein chimeras, and a reporter gene (Figure 1). The small molecule (Dex−Mtx) bridges the DNA-binding protein chimera (LexA−DHFR) and the transcription activation protein chimera (B42−GR) effectively reconstituting a transcriptional activator and stimulating transcription of a *lacZ* reporter gene.

Using standard β-galactosidase activity assays both on plates and in liquid culture,[25] we showed that Dex−Mtx can activate *lacZ* transcription *in vivo* (Figure 2 and Table 1). On the basis of previous studies showing that *lacZ* transcription levels correlate with the strength of protein−protein interactions in the yeast two-hybrid assay,[26] we expect β-galactosidase activity to be a good indicator of Dex−Mtx induced protein dimerization. In these assays, the extracellular concentration of Dex−Mtx ranged from 0.01 to 10 μM. Control experiments established that *lacZ* transcription was dependent on Dex−Mtx (Figure 2 and Table 1). Only background levels of β-galactosidase activity were detected when Dex−Mtx was omitted. A 10-fold excess of Mtx reduced Dex−Mtx-dependent *lacZ* transcription to near background levels. A 10-fold excess of Dex, however, did not effect Dex−Mtx-dependent *lacZ* transcription, and higher concentrations of Dex were toxic to the yeast cells. This result may be due to differences in cell permeability between Dex and Dex−Mtx or may suggest that LexA−DHFR, but not B42−GR, is the limiting reagent. In addition, when either or both receptors were deleted, only background levels of *lacZ* transcription were detected.

It is difficult to compare the effectiveness of different CIDs, as CID activity is assay-dependent and is not linear. It is interesting to note, however, that in the yeast three-hybrid assay the absolute levels of β-galactosidase synthesis are 150-fold higher for Dex−Mtx than for Dex-FK506 at 1 μM CID concentration based on liquid culture *lacZ* transcription assays (data not shown).

Although demonstrated using the yeast three-hybrid assay for protein dimerization, the heterodimeric Dex−Mtx molecule in principle can dimerize any two proteins fused to the hormone-binding domain of GR and DHFR. To extend the Dex−Mtx CID to organisms where Mtx is toxic, we are in the process of modifying Mtx so that it binds selectively to a DHFR mutant. This approach is well precedented in the AP−FKBP CID series.[6] Given the ease with which Dex−Mtx and potential analogues can be prepared and Dex−Mtx's high efficiency in protein dimerization, we anticipate that this Dex−Mtx protein dimerization system will find broad use in biochemistry and cell biology.

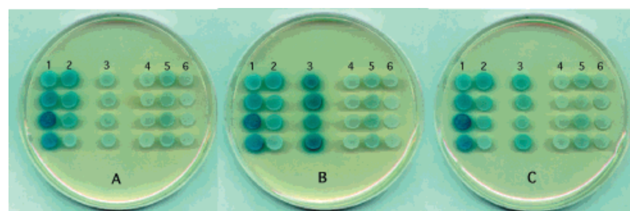


Figure 2. X-gal plate assay of Dex−Mtx-induced *lacZ* transcription. Yeast strains containing a *lacZ* reporter gene and different LexA- and/or B42-chimeras were grown on X-gal indicator plates with or without Dex−Mtx. Columns **1**−**6** on each plate correspond to yeast strains containing different LexA- and/or B42-chimeras: **1**, LexA−Sec16p, B42−Sec6p; **2**, LexA−Sec13, B42−Sec6p. **1** and **2** are direct protein−protein interactions used as positive controls.[27] **3**, LexA−DHFR, B42−GR; **4**, LexA−DHFR, B42; **5**, LexA, B42−GR; **6**, LexA, B42. X-gal plates **A**−**C** have different small molecule combinations: **A**, no Dex−Mtx; **B**, 1 μM Dex−Mtx; **C**, 1 μM Dex−Mtx, and 10 μM Mtx.

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Supporting Information Available: Experimental procedures for the synthesis of Dex−Mtx **1**, for the construction of the protein chimeras, and for the *lacZ* assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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