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An orthogonal dexamethasone–trimethoprim yeast three-hybrid system ☆

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The yeast three-hybrid assay is an important tool for the detection of protein–ligand interactions *in vivo* and has recently been used successfully for the discovery of novel drug targets and the directed evolution of enzymes [1–6]. Schreiber and co-workers [7] developed the first chemical inducer of dimerization (CID)[1], a dimer of the immunosuppressant FK506. Building from this work, a number of yeast three-hybrid systems based on different CIDs have been reported [2,8]. We previously developed and optimized a three-hybrid system built around the small molecule CID dexamethasone–methotrexate (Dex–Mtx) [9–11]. The Dex and Mtx ligands were chosen because of their high affinities for their respective protein receptors, the rat glucocorticoid receptor (GR) and *Escherichia coli* dihydrofolate reductase (DHFR), respectively [12,13]. However, we hypothesized that the cross-reactivity of Mtx with endogenous DHFR in the yeast cells could impair transcription activation by Dex–Mtx in the yeast three-hybrid assay. To overcome this partial limitation we set out to design a CID that would selectively bind to *E. coli* DHFR and not to endogenous yeast DHFR. As an alternative to Mtx, we chose the DHFR inhibitor trimethoprim (TMP), which is known for its selectivity for bacterial forms of DHFR [14]. Studies have confirmed that while Mtx inhibits growth of wild type *Saccrharomyces cerevisiae*, TMP does not [15], suggesting that TMP could be a superior CID in yeast. Here we report the design, synthesis, and *in vivo* activity of Dex–TMP in the yeast three-hybrid assay.

By analogy to our dexamethasone–methotrexate system, we chose to build a heterodimeric CID using the ligand–receptor pairs dexamethasone–rat glucocorticoid receptor (GR) and trimethoprim–*E. coli* dihydrofolate reductase. Both Dex and TMP can be modified without disrupting receptor binding, making them suitable CIDs [16–18]. Both ligands are cell permeable and commercially available. The ligand–receptor pair Dex–GR has a K_D of 5 nM and has been used successfully in yeast three-hybrid systems [9]. *E. coli* dihydrofolate reductase DHFR has a K_I of 1.3 nM for inhibition by TMP [14]. We anticipated that the two interactions would be sufficiently strong to induce protein dimerization and transcription activation in the yeast three-hybrid assay.

The design and synthesis of the Dex–TMP heterodimer is based on previous syntheses of Dex and TMP derivatives [16,18,19], with a linker analogous to that for the Dex–Mtx heterodimers most active in the yeast three-hybrid assay (Fig. 2c). Both ligands were coupled as their thiol derivatives to a di-iodo linker. First, Dex was converted to the carboxylic acid by oxidative cleavage using periodate and then derivatized with cystamine using standard peptide coupling reagents. The 4′-methoxy group of TMP was selectively cleaved in 48% hydrobromic acid. The resulting phenol was then derivatized with ethyl 5-bromovalerate using potassium tertbutoxide and converted to the corresponding carboxylic acid. Following saponification two equivalents of the TMP acid were reacted with cystamine dihydrochloride under standard peptide coupling conditions to generate the TMP disulfide. Finally, the Dex and TMP disulfides were reduced to their corresponding thiols using tri-*n*-butylphosphine. The thiol derivatives of the two ligands were coupled to the di-iodo linker sequentially, as for the previous Dex–Mtx CIDs. Thus, the Dex–TMP heterodimer

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[1] *Abbreviations used:* CID, chemical inducer of dimerization; Dex–Mtx, dexamethasone–methotrexate; Dex, dexamethasone; Mtx, methotrexate; GR, glucocorticoid receptor; DHFR, dihydrofolate reductase; TMP, trimethoprim; Dex–TMP, dexamethasone–trimethoprim; DBD, DNA-binding domain; AD, transcriptional activator domain; ONPG, o-Nitrophenyl-β-D-galactopyranoside; SLF, synthetic ligand for FK506-binding protein 12.

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was prepared from two components in a 10-step synthesis in 0.8% overall yield.

Dex–TMP was evaluated for its ability to activate transcription in the yeast three-hybrid assay using a LexA DNA-binding domain–DHFR protein chimera (LexA–DHFR), a B42 transcription activation domain–GR protein chimera (B42–GR) and a *lacZ* reporter gene under control of four tandem LexA operators (Fig. 1). Using standard *lacZ* transcription assays in both solid and liquid cultures [20], we showed that Dex–TMP can activate *lacZ* transcription in the yeast three-hybrid assay (Figs. 2a and b). X-gal plate assays were carried out as previously reported [10]. Yeast strains were grown on synthetic complete medium lacking histidine, uracil, and tryptophan and containing no glucose, but includes 2% galactose and 2% raffinose, and were grown with or without the small molecule. To determine how effectively Dex–TMP activates transcription in comparison to our previous Dex–Mtx system, we tested the two CIDs side by side at concentrations ranging from 1 to 10 μM in the external growth medium. Control experiments established that transcription activation was small-molecule dependent, and only background levels of *lacZ* transcription were detected when both Dex–Mtx and Dex–TMP were omitted. Activation over background levels was observed for both small molecules and at all concentrations, except for 1 μM Dex–TMP. The maximal levels of transcription were observed with 5 μM Dex–Mtx and 10 μM Dex–TMP. In addition, all three yeast strains were grown in liquid culture and assayed for β-galactosidase activity with ONPG as the substrate. In the liquid culture assays, growth medium containing 1, 5, or 10 μM

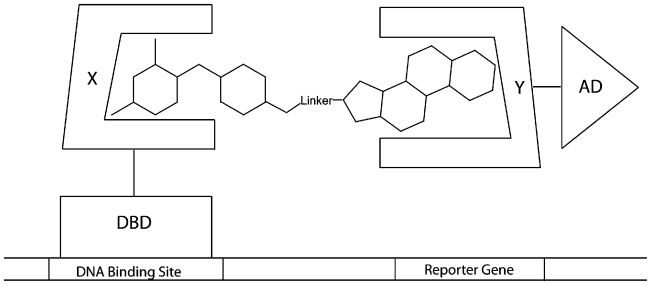

Fig. 1. Dexamethasone–trimethoprim (Dex–TMP) yeast three-hybrid system. A heterodimeric ligand (Dex–TMP) bridges a DNA-binding domain–dihydrofolate reductase fusion protein (DBD–DHFR) and a transcriptional activator domain–glucocorticoid receptor fusion protein (AD–GR), effectively reconstituting a transcriptional activator (DBD–AD) and activating transcription of a downstream reporter gene.

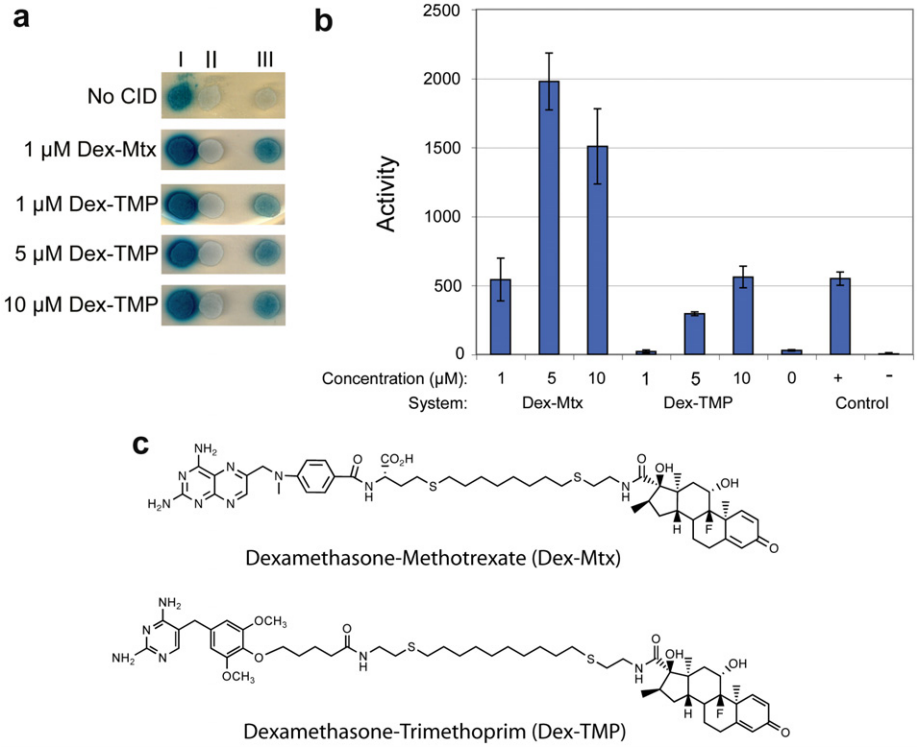

Fig. 2. *LacZ* transcription assays comparing the abilities of dexamethasone–trimethoprim (Dex–TMP) and dexamethasone–methotrexate (Dex–Mtx) to activate transcription in the yeast three-hybrid assay. (a) Columns I–III on each plate correspond to yeast strains containing different DNA-binding domain (DBD) and/or activation domain (AD) chimeras and a *lacZ* reporter gene: I, plasmid DBD–BAIT, plasmid AD–TARGET, plasmid *lacZ* reporter (I is a direct protein–protein interaction used as a positive control); II, integrated DBD–DHFR, plasmid AD, plasmid *lacZ* reporter (II has only the AD half of the AD–GR protein chimera and is used as a negative control); III, plasmid DBD–DHFR, plasmid AD–GR, plasmid *lacZ* reporter (III is the plasmid based yeast three-hybrid system). Plates were grown for 3 days with a concentration of small molecule ranging from 1 to 10 μM and compared to a background of no small molecule. (b) ONPG liquid assays of CID-induced *lacZ* transcription. The first three bars represent the data for the all-plasmid system with varying concentrations of Dex–Mtx, followed by varying concentrations of Dex–TMP and no CID. The last two rows are the positive and negative controls. (c) The structures of the two small molecules used in the study, Dex–Mtx and Dex–TMP.

Dex–Mtx resulted in a 22-, 79-, and 61-fold activation over background levels, respectively. The addition of 5 and 10 μM Dex–TMP resulted in a 12- and 23-fold increase in activation, respectively.

The new CID Dex–TMP can successfully dimerize the two halves of the transcriptional activator *in vivo* in the yeast three-hybrid assay, activating transcription of a *lacZ* reporter gene as shown using β-galactosidase activity assays. Thus, the ligand receptor pair TMP–*E. coli* DHFR provides a new CID for use in the three-hybrid assay and in other *in vivo* applications of CIDs. This pair may prove particularly useful for applications in mammalian cell lines or even animal studies, when the toxicity of Mtx may prove problematic. However, somewhat surprisingly, the new CID does not induce transcription activation as efficiently as Dex–Mtx. There is evidence, however, that TMP–SLF activates transcription in a yeast three-hybrid assay slightly better than Mtx–SLF (M. Schelle, C. Bertozzi, L. Miller, V. Cornish, unpublished results). These results point to the complexities of manipulating molecules at the cellular level. There are several plausible explanations for the difference in activity between Dex–TMP and Dex–Mtx. One possible reason for the disparity in activity is the large difference in affinities of the two for DHFR. Mtx binds *E. coli* DHFR with picomolar affinity (K_D = ca. 10 pM) [21], whereas TMP's affinity is much lower (K_I = 1.3 nM) [14]. Also, studies have found that, although yeast DHFR is not a target of TMP, the small molecule may bind to another yeast protein of unknown function [22]. Dex–Mtx may have more favorable cell permeability properties than Dex–TMP. In summary, this study provides a new CID pair, TMP–DHFR, which may be particularly advantageous for applications in mammalian cell lines and animal studies and illustrates the complexities of "engineering" at the cellular level.

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