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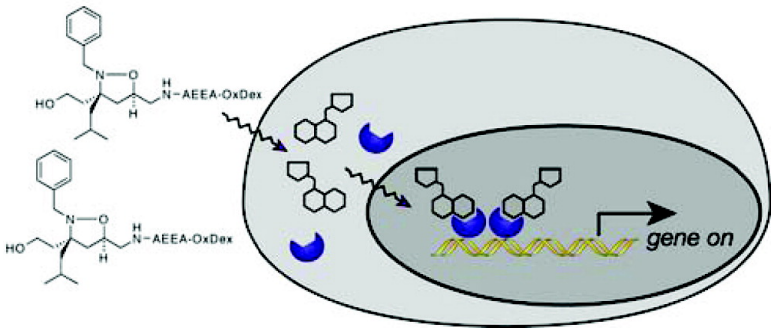

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Transcriptional Up-regulation in Cells Mediated by a Small Molecule

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Misregulated transcription is associated with many human diseases as either a cause or an effect.[1] In the case of acute promyelocytic leukemia, for example, a chromosomal translocation alters the function of a transcriptional activator, leading to a block in hemopoietic development of myeloid cells at the promyelocytic stage.[2] Thus, molecules that can reconstitute the function of transcriptional activators, so-called activator artificial transcription factors or activator ATFs, are highly desirable commodities as mechanistic tools with enormous therapeutic potential.[3] In particular, *small molecule* activator ATFs are valued for their likely advantages in terms of delivery, stability, and immunogenicity. Despite this motivation, progress toward small molecules that function as activators has been slow although peptidomimetic versions have been described.[3−6]

To up-regulate particular genes, an activator ATF must have two functions: the ability to interact with DNA in a tight and sequence-specific manner and the ability to interact with the transcriptional machinery, thus facilitating its assembly at the gene of interest. Although small molecules that reconstitute the DNA binding function of activators in vitro and in cells have been described,[7] the discovery of small molecules that function as transcriptional activation domains (TADs) in cells has been enormously challenging, perhaps in part because of the complex functional mechanism of these domains. Natural TADs, for example, must interact with a range of protein binding partners to stimulate chromatin remodeling and assemble the transcriptional machinery (see Figure 1).[3] Here we describe the first small molecule activation domain with function in living cells. This amphipathic molecular scaffold shows up to 80-fold levels of activity and functions at concentrations as low as 5 nM. This therefore represents an important step forward in the development of transcription-based therapeutics.

We recently described isoxazolidine **1**[4a] and related structures[8] as molecules that reconstitute the function of transcriptional activation domains in a cell-free system. These molecules exhibit good activity in a standard in vitro transcription assay (5 to 7-fold up-regulation), comparable to a well-characterized natural activation domain. In the cell-free assay, however, cellular permeability, nuclear localization, and cellular stability are not assessed. In addition, there is a much more limited range of potential protein binding partners relative to the cellular environment. This latter issue is particularly important since natural and non-natural TADs have been shown to exhibit promiscuous binding profiles, interacting with many different hydrophobic binding surfaces.[3] Thus, the transition from cell-free activity to cellular function can be quite challenging, illustrated by the absence of cell-active small molecule TADs.

To test small molecule TADs in cells, an appropriate DNA-targeting moiety was required. We chose a system developed by Kodadek and co-workers[5] in which a fusion protein consisting of the Gal4 DNA binding domain and the minimal ligand binding domain of the glucocorticoid receptor is constitutively expressed in the cells. The small molecule TADs under examination are thus

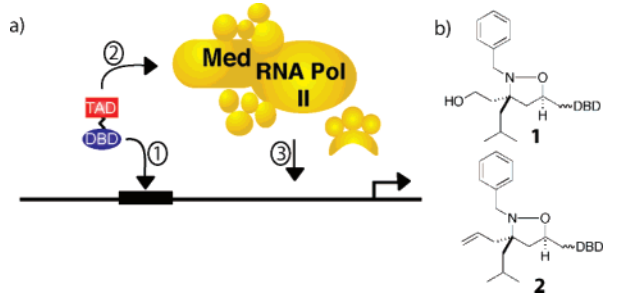
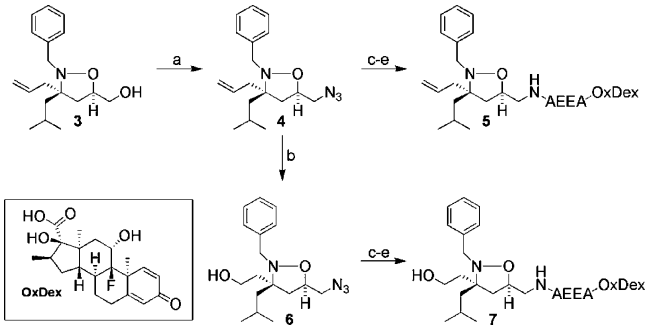


Figure 1. (a) As part of gene up-regulation, transcriptional activators ① bind to DNA using a DNA-binding domain (DBD), ② interact with the transcriptional machinery via the transcriptional activation domain (TAD), and ③ stimulate the assembly of the RNA polymerase II holoenzyme.[3] (b) Amphipathic isoxazolidine **1** functions as a TAD in a cell-free system whereas hydrophobic isoxazolidine **2** does not.[4a]

Scheme 1 [a]



[a] Conditions: (a) $(PhO)_2P(O)N_3$, DEAD, PPh_3, THF (71%); (b) (i) OsO_4, NMO, *t*BuOH/THF/H_2O (ii) $NaIO_4$, CH_3CN/H_2O (iii) $NaBH_4$, MeOH (76%); (c) PPh_3, H_2O, THF reflux (94%); (d) (i) Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEA), HOBT, HBTU, Et_3N, NMP; (ii) 20% piperidine; (e) OxDex, HOBT, HBTU, Et_3N, 2,6-lutidine, NMP.

tagged with a modified form of dexamethasone (OxDex); upon binding of the isoxazolidine-OxDex conjugate, the complex localizes the small molecule to binding sites for Gal4 that control the activity of a reporter gene, firefly luciferase. Thus, a measurement of luciferase activity provides direct information regarding the ability of the small molecule to function as a transcriptional activation domain.

For this study, our synthetic targets were derivatives of **1** and **2**; the more hydrophobic **2** does not function as a transcriptional activation domain in vitro and thus serves as a control.[4a] To prepare the targeted OxDex-isoxazolidine conjugates, racemic isoxazolidine **3**[4a] was first treated with diphenylphosphoryl azide, DEAD, and PPh_3 to transform the primary alcohol into an azide to produce isoxazolidine **4** in 71% yield (Scheme 1). A short PEG linker (Fmoc-8-amino-3,6-dioxaoctanoic acid, Fmoc-AEEA) was introduced following a Staudinger reduction of the azide moiety of **4**. Deprotection of the amine via treatment with piperidine and combination with the activated ester of OxDex provided isoxazolidine **5**; this conjugate and conjugate **7** were isolated and tested as

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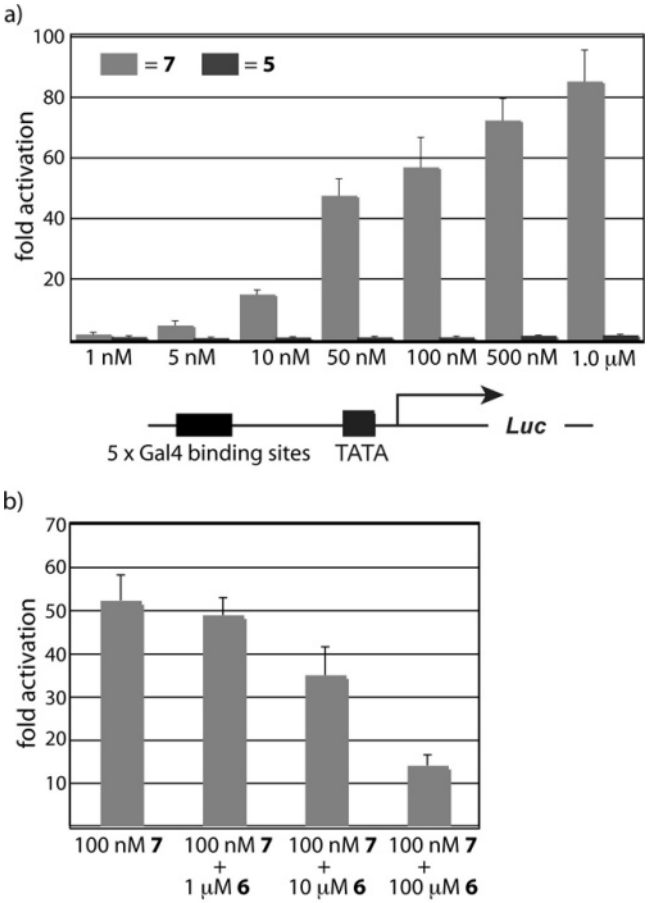

Figure 2. Results from luciferase assays in HeLa cell culture. (a) Activity of OxDex-isoxazolidine conjugates **5** and **7** expressed as fold activation relative to OxDex-AEEA alone. Briefly, HeLa cells were transfected with a plasmid expressing the Gal4 DBD-GR LBD fusion protein, a second plasmid bearing five Gal4-binding sites upstream of a firefly luciferase reporter gene, and a third plasmid expressing *Renilla* luciferase as a transfection control, as has been previously described.[5] Compounds were added to the cells as a DMSO solution 3 h after transfection such that the final concentration of DMSO in all wells was 1% (vol/vol). The firefly and *Renilla* luciferase activities were measured 40 h after compound addition. Fold activation was determined at each concentration by first dividing the firefly luciferase activity by that of *Renilla* luciferase. This value was then divided by the amount of activity observed with OxDex-AEEA. Each value is the average of at least three independent experiments with the indicated error (SDOM). (b) The transcriptional function of **7** is inhibited by the addition of increasing concentrations of isoxazolidine **6** (see Supporting Information for details).

a diastereomeric mixture due to the racemic isoxazolidine moiety. Our earlier studies demonstrated that the isoxazolidine enantiomers have identical transcriptional activity.[8] Azide **4** also served as an intermediate for the synthesis of the amphipathic target. Toward that end, the double bond appended to C3 was oxidatively cleaved to yield a primary alcohol at that position in 76% yield. Reduction of the azide followed by coupling to the AEEA linker and then to OxDex under the conditions described earlier provided amphipathic isoxazolidine **7** in 31% yield. The ability of the conjugates to activate transcription was then assessed in a standard dual-reporter luciferase assay (Figure 2).

As illustrated in Figure 2a, amphipathic isoxazolidine **7** exhibited measurable activity even at low nanomolar concentrations (5-fold at 5 nM) and a remarkable 80-fold activity at 1 μM. Over the range of concentrations tested, no phenotypic change was observed with the cells and the expression of the transfection control gene was unaffected. Analogous to data from cell-free experiments, hydrophobic isoxazolidine **5** does not function as a transcriptional activation domain at concentrations up to 1 μM. Evidently the general amphipathic character of **7** is important for overall activity, similar to natural transcriptional activation domains.[3,9] Notably, the EC_{50} of isoxazolidine **7** is 33 $\pm$ 6 nM, considerably lower than peptoid-based TADs ($\sim$10 μM).[5a]

One characteristic of natural transcriptional activators is that the DNA binding domain and the transcriptional activation domain function independently.[3] To test if the Gal4-GR LBD construct contributed to isoxazolidine transcriptional activity, a competitive inhibition or "squelching" experiment[10] was carried out. A constant concentration (100 nM) of isoxazolidine **7** along with increasing concentrations of isoxazolidine **6** (from 0 to 100 μM) was added to a series of HeLa cell aliquots. As illustrated in Figure 2b, at a concentration of 100 μM isoxazolidine **6** inhibited the activity of the isoxazolidine TAD by approximately 70%. This is consistent with the DNA binding domain serving as a transcriptionally inert promoter localization scaffold. The amphipathic isoxazolidine does, however, require DNA binding to function; isoxazolidine **6**, lacking an OxDex moiety, does not up-regulate transcription to detectable levels (see Supporting Information).

In summary, we have identified the first small molecule that functions as a transcriptional activation domain in living cells. Both our previous work[4a,8] and the results presented here suggest that these molecules function in a manner similar to natural transcriptional activation domains. The competitive inhibition experiments in particular suggest that the TAD is portable and should function when attached to alternative DNA targeting moieties. This therefore sets the stage for small molecule activator ATFs that target endogenous genes and our efforts in that direction will be reported in due course.

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Supporting Information Available: Details of the synthesis of isoxazolidines **4**−**7** and of the luciferase assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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