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Stereochemical Promiscuity in Artificial Transcriptional Activators

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The prevailing paradigm driving the discovery of small molecules that perturb biological functions requires ligands of high affinity and specificity for a particular macromolecule. This paradigm is most effectively applied in cases where a single, well-defined binding site influences the function of the macromolecule. However, many biological processes are carried out by macromolecular complexes whose assembly and function are initiated through combinations of weaker interactions (micromolar K_Ds) with functionally redundant binding surfaces; in this way, a relatively small number of proteins can reside in more than one complex and/or participate in multiple functions.[1] Small molecules that effectively regulate such systems will likely need to mimic the behavior of the endogenous participants.

Transcriptional activation is an example of a process initiated by a network of lower affinity interactions and is thus an ideal system in which to explore the development of small molecule regulators of macromolecular complexes. The activation domains (ADs) of endogenous transcriptional activators typically exhibit a low micromolar K_D, multipartner binding profile that is likely an essential functional contributor as the protein must mediate the assembly of the large transcriptional machinery complex on DNA (Figure 1a).[2] Several lines of evidence suggest that the AD binding sites within the transcriptional machinery are somewhat functionally redundant, and particular placements of specific side chains are not required for functionally productive binding interactions.[2,3] For example, ADs from VP16, Gcn4, and Gal4 target an overlapping group of transcriptional machinery protein targets despite exhibiting little sequence homology outside of a general amphipathic composition.[3c,4] We recently described the first small molecule AD, an isoxazolidine bearing functional groups seen in natural ADs (**1**, Figure 1b).[5] Here, we describe positional "mutagenesis" experiments in which we evaluated analogs of **1** bearing identical side chains in various locations within the isoxazolidine scaffold. The results reveal that the isoxazolidine small molecule ADs mimic the functional profile of natural ADs in that precise positioning of the amphipathic side chains is not a critical determinant for function. This finding has important implications for the design of future small molecule transcriptional regulators.

In our original experiments, all isoxazolidines were prepared as racemates and were tested as stereoisomeric mixtures.[5] We thus targeted each enantiomer of the original isoxazolidine (**3** and **4**) as well as a diastereomer (**5**) and two positional isomers (**6** and **7**) for this study (Figure 2a). The compounds contain the same functional groups found in the original active compound (**1**) but in varying three-dimensional orientations since significantly altering the hydrophobic and polar content of the molecules was found to decrease function.[5] Analogous to the natural system, we hypothesized that all of the molecules would function as transcriptional ADs.

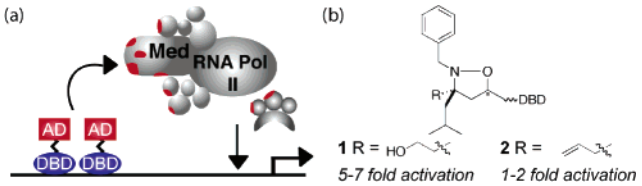

Figure 1. (a) Transcriptional activator-mediated gene up-regulation, with putative activation domain (AD) binding sites indicated in red. The DNA binding domain (DBD) localizes the activator to a specific DNA site. (b) **1** contains functional groups commonly observed in natural ADs and activates transcription well in vitro when localized to DNA.[5] Hydrophobic **2** functions poorly, consistent with studies of natural ADs.[5]

The key intermediate for the preparation of **3**, **5**, and **6** is isoxazoline **10**, isolated as a single enantiomer in 88% yield following a 1,3-dipolar cycloaddition reaction (Figure 2a).[6]

Toward **6**, installation of the C3 benzyl group was accomplished by silyl protection of the secondary alcohol of **10** followed by addition of benzylmagnesium chloride (80% yield; 10:1 dr).[6c] The major diastereomer was then treated with allyl bromide under microwave conditions to alkylate N2 and provide isoxazolidine **13** (65% yield). Oxidative cleavage of the double bond installed the requisite hydroxyl group on the N2 side chain, and treatment with TBAF unmasked the 1,2-diol that was cleaved to provide an aldehyde at C5; this sensitive intermediate was immediately combined with Mtx (Figure 2a) and the resulting conjugate **6** isolated by reversed-phase HPLC. For diastereomers **3** and **5**, allylmagnesium chloride was employed as the nucleophile in the initial addition reaction. Unlike the benzyl addition, the secondary alcohol was not protected in order to reduce the diastereoselectivity of the reaction and enable both diastereomers (**11** and **12**) to be isolated (71% combined yield, 5:1 dr). The two diastereomers were separated chromatographically, and each underwent installation of the N2 benzyl group via alkylation (81% yield) and oxidative cleavage of the C3 allyl group to provide **14** and **15**. Straightforward manipulations lead to the final conjugate targets **3** and **5**. Isoxazolidine **4** was prepared through an analogous reaction sequence starting with the enantiomer of **9**.

The function of the isoxazolidines was measured by their ability to up-regulate transcription in a standard in vitro transcription assay employing HeLa (human) nuclear extracts with the natural AD ATF14 as a positive control (Figure 2c).[5] The activity of enantiomers **3** and **4** is indistinguishable from that of AD **1** containing both enantiomers of the isoxazolidine ring (Figure 2c). Isoxazolidines **5**−**7** more significantly differ in the presentation of the amphipathic functional groups due to stereochemical changes (**5**) or positional changes within the ring (**6** and **7**). Nonetheless, all function well as transcriptional ADs, in line with our prediction. Isoxazolidine **7** showed the only noteworthy attenuation in activity, with 35% lower functional levels relative to **1** (∼4-fold). In sum,

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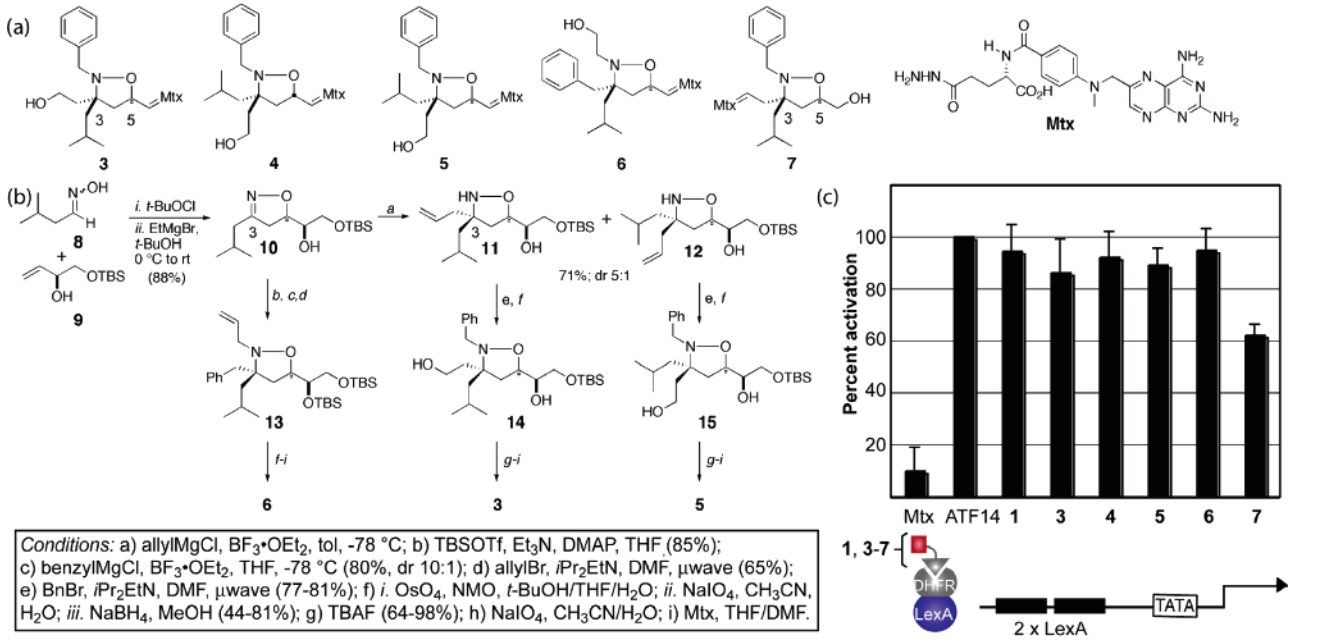

Figure 2. (a) Isoxazolidines with varying spatial orientations of polar and hydrophobic functional groups. (b) Synthetic scheme for isoxazolidines **3**, **5**, and **6**. An analogous series of reactions were used for the preparation of **4** and **7**.[5] (c) Results from in vitro transcription assays. The DBD is the fusion protein LexA-DHFR; the high affinity interaction between DHFR and methotrexate localizes the Mtx-tagged small molecules (50 nM) to DNA.[11] Each activity is the average of at least three independent experiments, with the indicated error (SDOM). The maximal activation is 7-fold relative to background. See Supporting Information for additional details.

the data indicate that precise positioning of functional groups is not the most important determinant of activator function in our system.

The conserved activity across amphipathic, isomeric isoxazolidines **3**−**7** parallels the functional behavior of the endogenous amphipathic ADs that this molecular class was originally designed to mimic. For example, the activity of **3** and **4** is consistent with an earlier report that the D and L-enantiomers of the natural AD ATF29 stimulate similar transcription levels in a cell-free system.[7] Among peptidic ADs, a variety of combinations of polar and hydrophobic amino acids function as ADs, but a hydrophobic/polar balance is conserved.[2,3a−c] Further, like our small molecules, endogenous ADs share a common structural motif; for natural ADs, structural studies suggest that formation of a helix occurs upon binding to a number of transcriptional machinery targets, although other secondary structures may play a role.[8] Also similar to the isoxazolidines,[5] mutations in natural ADs that disrupt the hydrophobic surface significantly decrease activation potential.[9] One remaining question is whether the similarities between the small molecules and natural ADs extend to the binding surfaces within the transcriptional machinery. Although the aggregate data are suggestive of an affirmative answer, cross-linking experiments will be required to provide a more definitive conclusion.

In sum, our data suggest that isoxazolidines are unlikely to be the only suitable scaffolds for the construction of small molecule transcriptional activation domains. Rather, a variety of appropriately functionalized conformationally constrained small molecules should also function well, a prediction currently under investigation. This strategy obviates the need to identify high affinity ligands for single protein targets and takes advantage of the remarkable functional flexibility of the endogenous transcriptional regulatory system. Given the increased interest in small molecule ADs as mechanistic probes and therapeutic agents,[10] this approach may find wide application.

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Supporting Information Available: Synthetic details for compounds **3**−**7** and complete refs 1b and 1c. This material is available free of charge via the Internet at http://pubs.acs.org.

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