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Transcriptional Tools: Small Molecules for Modulating CBP KIX-dependent Transcriptional Activators

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Abstract

Previously it was demonstrated that amphipathic isoxazolidines are able to functionally replace the transcriptional activation domains of endogenous transcriptional activators. In addition, *in vitro* binding studies suggested that a key binding partner of these molecules is the Creb Binding Protein (CBP), more specifically the KIX domain with this protein. Here we show that CBP and the KIX domain play an essential role in the ability of isoxazolidine transcriptional activation domains to activate transcription in cells. Consistent with this model, isoxazolidines are able to function as competitive inhibitors of the activators MLL and Jun, both of which utilize a binding interaction with KIX to up-regulate transcription. Further, modification of the N2 side chain produced two analogs with enhanced potency against Jun-mediated transcription, although increased cytotoxicity was also observed. Collectively these small KIX-binding molecules will be useful tools for dissecting the role of the KIX domain in a variety of pathological processes.

Introduction

Transcription factors, often characterized as undruggable, are nonetheless emerging as potentially attractive therapeutic targets due to their fundamental role in human disease.^{1–3} One class of transcription factors, transcriptional activators, plays a key regulatory role by binding to DNA and assembling the transcriptional machinery at a particular gene, thus stimulating gene expression. Miscued transcription caused by malfunctioning activators has been implicated in the onset of many cancers and this has spurred widespread interest in the development of small and large molecules that directly target misregulated genes.² The development of small molecule transcriptional activation domain (TAD) mimics is an advantageous strategy to modulate transcription because, similar to natural TADs, these molecules can function as activators when tethered to a DNA-binding domain (DBD) and as inhibitors when not localized to DNA (Figure 1a).³ TAD mimics are thus promising candidates for modulating gene transcription, for use as mechanistic probes, and as transcription-targeted therapeutics.³

Like their natural counterparts, small molecule TADs must interact with coactivators in the transcriptional machinery in order to function. This presents a formidable challenge since the number of putative coactivator targets of activators is large, with few validated as physiologically relevant.⁴ One target of importance is the CREB binding protein (CBP), a large (265 kDa) multidomain coactivator and histone acetyl transferase.⁵ CBP integrates transcriptional signals from >100 transcription factors and is an essential protein for cell growth and development. In contrast to most coactivators, the individual domains of CBP

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have proven amenable to structural characterization and, concomitantly, have been attractive targets for the development of small and large molecule transcriptional regulators.⁶ A domain that has attracted much attention in this regard is the KIX domain, an 87-residue module whose solution structure has been elucidated.⁷ Despite its relatively small size, the KIX domain contains at least two activator binding sites, the CREB/Myb site and the MLL/Jun/Tat/Tax site (Figure 1b).⁸ Screening against the KIX domain has led to the discovery of both peptides and peptidomimetic TADs that function well in cells;^{6b,9} derivatives of these peptides have been useful tools for defining characteristics of activator binding sites.¹⁰ Not just useful for creating activators, a small molecule screen against the KIX domain has also yielded naphthol AS-E phosphate and related derivatives that competitively inhibit the KIX-CREB binding interaction.^{6e,6h} Complementary to genetic strategies, compounds that inhibit KIX-binding activators will be useful tools in dissecting the role of the KIX domain in physiological processes such as cell differentiation and hematopoiesis.¹¹ Furthermore, both MLL and Jun have been implicated in several cancers, including leukemias and solid tumors;¹² thus compounds that block these proteins from forming key interactions with CBP may prove useful in the development of transcription-targeted therapeutics.^{1-5, 15}

We identified several isoxazolidine-based TADs through a top-down discovery strategy that reconstitute the function of a natural activator when localized to a promoter (e.g. **1a**, Figure 1c).¹³ Subsequent *in vitro* binding studies revealed that one target of these small molecules is the KIX domain of CBP, more specifically the MLL/Jun/Tat/Tax site within that module (Figure 1b).^{6c} What was not clear from these studies was if this interaction is essential for function in a cellular context. Here we provide evidence that the isoxazolidine-KIX interaction is required for cellular activity. Consistent with this model, in the absence of DNA binding, isoxazolidine **1** is able to competitively inhibit transcription mediated by the KIX-dependent activators MLL and c-Jun. Based on natural peptide ligands for KIX, alteration of the aromatic side chain of **1** led to the identification of two molecules with enhanced potency, although increased cytotoxicity was also observed.

Results and Discussion

Cellular activity of **1** depends upon CBP

Previously we demonstrated that amphipathic isoxazolidine **1** activates transcription and interacts with the KIX domain of CBP, while **2**, bearing an alkene in place of the hydroxyl functional group at C3, does not activate nor does it interact with KIX.^{6c,13} To assess if CBP is required for **1**-mediated transcriptional activity, the impact of lowering CBP concentrations via shRNA knockdown was examined in the presence of **1** conjugated to the glucocorticoid receptor (GR) ligand OxDex (**1a**).¹⁶ In these experiments, cells were transfected with a luciferase reporter bearing five Gal4 binding sites and a plasmid encoding a fusion protein of the Gal4 DBD and the minimal ligand binding domain of GR in the presence of **1a**. In this two-hybrid assay, molecules tagged with the GR ligand OxDex will interact with the fusion protein and thus be localized to DNA.^{6b} The knockdown of CBP with shRNA resulted in an $86 \pm 8\%$ decrease (from 70-fold to 12-fold) in **1a**-driven luciferase expression, while a scrambled shRNA had no effect (see SI Figure S1). Additionally, the expression of exogenous CBP partially rescued the activity of **1a** (from 12- to 50-fold, see SI Figure S3). In contrast, increased CBP concentration had no impact on the ability of isoxazolidine **2a** to activate transcription, suggesting there is a direct link between CBP concentration and **1a**-driven activation. Additionally, a change in CBP concentration had no effect on expression of a luciferase gene driven by a CMV promoter that does not contain Gal4 binding sites (see SI Figure S2). Taken together, these data reveal that the activity of **1a** is directly impacted by CBP concentration in the cell.

Another common strategy for altering cellular CBP availability is to use the viral protein E1A to sequester it.¹⁷ As shown in Figure 2, there is an $80 \pm 3\%$ decrease (from 70-fold to 15-fold) in isoxazolidine **1a**-mediated activation when E1A is present. As outlined earlier, our previous *in vitro* studies revealed that **1b** interacts with the KIX domain in particular;^{6c} to further probe the importance of the isoxazolidine-KIX interaction for transcriptional activity, the activity experiments were carried out in the presence of over-expressed KIX domain and this led to a $60 \pm 3\%$ decrease (to 30-fold) in activation by **1a** (Figure 2). This is consistent with previously reported squelching experiments of Tax, a viral KIX-targeting activator, with a 50% decrease in Tax-driven transcription observed when KIX is over-expressed.¹⁸ These squelching data indicate that that KIX binding is critical for the transcriptional activity of **1a**. However, the inability of over-expressed KIX to completely abrogate the activity of **1a** suggests that other binding partners are likely also important.^{6c} In sum, modulation of transcriptional activity by affecting the availability of CBP supports the model that **1a** functions as a transcriptional activator through binding the KIX domain of CBP.

Inhibition of MLL and Jun

Although a number of natural TADs interact with the KIX domain,^{19,21,44,45} the isoxazolidine TADs were the first examples of small molecules that interact with the site utilized by the amphipathic TADs of MLL, Jun, Tat and Tax.^{6c} The overlapping binding footprint of **1b** with that of MLL and Jun should enable the small molecule to function as a competitive inhibitor of these activators. Towards this end, a series of activator competition studies were performed using proteins composed of the MLL and Jun TADs and the DBD of Gal4. Although **1b** is considerably smaller than the minimal TADs for MLL and Jun, it was able to inhibit the activation of both MLL and Jun in a dose-dependent manner (Figure 3a). The EC_{50} for inhibition of Jun by **1** is $19 \mu\text{M}$ and for inhibition of MLL it is $36 \mu\text{M}$. In addition, ESX, an activator that is not known to bind the KIX domain was not inhibited in a similar series of experiments. This is consistent with previous results performed in two ESX-driven cell lines where **1** exhibits inhibition only at high concentrations ($> 50 \mu\text{M}$).¹⁹

The previous reporter gene experiments demonstrated that **1b** inhibits the minimal TAD of Jun. It was unclear, however, if the isoxazolidine would function in a similar manner with the full-length Jun protein in an endogenous promoter context. To assess if inhibition of native Jun could be accomplished with isoxazolidine **1b**, we chose to examine expression of the cyclin D1 protein, a key component of the cell cycle machinery.²¹ Cyclin D1 expression is regulated by the AP-1 transcription factor that is a Jun homo- or a Jun-Fos heterodimer.^{12f,22} As illustrated in Figure 3b, treatment of Jun-expressing MCF-7 breast cancer cells with $40 \mu\text{M}$ **1b** (a concentration two-fold higher than the EC_{50}) led to a significant decrease in cyclin D1 expression. In the same time course, expression of Jun was minimally impacted while the control, GAPDH, was unchanged. Thus, isoxazolidine **1b** exerts an inhibitory effect on expression of a Jun-regulated gene not only in an artificial reporter system, but also in a native context. The demonstration of isoxazolidine **1b**-mediated inhibition of a Jun-regulated gene is a key step in developing a new chemical tool for probing the Jun pathway.

Inhibition of MLL and Jun with N2 Analogs

Although the results of Figure 3a illustrate that isoxazolidine **1b** serves as a transcriptional inhibitor, concentrations $>20 \mu\text{M}$ are required. For these molecules to serve as probes of KIX function, an increase in potency is highly desirable. Like a peptide, the isoxazolidine is a modular scaffold, amenable to functionalization at the N2, C3, C4, and C5 positions. Thus, to design new molecules with increased potency, we looked to natural KIX-binding peptides for functional groups. The sequences of several natural TADs that bind the MLL/Jun/Tat/Tax site of the KIX domain (Figure 4a) possess diverse amphipathic sequences. Whereas

Tax and Jun only possess aliphatic hydrophobic side chains, Tat and MLL TADs contain aromatic side chains from tryptophan and phenylalanine, respectively. These sequence differences may contribute to the range of dissociation constants observed for these activators (MLL = 2.5 μ M, Tat = 11 μ M, Jun = 30 μ M⁵⁴), though this has not been determined. In the case of the isoxazolidine scaffold, an isoxazolidine bearing a biphenyl substituent at the N2 position (**3**) does not bind the KIX domain or activate transcription; **6c**, **23** thus we used biphenyl as an upper limit on steric modification. Early in our studies, we observed that a smaller substituent, *p*-hydroxyphenyl (**4**), did not negatively impact KIX binding by ¹H-¹⁵N-HSQC binding experiments (SI Figure S4). Thus, to assess the functional impact of aromatic and aliphatic substituents at the N2 position of **1**, a small series of isoxazolidines was prepared bearing both functionality similar to that of natural activators (**4**, **7–9**) and small steric (**5**) and electronic (**6**) modifications.

While **1b** and **4** exhibit no measurable toxicity at concentrations \leq 50 μ M, the remaining analogs inhibited cell growth at concentrations above 10 μ M, limiting the range of concentrations that could be examined (see SI Figure S3). As expected, biphenyl **3** did not affect Jun-driven transcription; in addition, compounds **4**, **8**, and **9** exhibited little activity. However, as shown in Figure 4c and d, isoxazolidines **5**, **6** and **7** inhibited Jun transcriptional activity in a dose-dependent manner. Indeed, these three compounds are more effective than **1** (1.3–2 fold greater inhibition at 10 μ M). The levels of inhibition achieved by these molecules is higher for Jun than for MLL, perhaps a reflection of the >10 fold difference in the dissociation constants of these two activators (SI Figure S5).

Despite the improved activity of isoxazolidines **5–7** relative to **1**, none of the three exhibit sub-micromolar EC₅₀s; this highlights one of the challenges of designing small molecule transcriptional modulators, which possess a much smaller surface area relative to their peptidic counterparts.²⁴ While the isoxazolidine shares an overlapping binding surface with that of Jun and MLL, the peptidic ligands for KIX interact with a significantly larger area of the domain and make more intermolecular contacts that contribute both to binding affinity and specificity.^{6c,8b,8d} To more substantially increase potency, it may be necessary to utilize a small molecule framework that can incorporate additional functional groups for interaction with the KIX domain, such as dimeric isoxazolidines, for example.^{6c} In addition, we note that recent advances with cell-permeable, constrained peptides have shown promising results in inhibiting activators that interact with other domains of CBP.^{6f}

In summary, here we have demonstrated that the isoxazolidine transcriptional activation domain mimic **1** requires the KIX domain of CBP for cellular function and, consistent with this mechanism, **1** can competitively inhibit the ability of the native KIX-dependent activators Jun and MLL to function. Investigation of analogs of **1** led to the identification of two additional Jun and MLL-inhibitors with improved potency. These inhibitors will be useful tools for further dissecting the role of the KIX domain in a variety of physiological and pathological processes either alone or in combination with molecules that target other sites within CBP and/or its HAT activity. ^{5a,6e,25} The answers to these questions will further define the role of CBP in small molecule-mediated gene expression and provide a platform for development of new classes of small molecule transcriptional modulators.

Materials and Methods

Plasmids

The murine-CBP expression plasmid pRC/RSV-m CBP-HA26 and the 12S E1A expression plasmid, pBabe 12S E1A27 were purchased from Add gene. The pCI-KIX-NLS plasmid was constructed by PCR amplification of the KIX domain (residues 586-672) from the previously described pRSET-B-His6-KIX(586-672) expression plasmid by using the PCR

primers (5'-CTGTCGACGGTGTTCGAAAAGGCTGGCA-3' and 5'-CAAGGCGGCCGCTATAAACGTGACCTCCGC-3').^{6c} The PCR product was digested with *NotI* and *SalI* and ligated into the multiple cloning site of the pCI mammalian expression vector (Promega) using standard molecular biology techniques. The SV40 nuclear localization sequence (126-132) was subsequently incorporated into pCI-KIX plasmid C-terminal to the KIX domain via site-directed mutagenesis (Quik-change kit, Stratagene). Plasmid Gal4-MLL(2829-2883) was constructed by PCR amplification of the MLL minimal activation domain (residues 2829-2883)^{17d} from a full length MLL plasmid containing an N-terminal flag tag, (F-MLL, a gift from Dr. Jay Hess) using PCR primers (5'-GACTGGATCCCTGAAATCAGATTCAGACAATAAC-3' and 5'-CAAGGCGGCCGCAAGACCCAATCCTTCACCAAG-3'). The PCR product was digested with *BamHI* and *NotI* and ligated into the plasmid pCMV-Gal4 using standard molecular biology techniques. The plasmid pTKGG encoding the DNA binding domain of Gal4 fused to the minimal ligand binding domain of the glucocorticoid receptor was the generous gift of Dr. Tom Kodadek. The plasmid encoding the Jun TAD fused to the Gal4 DBD and the luciferase reporter plasmids, pG5luc and pRLSV40, were purchased from Promega. The CBP shRNA construct was prepared as previously described.¹⁶

Transcriptional inhibition assay

HeLa cells (10,000 per well) were transfected with luciferase reporter and *Renilla* control plasmids in addition to a plasmid encoding for either the MLL or Jun activation domain fused to the DNA-binding domain of Gal4. Compound was added four hours post-transfection and the luminescence was assessed after 24h as described above.

Western blot

HeLa cells (10000 per well) were lysed using passive lysis buffer (Promega) with Complete[®], EDTA-free protease inhibitors (Invitrogen) for 10 minutes at room temperature on an orbital shaker. Lysates were centrifuged at 14000 rpm for 20 minutes and the supernatant was mixed with 4× loading dye (Invitrogen) and BME (final concentration of 1%). The samples were then heated at 95°C for 10 minutes, separated by SDS-PAGE, transferred to a PVDF membrane and probed using standard conditions. Jun (sc-1694, 1:1000), cyclin D1 (sc-718, 1:1000), CBP (sc-7300, 1:500), and GAPDH (sc-47724, 1:2000) antibodies were all purchased from Santa Cruz Biotechnology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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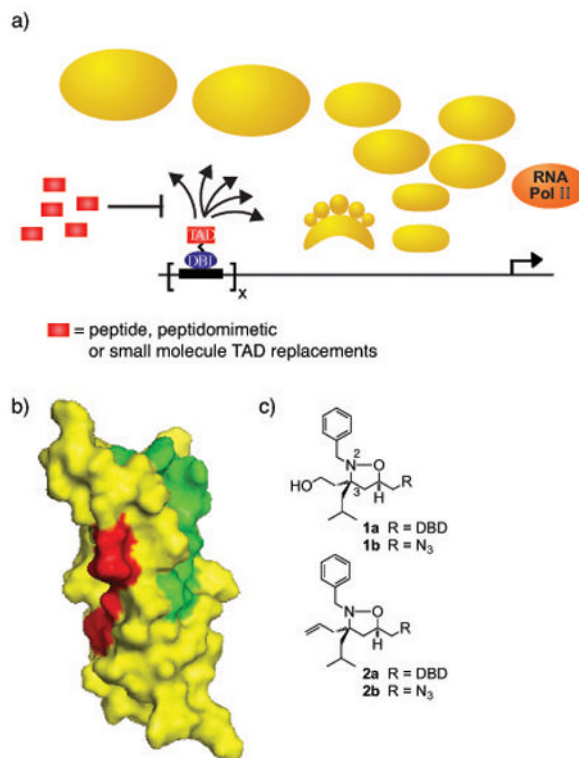


Figure 1.

a) Transcriptional activators, minimally comprised of a DNA-binding domain (DBD; blue oval) and a transcriptional activation domain (TAD; red rectangle), upregulate transcription by stimulating chromatin remodeling and facilitating the assembly of the RNA polymerase II holoenzyme at a promoter.¹⁴ The TAD is primarily responsible for the direct binding interactions with the transcriptional machinery (gold) in order to accomplish this. Potential binding partners of TADs within the transcriptional machinery include enzymes that modify chromatin, the proteasome, and/or coactivators. Small or large molecule mimics of TADs can serve as competitive inhibitors of activators, thus down-regulating transcription. b) The two activator-binding sites within the KIX domain of CBP/p300. Highlighted in green in the space-filling diagram of the KIX domain are the residues that experience the greatest chemical shift perturbation upon interacting with the TAD of MLL as measured by ^1H - ^{15}N -HSQC experiments;^{8b} the TADs of Tat, Tax and Jun occupy a similar binding site.^{8d-f} In red are the amino acids that change when interacting with Myb.¹⁵ Pymol figures were generated from 1 kdx. c) Isoxazolidine-based mimics of transcriptional activation domains. DBD = OxDex conjugated to an ethylene glycol linker (AEEA).¹³

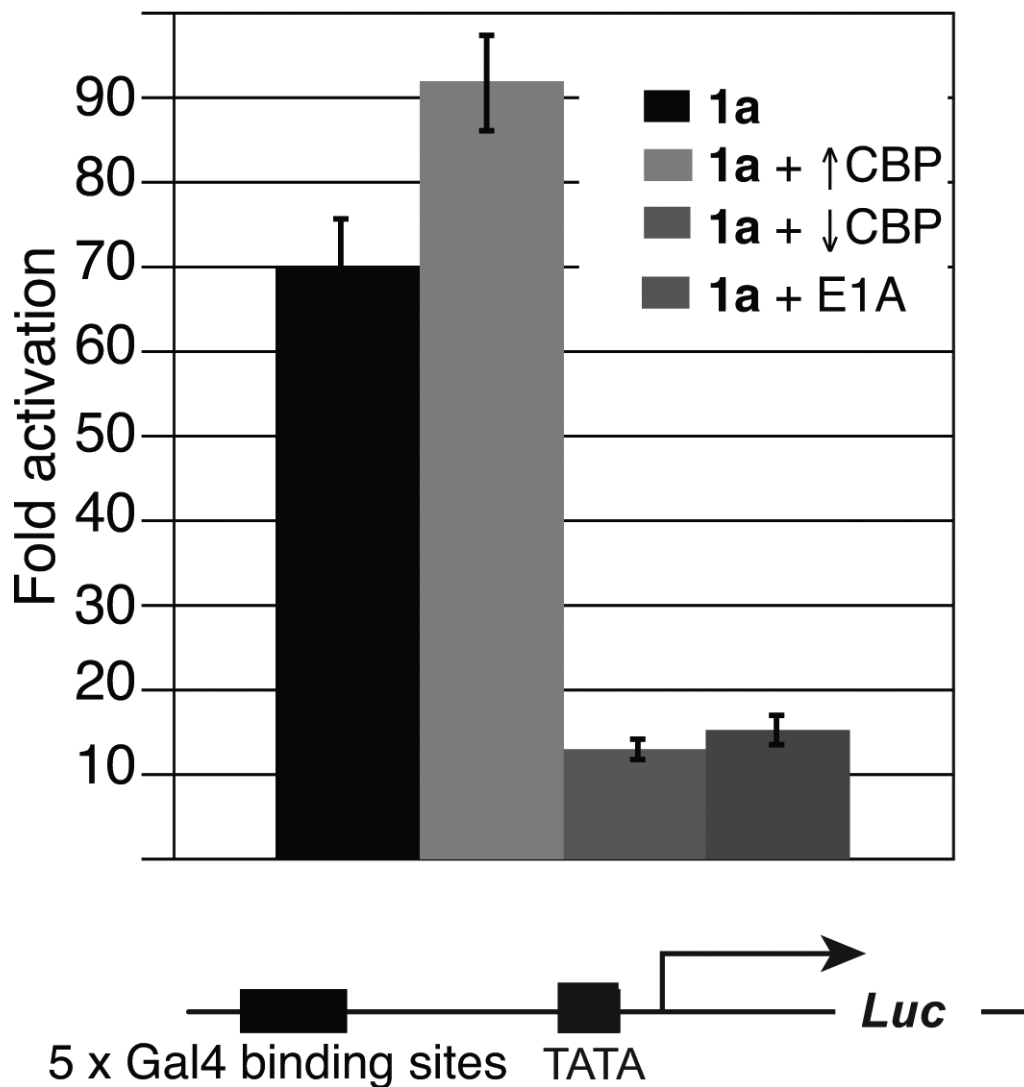


Figure 2. Isoxazolidine-driven gene expression requires CBP and the KIX domain

HeLa cells were transfected with a plasmid encoding the DBD of Gal4 fused to the ligand-binding domain of the glucocorticoid receptor, a firefly luciferase reporter plasmid containing five Gal4 binding sites, and a *Renilla* transfection control. Additional plasmids were transfected as indicated for CBP, CBP shRNA, KIX, and E1A experiments. Four hours post-transfection, 10 μ M **1a** was added as a solution in DMSO such that the final concentration of DMSO was 1% (v/v). Luciferase output was measured 24h after addition of compound; fold activation is a ratio of firefly and *Renilla* luminescence divided by the ratio of firefly and *Renilla* signal observed with DBD alone. Each value represents at least three individual experiments with the indicated error (standard deviation of the mean, SDOM).

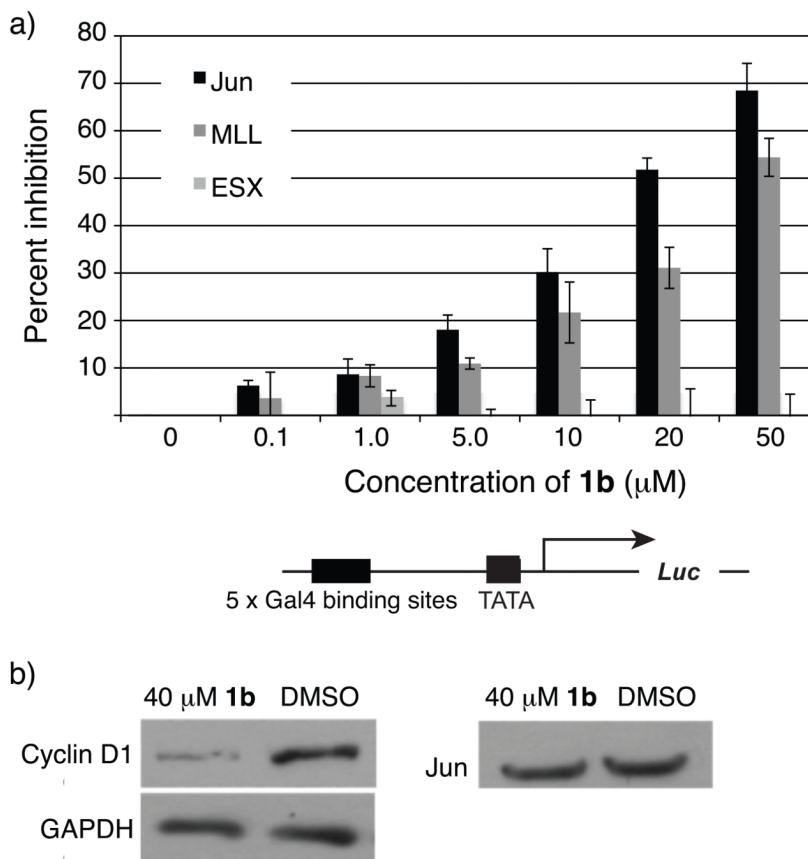


Figure 3. Inhibition of KIX-targeting TADs by 1b

a) Isoxazolidine **1b** decreased Jun and MLL minimal TAD-driven activation in a dose-dependent manner while not affecting ESX, an activator that is not known to bind the KIX domain. Cells were transfected with Firefly and Renilla luciferase plasmids in addition to plasmids encoding the DBD of Gal4 fused to the minimal activation domains of either MLL, Jun, or ESX. Four hours post-transfection cells were treated with increasing concentration of **1b** (0 \rightarrow 50 μM) as a solution in DMSO such that the final DMSO concentration was 1% (v/v). Percent inhibition is the ratio of fold activation of Jun at each concentration of compound and the fold activation of Jun in a DMSO-treated control. Each bar represents at least three independent experiments with the indicated error (SDOM). b) MCF-7 cells were treated with 40 μM **1b** (as a solution in DMSO such that the final concentration of DMSO was 1% v/v) for 24 h, at which time the cells were lysed and the lysates separated by SDS-PAGE. Western blots were performed using commercial antibodies against GAPDH, Jun, and cyclin D1.

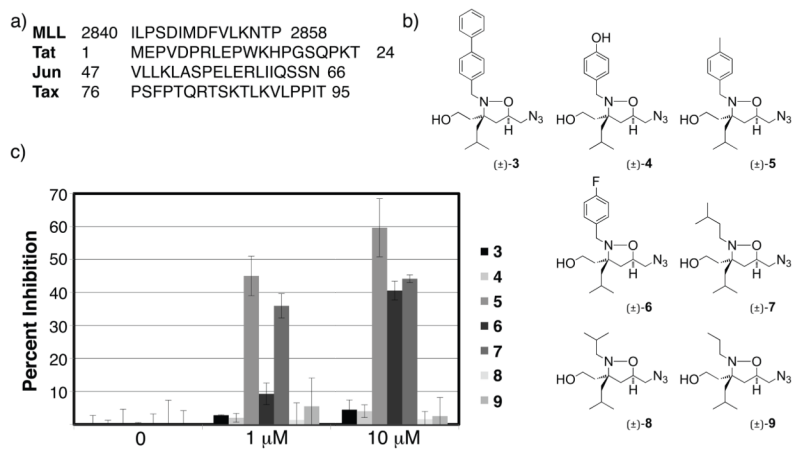


Figure 4.

a) Sequences of KIX-binding TADs. b) Analogs of **1b** tested for MLL and Jun inhibition; see SI for complete details of synthesis and characterization. c) Inhibition of Jun with isoxazolidine analogs. Inhibition assays were carried out as described in Figure 3a.