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4,5-Disubstituted oxazolidinones: High affinity molecular effectors of RNA function

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ABSTRACT

The T box transcription antitermination system is a riboswitch found primarily in Gram-positive bacteria which monitors the aminoacylation of the cognate tRNA and regulates a variety of amino acid-related genes. Novel 4,5-disubstituted oxazolidinones were identified as high affinity RNA molecular effectors that modulate the transcription antitermination function of the T box riboswitch.

Identifying RNA ligands that modulate transcription regulation is an important area for drug discovery that has been only minimally explored to date. One potential therapeutic target is the T box transcription antitermination mechanism. This mechanism regulates many amino acid-related genes, including aminoacyl-tRNA synthetase genes, and is found predominantly in Gram-positive bacteria.[1] The T box RNAs are members of the 'riboswitch' family in which nascent RNAs directly sense effector molecules to control gene expression.[2–4] The T box genes contain a complex set of structural elements within the 5′ untranslated region of their mRNAs (the 'leader region'). These elements include a transcription termination signal that abrogates synthesis of the full-length mRNA and a competing antiterminator element. Read-through of the terminator, and expression of the downstream gene, is dependent on binding of a specific uncharged tRNA to the nascent RNA transcript; each gene in the T box family responds independently to the cognate uncharged tRNA.[5] The T box antitermination mechanism can function in the absence of additional cellular factors,[6] and the antiterminator RNA element is a critical component of the mechanism.[5] The leader RNA–tRNA interaction stabilizes the antiterminator element, thereby preventing formation of the competing terminator element (Fig. 1). The antiterminator element is highly conserved and has been extensively characterized by genetic, biochemical, and structural biology approaches.[7–9]

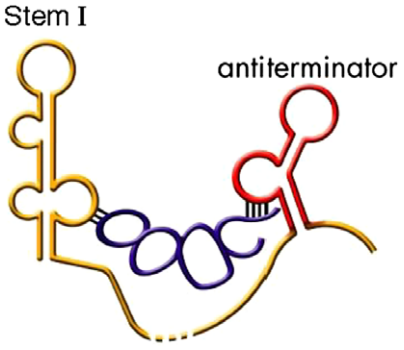

Figure 1. Schematic of tRNA (blue) binding to T box leader RNA. The tRNA anticodon loop base pairs with the Specifier Sequence in Stem I while the tRNA acceptor end base pairs with nucleotides in the antiterminator (red).[1]

A significant challenge in rational ligand design for RNA structure-specific binding is to achieve both high affinity and excellent tertiary structure specificity. Aminoglycosides, the most widely studied RNA ligands, bind primarily in divalent cation binding sites.[10–12] The electrostatic attraction between the multiple protonated amino groups and the negatively charged RNA phosphate backbone leads to very high affinities. However, due to the ubiquitous presence of divalent cation binding sites in RNA, primarily for tertiary fold stabilization,[13] the aminoglycosides readily bind many RNAs[14] thus reducing their utility for RNA structure-specific ligand

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design. A variety of other RNA ligands have been investigated,[15–21] but few have met the challenge of achieving high specificity for unique RNA structures and high affinity without relying on significant electrostatic interactions. A preliminary investigation of 4,5-disubstituted oxazolidinones[22] demonstrated that these ligands may have the potential for specific structural recognition of T box antiterminator RNA.

Functionally relevant models of the T box antiterminator element have been developed.[8] AM1A is a model of the wild-type antiterminator and is fully functional in vivo in the context of the full-length leader sequence.[7] C11U is a reduced function variant; the corresponding C to U change in the full T box leader sequence results in a significant decrease in antitermination efficiency in vivo[23] and in vitro (N.G. and T.H., unpublished). The C11U model RNA exhibits reduced affinity for tRNA in vitro, indicating a strong correlation between structural recognition in the tRNA complex in vitro and antitermination efficiency.[8,24] The reduction in activity and affinity along with evidence for structural differences compared to AM1A[9] make C11U an excellent specificity control.

We recently reported a novel class of 4,5-disubstituted oxazolidinones (**1**, Fig. 2)[25] that bind RNA without extensive reliance on electrostatic interactions.[22] These compounds exhibited good binding specificity and affinity for AM1A compared to C11U, but they have relatively poor water solubility. We sought a method to improve water solubility and retain the affinity and specificity previously observed. Our solution was to link a series of alkyl/aryl groups to the oxazolidinone ring via a basic amine (e.g., **2**). We predicted that this could be accomplished via the reaction of aziridine **3** with an appropriate amine (Scheme 1). Reaction of aziridine **3**[25] with a secondary amine provided oxazolidinones **4a**–**4d** in good yields. A small group of amines was initially chosen. As our initial lead compound had a simple phenyl ring in place of the NR_2 group, we wanted to include aromatic rings on the amine. This group of amines includes *N*-methyl aniline, *N*-methyl phenethyl amine, *N*-phenyl piperazine, and a single non-aryl substituted amine, morpholine. Reaction of **4** with an excess of an acid chloride provided compounds **2a**, **2b**, **2c**, **2e**, and **2f** directly.[26] For the majority of these compounds we chose R′ = $PhCH_2$ as this was the optimal ester in our previous lead compound. We chose two other R′ groups, and one R′ = nC_7H_{15} was linked to the *N*-methyl aniline derivative **2c** to provide another direct analog of **1**. We also included a carbamate in place of the ester to improve water solubility and potentially provide additional sites for non-covalent contacts with the RNA target. This compound (**2d**) was readily prepared by treatment of **4b** with TFA, followed by neutralization and reaction with 4-acetylphenylisocyanate. All compounds were converted to their hydrochloride salts prior to biological evaluation.

These compounds differ significantly in substitution pattern from other known oxazolidinone RNA ligands (e.g., 3,5-disubstituted oxazolidinones such as the antibiotic linezolid).[27–29] While there have been structure–activity relationship studies of the antibacterial activity of linezolid analogs,[30–34] little has been reported regarding RNA recognition.

RNAs 3′-Fl-18-Rh-AM1A and 3′-Fl-18-Rh-C11U were prepared and a fluorescence resonance energy transfer (FRET) binding assay[22,35] was performed (Table 1). Compounds **2a** and **2b** are the closest structurally to **1** with only an amine inserted between the oxazolidinone and the phenyl ring. These two compounds differ at the ester substitution, with **2a** retaining the phenyl acetate ester of **1** and **2b** having an octanoate ester. Compound **2a** shows similar affinity to C11U as **1** while showing somewhat worse affinity to AM1A. Compound **2b** shows excellent and roughly equal affinity to both AM1A and C11U. The phenylpiperazine substituted oxazolidinones (**2c**, **2d**) both show excellent affinity to AM1A. Oxazolidinones **2b**, **2c**, and **2d** bound T box antiterminator model RNA with low micromolar to nanomolar K_d values. These K_d values rival those of the aminoglycosides for binding to AM1A (neomycin K_d = 8 μM).[35] This high affinity without extensive reliance on electrostatic attraction (only two protonatable amines vs. six for neomycin) points to the importance of non-electrostatic interactions in the binding of oxazolidinones to RNA. Compound **2c** bound to AM1A with 8-fold greater affinity than C11U, while **2d** bound to both model RNAs with roughly similar affinity. While structural differences between **2c** and **2d** are minimal, the carbamate moiety of **2d** may provide both additional hydrogen bonding capabilities and restricted rotation around the C–N bond relative to **2c**. Compound **2e** in which the ring of the *N*-substitution is an *N*-methyl phenethyl amine can be viewed as a conformationally less constrained *N*-phenyl piperazine. This compound showed substantially less affinity to AM1A relative to either **2c** or **2d**. Compound **2f** in which the aromatic ring on the amine is completely gone shows reasonable affinity to AM1A. Given the good affinity and selectivity of **2c** and the good affinity and poor selectivity of **2d**,

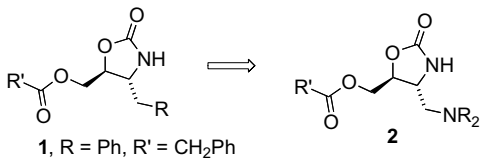
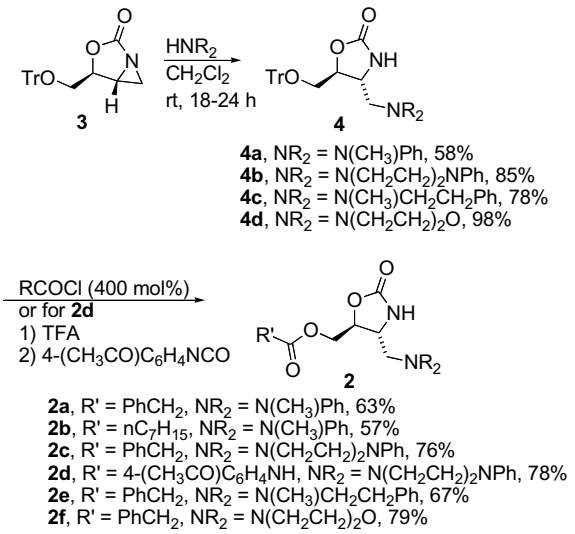


Figure 2. Previously prepared oxazolidinone RNA binding agents **1**, and proposed new analogs **2**.



Scheme 1. Synthesis of amine substituted oxazolidinones **2a**–**2f**.

Table 1
Oxazolidinone affinity for antiterminator model RNA[a]

Compound	AM1A K_d	C11U K_d
1[b]	9 (±4.5)	125 (±9)
2a	61 (±15)	122 (±18)
2b	6.6 (±1.7)	4.1 (±1.1)
2c	13 (±4)	100 (±30)
2d	0.9 (±0.4)	<1[c]
2e	114 (±31)	NT
2f	42 (±6)	NT

[a] K_d values (μM) determined using FRET-derived binding assay with 100 nM labeled RNA.[22] All R^2 values > 0.9 unless otherwise noted. NT, not tested.
[b] Data from Ref. 22.
[c] Poor fit for single- and two-site binding; K_d below detection limits of assay.

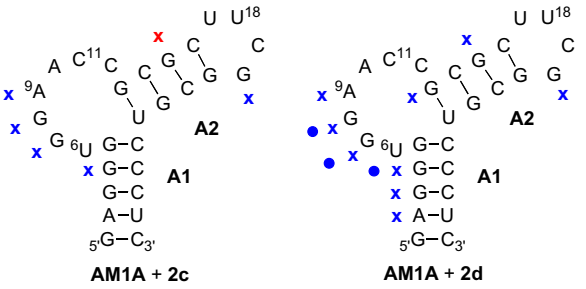


Figure 3. Summary of enzymatic probing assays of AM1A in the presence of **2c** and **2d** with RNase T1 (×) and RNase A (circle) indicating enhanced (red) or reduced (blue) cleavage in the presence of the ligand.

we chose to further examine both of these compounds in further studies. These two molecules differ only in the acyl substitution suggesting that significant non-covalent contacts are mediated through this substituent.

With the FRET-labeled antiterminator RNA used in the binding studies, the maximal change in relative fluorescence (F_{rel}) correlates with structure-specific conformational changes in the RNA induced upon ligand binding.[35] There was a significant difference in the maximal F_{rel} for **2c** binding to AM1A (max F_{rel} = 0.2) compared to **2d** (max F_{rel} = 0.3), strongly indicating that the two oxazolidinones bind AM1A in different manners.

Further evidence for different binding modes was observed in enzymatic cleavage assay patterns (Fig. 3). No significant change in the RNase A cleavage of AM1A was observed in the presence of oxazolidinone **2c**. However, in the presence of **2d** the relative band intensities decreased for positions 6 (37%), 7 (69%), and 8 (47%). With RNase T1 cleavage of AM1A in the presence of **2c**, there was enhanced cleavage at position 15 (70%) and reduced cleavage in several locations, most notably positions 7 (44%), 8 (41%), and 9 (57%). With **2d**, however, there was an overall decrease in RNase T1 enzymatic cleavage. The differential enzymatic cleavage patterns of **2c** compared to **2d** may be due to different RNA binding modes.

The effect of **2c** and **2d** on antiterminator RNA function was tested using an in vitro transcription antitermination system. Transcription of the *Bacillus subtilis glyQS* T box gene with *B. subtilis* RNA polymerase in the absence of tRNA resulted in efficient termination (Fig. 4); addition of uncharged $tRNA^{Gly}$ resulted in a 20-fold increase in read-through of the termination site, as previously reported.[6] Addition of **2c** (1.3 mM) caused a 40% reduction in the efficiency of $tRNA^{Gly}$-dependent antitermination, but had no effect on transcription of a non-T box DNA template (data not shown). The reduction in antitermination is likely to be due to competitive inhibition since the affinity of **2c** for binding to AM1A decreased 5-fold in the presence of cognate tRNA (1.25 μM) to a K_d of

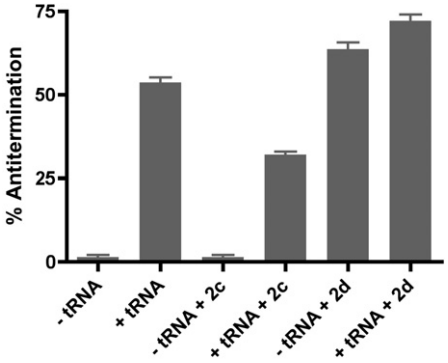


Figure 4. Effect of **2c** and **2d** on *B. subtilis glyQS* antitermination activity in vitro (n = 3).

64 ± 16 μM (R^2 = 0.9). In contrast, **2d** (0.8 mM) resulted in increased antitermination in the presence or absence of tRNA, suggesting that this compound stabilizes the antiterminator element, obviating the requirement for tRNA binding. The different effects of **2c** and **2d** on antitermination are consistent with different modes of interaction with the antiterminator model RNAs. The relatively high concentration of **2c** required to compete with the acceptor end of tRNA for binding the antiterminator and to inhibit antitermination is reasonable given the strong tRNA-model antiterminator RNA affinity (K_d = 0.02 μM)[24] and given that the tRNA can pre-bind the leader at the Specifier Sequence before the antiterminator is transcribed,[36] thus likely further enhancing affinity in the context of the in vitro transcription antitermination assay.

In this report, we have identified 4,5-disubstituted oxazolidinones that both bind to the T box antiterminator RNA element and directly affect antitermination. These compounds are amine substituted analogs of previously reported oxazolidinones. The inclusion of the basic amine in the compound significantly enhances the affinity. The affinity and structural specificity was sufficiently high that **2c** acted as an inhibitory molecular effector and disrupted in vitro antitermination. Compound **2d** acted as an enhancing molecular effector leading to tRNA-independent antitermination in vitro. The differing binding modes of **2c** and **2d** with antiterminator RNA provides strong evidence that rational drug design strategies can be used to selectively develop high affinity ligands (rivaling the nM affinity observed with **2d**) that retain the antitermination inhibitory activity of **2c**.

Acknowledgments

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Supplementary data

Representative binding isotherms and enzymatic probing data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.015.

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