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Structure–activity studies of oxazolidinone analogs as RNA-binding agents

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Abstract—We have synthesized and tested a series of novel 3,4,5-tri- and 4,5-disubstituted oxazolidinones for their ability to bind two structurally related T box antiterminator model RNAs. We have found that optimal binding selectivity is found in a small group of 4,5-disubstituted oxazolidinones.

A variety of ligands, including both organic and inorganic compounds, intercalators and non-intercalators, are known to bind RNA.[1,2] While some interact with very specific RNA structural features, many RNA-binding ligands have only minimal selectivity due to a reliance on electrostatic binding interactions.[1,3–10] Simple polycationic compounds with limited conformational flexibility preferentially bind RNA over DNA via major groove recognition.[11] Cationic diphenyl furans recognize specific shapes of RNA.[12] Phenothiazine derivatives bind irregular tertiary RNA folds.[13] Larger antibiotics such as the macrolide erythromycin interact specifically with bacterial 23S rRNA.[14] Aminoglycosides are the best studied class of small molecules that bind RNA. Binding involves specific shape recognition as well as interaction with cationic binding sites.[3,15,16] The enhanced RNA affinity of modified aminoglycosides when additional amino[17] or guanidino[18] groups are added illustrates the importance of electrostatic interactions.

While electrostatic attraction plays a major role in ligand binding, there are examples of RNA ligands where hydrogen bonding, in the absence of significant electrostatic attraction, plays a critical role in oligonucleotide recognition.[19–21] Non-ionic regions of RNA ligands have also been shown to be important for conferring binding specificity.[22] Recent microbial product screens have led to the discovery of nonionic small molecules, oxopolyenes, that bind specific RNAs.[23,24]

Oxazolidinones are an additional class of less-highly charged compounds that have been found to bind RNA. In particular, the 3,5-disubstituted oxazolidinones interact specifically with rRNA, forming unique contacts in domain V of 23S rRNA.[25–30] Oxazolidinone binding leads to inhibition of translation at the initiation stage of protein synthesis.[31] Little is known about the structure–activity relationships of the binding of oxazolidinones to RNA.

The challenge of RNA-targeted drug discovery is to develop ligands with high affinity and high specificity that can distinguish between closely related RNA tertiary structures. To address this challenge, a series of oxazolidinones was prepared and the oxazolidinones' selective affinities for binding two structurally related T box antiterminator model RNAs were investigated.

The T box antiterminator RNA is a key component of the T box transcription antitermination regulatory element found in many Gram-positive bacteria.[32] Over 500 genes, primarily encoding aminoacyl-tRNA synthetases and amino acid biosynthetic enzymes, have been identified that are regulated by this mechanism.[33] The 5′ untranslated region (5′ UTR) of the T box genes is characterized by conserved primary and secondary structures including the 14-nucleotide region called the T box.[34] The formation of two alternative secondary structures, terminator and antiterminator, in this region

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controls the expression of the gene. The molecular effector that determines which structure forms is uncharged cognate tRNA. The tRNA interacts with the 5′ UTR in at least two different places. Base-pairing of the tRNA anticodon with a codon sequence (the 'Specifier Sequence') positioned at a specific location in the 5′ UTR of the gene provides the specificity to the antitermination mechanism.[32] Base-pairing of four nucleotides of the acceptor stem of the tRNA with complementary nucleotides in a seven-nucleotide bulge within the antiterminator allows the system to respond specifically to uncharged tRNA.[35] The interaction of the cognate tRNA with the 5′ UTR can occur in the absence of additional cofactors[36] and is highly sensitive to minor sequence changes within the antiterminator bulge.[37] This regulatory system is very unique and recent aminoglycoside binding studies indicate the T box antiterminator is a potential target for specific ligand binding.[38]

We had previously reported on the synthesis of a small library of 3,4,5-trisubstituted oxazolidinones (**4**).[39] We wished to examine the RNA binding ability of this class of oxazolidinones in order to begin to develop a structure–activity relationship on oxazolidinone-RNA binding. We initiated the synthesis of these compounds by reaction of aziridine **1** with a series of 3 organocuprate reagents to provide oxazolidinone **2**. The N–H of the oxazolidinone was alkylated or arylated to provide a second group of di-substituted oxazolidinones. The trityl group was concomitantly removed and the resulting alcohol acylated[40] to provide the library of tri-substituted oxazolidinones **4** (Table 1).

The two antiterminator model RNAs used for the oxazolidinone binding studies were based on the wildtype model (Fig. 1), AM1A, and a reduced function model, AM1A(C11U).[41] The design of the model RNAs was based on in vivo mutational and phylogenetic studies.[37,42] The antiterminator is composed of two helices that flank a seven-nucleotide bulge. The first four bases of the bulge (corresponding to positions 6–9 in AM1A)

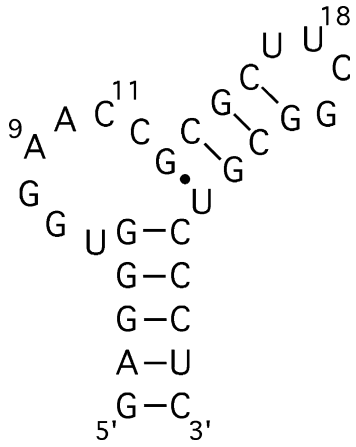


Figure 1. Model RNA AM1A.

Table 1. Oxazolidinones prepared and normalized 9-Ap-F_{rel} values

Compound	R^1	R^2	R^3	AM1A[a]	AM1A(C11U)[a]
4a	Ph	2-(CO_2Me)Ph	Bn	1.00	1.47
4b	Ph	2-(CO_2Me)Ph	cC_6H_{11}	0.64	1.19
4c	Ph	2-(CO_2Me)Ph	nC_7H_{15}	1.19	1.06
4d	Ph	4-(OMe)Ph	Bn	0.81	0.23
4e	Ph	4-(OMe)Ph	cC_6H_{11}	0.73	0.77
4f	Ph	4-(OMe)Ph	nC_7H_{15}	0.43	0.68
4g	Ph	nC_4H_9	Bn	0.48	0.36
4h	Ph	nC_4H_9	cC_6H_{11}	0.48	0.59
4i	Ph	nC_4H_9	nC_7H_{15}	1.16	1.37
4j	$PhCH_2CH_2$	2-(CO_2Me)Ph	Bn	0.79	1.12
4k	$PhCH_2CH_2$	2-(CO_2Me)Ph	cC_6H_{11}	0.71	0.78
4l	$PhCH_2CH_2$	2-(CO_2Me)Ph	nC_7H_{15}	1.21	0.76
4m	$PhCH_2CH_2$	4-(OMe)Ph	Bn	0.75	1.00
4n	$PhCH_2CH_2$	4-(OMe)Ph	cC_6H_{11}	0.64	0.70
4o	$PhCH_2CH_2$	4-(OMe)Ph	nC_7H_{15}	0.65	0.72
4p	$PhCH_2CH_2$	nC_4H_9	Bn	0.72	0.70
4q	$PhCH_2CH_2$	nC_4H_9	cC_6H_{11}	0.65	0.78
4r	$PhCH_2CH_2$	nC_4H_9	nC_7H_{15}	0.56	0.69
4s	cC_6H_{11}	2-(CO_2Me)Ph	Bn	0.39	0.77
4t	cC_6H_{11}	2-(CO_2Me)Ph	cC_6H_{11}	0.61	0.80
4u	cC_6H_{11}	2-(CO_2Me)Ph	nC_7H_{15}	0.47	0.97
4v	cC_6H_{11}	4-(OMe)Ph	Bn	0.71	1.05
4w	cC_6H_{11}	4-(OMe)Ph	cC_6H_{11}	0.61	0.82
4x	cC_6H_{11}	4-(OMe)Ph	nC_7H_{15}	0.58	0.78
4y	cC_6H_{11}	nC_4H_9	Bn	0.58	0.78
4z	cC_6H_{11}	nC_4H_9	cC_6H_{11}	0.54	0.70
4aa	cC_6H_{11}	nC_4H_9	nC_7H_{15}	0.73	1.05

[a] Normalized 9-Ap-F_{rel} values arbitrarily set to 1 for **4a** with AM1A, where 9-Ap-F_{rel} = $F_{(9ApRNA+ligand)}$ − $F_{(ligand)}$. All other values relative to **4a** with AM1A. Assays contained 100 nM 9-Ap-RNA in 25 mM Tris–borate, pH 8, 0.5 mM EDTA, 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM ligand. Data were collected using a JY Horiba SPEX Fluoromax-2 Spectrofluorometer.

base pair with the acceptor end nucleotides of the tRNA. The exact function of the remaining, highly conserved, nucleotides remains unknown. The NMR solution structure of the AM1A model RNA indicates that the seven-nucleotide bulge introduces a bend in the structure, and that the C11U substitution in the bulge alters the structure in this part of the molecule.[43]

A sensitive method for monitoring ligand binding to RNA is to utilize 2-aminopurine fluorescently labeled RNA. In AM1A the 3′ end of the bulge nucleotides, A9 through C12, is involved in extensive stacking and the 2 position of A9 projects into an aromatic lined pocket.[43] Consequently, a 2-aminopurine base at position 9 would likely experience an environment change upon ligand binding resulting in significant, detectable changes in the fluorescence emission. The 2-aminopurine base has been used in studying other RNA–ligand interactions and acts as a replacement for A, including the ability to base pair with U.[44–48] The appropriately 2-aminopurine labeled model RNAs (9-Ap-AM1A, 9-Ap-AM1A(C11U)) were purchased as purified material (Dharmacon) and dialyzed prior to use.

In preliminary screens of ligand binding to 9-Ap-AM1A and 9-Ap-AM1A(C11U), addition of ligand led in all cases to an enhancement in fluorescence (data not shown). The extent of enhancement varied depending on the ligand and RNA model (see normalized F_{rel} in Table 1). The relative fluorescence ratio is summarized in Figure 2 to highlight selectivity differences of the compounds for the two different antiterminator model RNAs.

Most compounds showed no significant difference in binding between AM1A and AM1A(C11U); however, there were a few notable exceptions. Compounds **4b**, **4s**, and **4u** exhibited a 2-fold preference for AM1A-(C11U) relative to AM1A (Table 1 and Fig. 2). Structurally the primary common elements are the 2-carbomethoxyphenyl group at the nitrogen of the oxazolidinone and a cyclohexyl group at either R^1 or R^3 (but not both). Compounds **4d** and **4l** showed a preference for AM1A over AM1A(C11U), with that preference being most notable (close to 4-fold) for compound **4d**.

Titration experiments of the 9-Ap-RNA with **4d** confirmed the qualitative results of the 1 mM screen (Fig. 3) in that a significant signal enhancement was observed for AM1A compared to little change for AM1A-(C11U). However, the concomitant red shift in the emission maximum from 365 to 375 nm upon addition of **4d** precluded using a detailed ligand titration of 9-Ap-AM1A to determine K_d's. The emission maximum of 2-aminopurine can be affected by local environment polarity.[49,50] The red shift in the emission profile along with the enhancement are strong indicators that **4d** is binding in the bulge region affecting the local RNA environment (e.g., base stacking). A fluorescence resonance energy transfer (FRET)[44–47] antiterminator model system was used instead to determine detailed binding constants.[38]

The FRET-labeled model RNAs contained a fluorescein (the donor fluorophore) on the 3′ end and a rhodamine (the acceptor fluorophore) on U18 in the UUCG loop. The precursor 3′-Fl-18-(2′-amino)-AM1A (or AM1A-

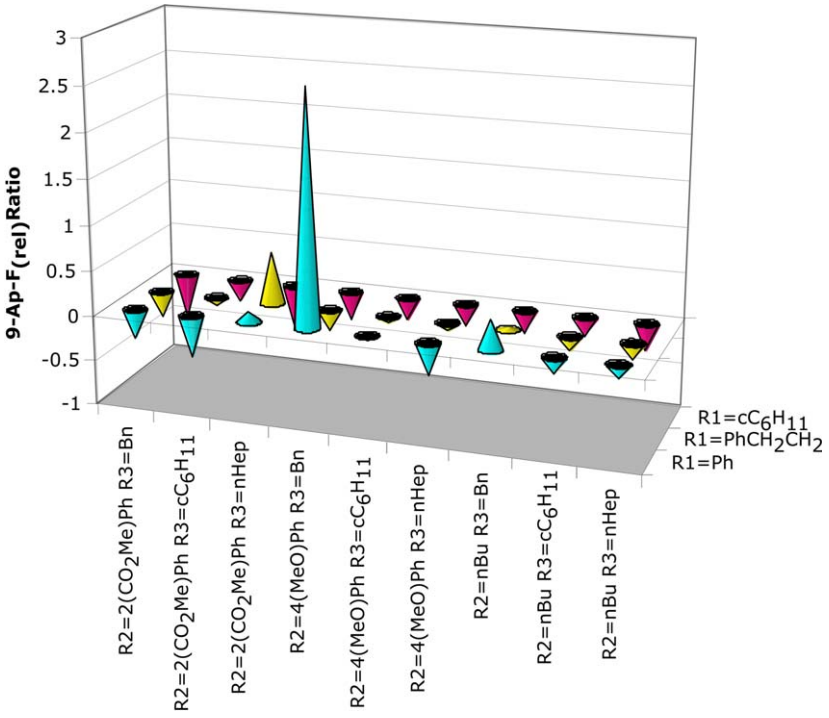


Figure 2. Relative fluorescence ratio of compounds with AM1A versus AM1A(C11U) highlighting selectivity differences where 9-Ap-F_{rel} ratio = (9-Ap-$F_{rel(AM1A)}$/9-Ap-$F_{rel(AM1A(C11U))}$) − 1. Positive values correspond to preferential binding effect for 9-Ap-AM1A, while negative values correspond to preferential binding effect for 9-Ap-AM1A(C11U). See Table 1 for conditions.

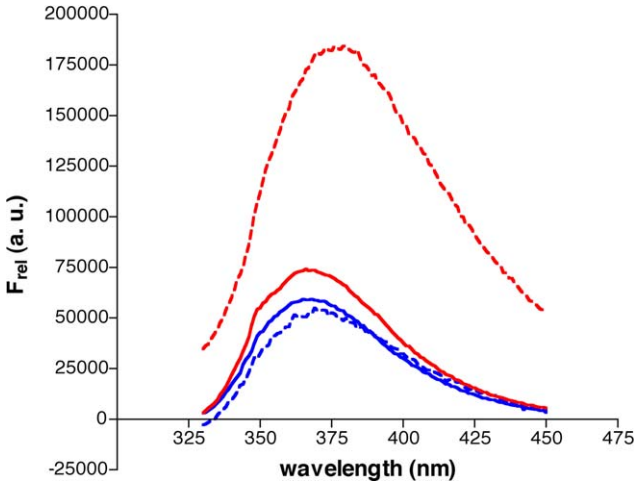
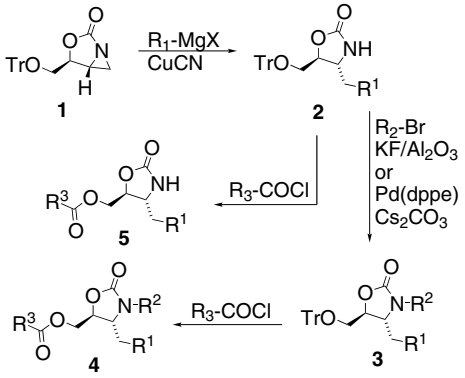


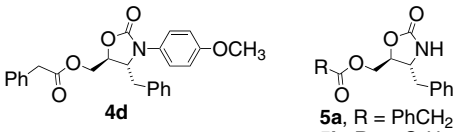
Figure 3. Fluorescence emission spectra for **4d** added to 9-Ap-RNA. Concentrations are 0 (solid) and 40 (dashed) μM **4d** for binding to 9-Ap-AM1A (red) and 9-Ap-AM1A(C11U) (blue), where $F_{rel} = [F_{(9ApRNA+drug)} - F_{(RNA+drug)}]$ in 25 mM Tris–borate, pH 8, 0.5 mM EDTA, 50 mM NaCl, and 5 mM $MgCl_2$.

(C11U)) was purchased (Dharmacon) and labeled with the *N*-hydroxysuccinimide ester of carboxytetramethylrhodamine using an established protocol[51] to prepare the FRET-labeled model RNAs 3′-Fl-AM1A-Rhd and 3′-Fl-AM1A(C11U)-Rhd. Ligand binding-induced changes in the bend angle between the helices flanking the bulged nucleotides result in changes in the FRET observed and can readily be used to determine K_d's.[38]

Since the initial ligand binding assay indicated preferential binding of **4d** to AM1A compared to AM1A(C11U), we prepared three simpler analogs of **4d** in which the nitrogen substituent was replaced with an H. Modification of the nitrogen substituent of related oxazolidinone antibacterial agents (e.g., linezolid) has a significant effect on antibacterial activity.[52–55] Consequently, compounds **5a**–**5c** were prepared as shown in Scheme 1, except that the nitrogen alkylation step was omitted.[56] Detailed K_d determinations were done using the FRET-labeled antiterminator RNA and compared to the preliminary lead compound **4d** (Fig. 4).[57]



Scheme 1.



	AM1A K_d (μM)	AM1A(C11U) K_d (μM)
4d	3.4 ±1.9	25 ±10
5a	9 ± 4.5	125 ± 9
5b	NB[a]	NB[a]
5c	10 ± 2.5	14.3, 49.1[b]

Figure 4. Structures and K_d values of selected oxazolidinones. [a]No binding detected up to 200 μM. [b]Data best fit two site binding, both K_d values reported.

The detailed K_d determination for **4d** is consistent with the preliminary aminopurine screens in that **4d** bound AM1A more tightly (by 7-fold) than AM1A(C11U). This selectivity is significant because the two RNAs are structurally very similar.[43] The selectivity was further enhanced in **5a** to a 14-fold preference for binding AM1A. No change in the FRET was observed for **5b**, indicating either that this analog does not bind at the concentrations investigated or that the binding is FRET-silent and does not induce a significant change in factors affecting the FRET efficiency (e.g., the distance between the donor and acceptor fluorophore). The low micromolar K_d for **5a** along with the significant RNA selectivity indicate that oxazolidinones can potentially be developed for very high affinity, high specificity binding to complex RNA targets.

It is evident that simple modifications to the oxazolidinone nucleus can have profound effects upon the binding and selectivity of oxazolidinones binding to RNA. Compounds **4d**, **5a**, and **5c** exhibit affinities among the highest of any reported non-electrostatic RNA-binding ligands. The discovery of simpler oxazolidinone analogs (compounds **5a** and **5c**) should enhance our ability to determine the SAR for small molecule·RNA binding.

Acknowledgments

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56. Typical procedure for the conversion of **2**–**5**. A solution of **2** (0.2 g, 0.45 mmol) was dissolved in CH_2Cl_2 (1.0 mL), phenylacetyl chloride (0.22 mL, 1.65 mmol) was added, and the reaction was stirred at rt for 24 h. The reaction was diluted with CH_2Cl_2 (10 mL), washed with saturated aq. $NaHCO_3$, brine, and dried over $MgSO_4$. After filtration and concentration, the crude product was purified by chromatography (35% EtOAc:hexanes) to provide 111 mg of **5a**, 76%. ^1H NMR (250 MHz, $CDCl_3$): δ 7.37–7.24 (8H, m), 7.05 (2H, dd, $J = 8.0$, 2.1 Hz), 5.46 (1H, br s), 4.45 (1H, q, 5.7 Hz), 4.16–4.08 (2H, m), 3.75–3.65 (1 H, m), 3.61 (2H, s), 2.80 (2H, d, $J = 9.3$ Hz); ^{13}C NMR: δ 171.0, 157.8, 135.3, 133.4, 129.2, 129.0, 129.0, 128.7, 127.4, 127.3, 78.4, 63.9, 55.7, 41.4, 41.0.
57. Binding assays consisted of 50 mM sodium phosphate, pH 6.5, 5 mM $MgCl_2$, 50 mM NaCl, and 0.01 mM EDTA with 100 nM of either 3′-Fl-AM1A-Rhd or 3′-Fl-AM1A (C11U)-Rhd with increasing concentration of the small molecules. Data were collected 10 min after ligand addition except for **5c** with AM1A(C11U), which was at 120 min. The K_d values were determined by plotting FRET F_{rel} versus ligand concentration where FRET $F_{rel} = |F - F_0|/F_0$. The F and F_0 were the fluorescence at 585 nm of the FRET-labeled RNA with and without ligand, respectively. All binding experiments were done in duplicate and averaged during analysis in Prism (GraphPad). Data were collected using a Molecular Devices FlexStation 96-well array spectrofluorometer.