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Anisotropy studies of tRNA-T box antiterminator RNA complex in the presence of 1,4-disubstituted 1,2,3-triazoles

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Abstract

The binding of tRNA to the T box antiterminator RNA element is a critical component of the T box riboswitch mechanism that regulates essential genes in many Gram-positive bacteria. A series of 1,4-disubstituted 1,2,3-triazoles was screened for disruption of the tRNA-T box antiterminator RNA interaction using a fluorescence anisotropy-based assay. Several compounds reduced the anisotropy greater than 50% likely indicating significant competition for binding antiterminator RNA. General structure-activity trends indicated that the substituents at both N-1 and C-4 likely are involved in ligand binding. In addition, the anisotropy of the complex was significantly decreased not only by ligands with the possibility for electrostatic interactions with the RNA, but also by ligands with the potential for π - π stacking or other hydrophobic interactions indicating that these nonelectrostatic interactions could possibly be utilized in the future development of compounds that target and disrupt the function of this medicinally important riboswitch.

The T box riboswitch is found in the 5'-untranslated region (5'-UTR) of the mRNA of many aminoacyl tRNA synthetases, amino acid biosynthesis and transport genes in Gram-positive bacteria.¹ This regulatory riboswitch is an intriguing potential medicinal target given that it is found in many pathogenic bacteria and that it contains highly conserved structural and sequence elements.²⁻⁴ Two of these elements are required for tRNA binding and riboswitch function. The anticodon of the tRNA base pairs with a triplet sequence contained in the specifier loop at the beginning of the 5'-UTR and the tRNA acceptor end nucleotides base pair with four nucleotides in the bulge of the antiterminator element found near the end of the 5'-UTR.¹ This latter base pairing only occurs with non-aminoacylated (uncharged) tRNA. In the absence of uncharged tRNA, an alternative mRNA structural element forms, the terminator, and transcription terminates. Binding of uncharged tRNA to the antiterminator stabilizes the antiterminator structural element and precludes the formation of the terminator.¹ This represents the actual "switch" of the riboswitch since the antiterminator and terminator share common nucleotides and cannot coexist. Consequently, development of a high throughput assay to monitor the ability of ligands to disrupt tRNA binding to the antiterminator structural element (Figure 1a) is a critical step towards developing medicinal agents that target the T box riboswitch.

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Supplementary Material

A summary of Z' factors is provided in the supplementary material.

Recently, we reported ligand binding trends for a series of 1,4-disubstituted 1,2,3-triazoles binding to T box antiterminator model RNA.⁵ While the FRET binding assay used for this and other binding studies^{6–10} readily identifies potential high-affinity ligands, a complementary assay is needed that can identify which ligands bind in a manner that disrupts tRNA binding to the antiterminator RNA. We chose to investigate the potential of a fluorescence anisotropy-based assay for monitoring inhibition of tRNA binding to antiterminator model RNA. Anisotropy has been used to screen for ligand disruption of peptide-protein complexes¹¹ and RNA-peptide/protein complexes.^{12, 13} In this paper, we report the development of a unique fluorescence anisotropy assay and the structure-activity relationship of 1,4-disubstituted 1,2,3-triazoles ligands (Figure 1b) that disrupt tRNA binding to the T box riboswitch antiterminator model RNA.

Previous studies have demonstrated that complementary tRNA^{Tyr}(A73U) binds antiterminator model RNA AM1A^{14, 15} in a functionally relevant manner,¹⁴ thus indicating the functional relevance of using the tRNA-antiterminator model RNA complex in a ligand disruption assay. In the T box anisotropy assay (Figure 1a), when tRNA binds to fluorescently labeled AM1A, the AM1A/tRNA complex (positive control) molecular weight is ~4 times larger than AM1A alone (negative control), generating a greater anisotropy value. When a ligand competes with tRNA for binding the antiterminator, the anisotropy decreases from that of the complex since the ligand molecular weight when bound to the fluorescently labeled AM1A is not as significant.

The fluorescence anisotropy binding assay was optimized by monitoring tRNA^{Tyr}(A73U)¹⁴ binding to 5'-Rhd-AM1A (5'-tetramethylrhodamine-GAGGGUGGAACCGCGCUUCGGCGUCCCUC-3', Dharmacon, Inc.). Anisotropy values were obtained and the binding isotherms analyzed (*Prism* 4.0) resulting in an apparent $K_d = 1.4 \pm 0.4 \mu\text{M}$ ($R^2 = 0.9$) (Figure 2a). To prove that the increased anisotropy was caused by the AM1A/tRNA complex formation, unlabeled AM1A was used as a competitive inhibitor. Addition of unlabeled AM1A to the 5'-Rhd-AM1A/tRNA complex led to a significant decrease in the anisotropy (Figure 2b).

To find the optimal assay conditions for ligand screening, the screening window coefficient (Z' -factor) was determined. The Z' -factor is a characteristic parameter for quantifying the quality of high throughput assays.¹⁶ Z' -factors were measured at four different tRNA concentrations, 1.4 μM , 2.5 μM , 3.0 μM and 4.2 μM , which, based on the binding isotherm (Figure 2a), increase the anisotropy of 5'-Rhd-AM1A to 50%, 69%, 75%, and 85% of the maximal observed anisotropy respectively. As expected, higher tRNA concentrations provided a larger signal window between negative control and positive control thus resulting in a better Z' -factor (see Supplementary Material). Ideally, a competitive ligand screening assay would utilize [tRNA] $\approx 1.4 \mu\text{M}$ (the observed K_d for tRNA binding 5'-Rhd-AM1A) to allow estimation of the ligand K_i from an observed IC_{50} . However, the Z' -factor at this concentration was unacceptable (-1.05) due to the narrow window between the positive and negative control anisotropy values. In contrast, at [tRNA] = 4.2 μM the $Z' = 0.52$ is excellent for a high throughput assay,¹⁶ but this represents a tRNA concentration that leads to 85% bound AM1A which is not optimal for screening for competitive inhibitors. Consequently, a balance between these two extremes was chosen and the screening assays were performed with [tRNA]=2.5 μM ($Z' = 0.29$).

Using the optimized anisotropy assay, the ligand-induced anisotropy changes (Δr) were determined for a series of 1,4-disubstituted 1,2,3-triazoles (Figure 1b) to assess their ability to disrupt the antiterminator/tRNA complex. The majority of the compounds caused a decrease in anisotropy (Table 1, [ligand] = 100 μM). In particular, six compounds led to > 50% decrease (GHB-7, GHB-11, GHB-23, GHB-44, GHB-60, GHB-76) with two of these

decreasing the anisotropy > 70% (GHB-7 and GHB-76, Figure 3). This significant decrease indicates that the ligands are likely competing with tRNA for binding AM1A. It is possible that the observed ligand-induced dissociation of the complex may be due to the ligand binding the tRNA rather than binding the AM1A (see brackets in Figure 1a), however, this is not likely since this series of triazoles has previously been shown to bind AM1A directly as monitored by a fluorescence resonance energy transfer assay.⁵ A small subset of compounds resulted in a slight increase in anisotropy with three leading to > 10% increase (GHB-62, GHB-66, GHB-67, Figure 3). These compounds may stabilize the antiterminator/tRNA complex and/or reduce local mobility of the 5'-Rhd fluorophore.

While the structure-activity relationship of the triazole derivatives is complex and dependent upon several substituents, multiple trends can be noted. In an examination of R², several substituents were used, these include an octanoate and benzoate ester, a butyl and benzyl carbamate, a benzyl ether and a phenyl and butyl alkyl chain. Of these the benzoate ester derivatives had uniformly the least change in anisotropy (smallest Δr values) regardless of the NR₂ substituents. All of the other substituents at R² had a relatively wide range of Δr values across the set of NR₂ substituents. Of these the benzyl carbamate derivatives were uniformly the highest across a range of NR₂ groups. This R² group contains the analog (GHB-7) with the largest Δr value (−79%). Direct comparisons of the benzyl and butyl carbamate series (e.g. GHB-28 with GHB-68) show a general preference for the benzyl carbamate series. The last set of R² derivatives has R²/R³ being a cyclohexane ring. Again a wide range of Δr values can be seen across a range of NR₂ and R¹ substituents. Within this class of compounds are three of nine derivatives in which positive Δr values are observed and only where NR₂ = morpholine. Among this set of compounds, lower Δr values were observed for R¹ = H.

Comparison of the NR₂ groups across the library is again difficult as the Δr is strongly dependent upon the interplay between R² and NR₂, nonetheless, several trends can be noted. In an examination of the NR₂ group, both cyclic amine and acyclic amines were used in the library synthesis. Of the cyclic amines, morpholine proved to show the least overall Δr values. A comparison of *N*-phenyl piperazine and 4-phenyl piperidine showed roughly similar Δr values across a range of R² substituents although there are several exceptions. For example GHB-61 (R² = CH₂OC(O)NHBn, NR₂ = 4-phenylpiperidine) and GHB-57 (R² = CH₂OC(O)NHBn, NR₂ = *N*-phenyl piperazine) show almost identical Δr values while a similar pair GHB-19 and GHB-13 where R² = *n*-C₄H₉ are different by 30 units. Comparing these two NR₂ substituents with 3-carboethoxypiperidine shows roughly similar values across similar R² groups. This suggests that the observed Δr values of *N*-phenyl piperazine and 4-phenyl piperidine are not simply due to π - π stacking. Five different acyclic NR₂ groups were used, N(*n*-C₄H₉)₂, N(Me)CH₂(*c*-C₆H₁₁), N(Me)(CH₂)₂Ph, N(Me)(CH₂)₃Ph, and N(Me)(CH₂)₂CH(CH₃)₂. One observable trend involves N(Me)(CH₂)₂Ph, and N(Me)(CH₂)₃Ph. In general the phenylpropyl chain appears to have a larger Δr value than the phenylethyl chain suggesting placement of the phenyl ring is significant for disruption of the AM1A/tRNA complex.

A reasonable interpretation of this complex structure-activity relationship is that subsets within this series of compounds are binding in different ways to AM1A. Recent studies of oxazolidinone ligands binding to AM1A have indicated that even enantiomers can bind to the antiterminator RNA with similar affinity by making comparable binding contacts with different functional group partners.¹⁷ Further studies are underway to characterize the specific binding interactions for the lead 1,4-disubstituted 1,2,3-triazoles binding to antiterminator model RNA AM1A. A key factor to determine is whether this series of compounds binds a single, common site or multiple sites within the T box riboswitch antiterminator RNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Scheme of ligand-induced disruption of T box antiterminator model RNA binding to tRNA. The antiterminator model RNA AM1A is fluorescently labeled with tetramethylrhodamine (red dot) at the 5' end. b) 1,4-disubstituted 1,2,3-triazoles.

Figure 2.

a) Fluorescence anisotropy binding isotherm of tRNA^{Tyr}(A73U) binding to 5'-Rhd-AM1A (0.1 μM) in 50 mM NaH₂PO₄ pH 6.5, 50 mM NaCl, 0.01 mM EDTA, 15 mM Mg²⁺, 5% (v/v) DMSO. $\lambda_{\text{ex}} = 545$ nm, $\lambda_{\text{em}} = 590$ nm and G factor = 0.958. b) Disruption of 5'-Rhd-AM1A (0.1 μM) / tRNA (2.5 μM) complex by unlabeled AM1A.

Figure 3.
Select 1,4-disubstituted 1,2,3-triazoles that illustrate Δr structure-activity trends (see text).

Table 1

Ligand-Induced Anisotropy Changes (Δr) of 1,4-Disubstituted 1,2,3-triazoles^a

Ligand	NR ₂	R ¹	R ²	R ³	Δr (%)
GHB-7	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O)NHCH ₂ Ph	H	-79
GHB-9	N(CH ₃)(CH ₂) ₃ Ph	H	<i>n</i> -C ₄ H ₉	H	-37
GHB-10	4-Ph-piperazine	H		(CH ₂) ₄	-47
GHB-11	3-(CO ₂ Et)-piperidine	H	<i>n</i> -C ₄ H ₉	H	-53
GHB-12	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	<i>n</i> -C ₄ H ₉	H	-47
GHB-13	4-Ph-piperazine	H	<i>n</i> -C ₄ H ₉	H	-47
GHB-14	3-(CO ₂ Et)-piperidine	H		(CH ₂) ₄	-16
GHB-15	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H		(CH ₂) ₄	-42
GHB-16	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	<i>n</i> -C ₄ H ₉	H	-47
GHB-17	N(CH ₃)(CH ₂) ₂ Ph	H		(CH ₂) ₄	-21
GHB-18	N(<i>n</i> -C ₄ H ₉) ₂	H		(CH ₂) ₄	-32
GHB-19	4-Ph-piperidine	H	<i>n</i> -C ₄ H ₉	H	-16
GHB-20	4-Ph-piperidine	H		(CH ₂) ₄	-42
GHB-21	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H		(CH ₂) ₄	-47
GHB-22	4-Ph-piperazine	H	Ph	H	-32
GHB-23	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	Ph	H	-58
GHB-24	4-Ph-piperazine	H	CH ₂ OCH ₂ Ph	H	-37
GHB-25	3-(CO ₂ Et)-piperidine	H	CH ₂ OCH ₂ Ph	H	-26
GHB-26	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OC(O)NHCH ₂ Ph	H	-42
GHB-27	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)NHCH ₂ Ph	H	-42
GHB-28	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)NHCH ₂ Ph	H	-47
GHB-29	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O)NHCH ₂ Ph	H	-26
GHB-30	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)NHCH ₂ Ph	H	-32
GHB-31	4-Ph-piperidine	C(O)Ph		(CH ₂) ₄	-16
GHB-32	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)Ph		(CH ₂) ₄	-32
GHB-33	N(CH ₃)(CH ₂) ₃ Ph	C(O)CH ₂ OPh		(CH ₂) ₄	-42

Ligand	NR ₂	R ¹	R ²	R ³	Δr (%)
GHB-34	4-Ph-piperidine	C(O)CH ₂ OPh		(CH ₂) ₄	-42
GHB-35	4-Ph-piperazine	C(O)CH ₂ OPh		(CH ₂) ₄	-37
GHB-36	N(CH ₃)(CH ₂) ₂ Ph	C(O)CH ₂ OPh		(CH ₂) ₄	-32
GHB-37	N(<i>p</i> -C ₄ H ₉) ₂	C(O)CH ₂ OPh		(CH ₂) ₄	-26
GHB-38	3-(CO ₂ Et)-piperidine	C(O)CH ₂ OPh		(CH ₂) ₄	-21
GHB-39	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)CH ₂ OPh		(CH ₂) ₄	-37
GHB-40	4-Ph-piperazine	H	CH ₂ OC(O)NH(<i>p</i> -C ₄ H ₉)	H	-11
GHB-41	4-Ph-piperazine	H	CH ₂ OC(O)Ph	H	-11
GHB-42	morpholine	H	CH ₂ OC(O)NH(<i>p</i> -C ₄ H ₉)	H	-5
GHB-43	morpholine	H	CH ₂ OC(O)Ph	H	-5
GHB-44	N(<i>p</i> -C ₄ H ₉) ₂	C(O)CH ₂ Ph		(CH ₂) ₄	-53
GHB-45	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)CH ₂ Ph		(CH ₂) ₄	-5
GHB-46	morpholine	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	-11
GHB-47	morpholine	C(O)CH ₂ OPh		(CH ₂) ₄	-5
GHB-48	3-(CO ₂ Et)-piperidine	C(O)CH ₂ Ph		(CH ₂) ₄	-21
GHB-49	3-(CO ₂ Et)-piperidine	C(O)Ph		(CH ₂) ₄	-11
GHB-50	N(CH ₃)CH ₂ Ph	H	Ph	H	-32
GHB-51	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	C(O)Ph		(CH ₂) ₄	-26
GHB-57	4-Ph-piperazine	H	CH ₂ OC(O)NHCH ₂ Ph	H	-32
GHB-58	4-Ph-piperazine	C(O)Ph		(CH ₂) ₄	-21
GHB-59	4-Ph-piperazine	H	CH ₂ OC(O) <i>p</i> -C ₇ H ₁₅	H	-26
GHB-60	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	C(O)CH ₂ OPh		(CH ₂) ₄	-59
GHB-61	4-Ph-piperidine	H	CH ₂ OC(O)NHCH ₂ Ph	H	-29
GHB-62	morpholine	H		(CH ₂) ₄	24
GHB-63	morpholine	H	<i>p</i> -C ₄ H ₉	H	0
GHB-64	morpholine	H	Ph	H	6
GHB-65	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OCH ₂ Ph	H	-6
GHB-66	morpholine	H	CH ₂ OCH ₂ Ph	H	12
GHB-67	N(<i>p</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)NH(<i>p</i> -C ₄ H ₉)	H	18

Ligand	NR ₂	R ¹	R ²	R ³	Δr (%)
GHB-68	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-24
GHB-69	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-24
GHB-70	N(CH ₃)CH ₂ (<i>α</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-12
GHB-71	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	-12
GHB-72	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	6
GHB-73	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	0
GHB-74	N(CH ₃)CH ₂ (<i>α</i> -C ₆ H ₁₁)	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	-18
GHB-75	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	-12
GHB-76	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	-71
GHB-77	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O)Ph	H	-12
GHB-78	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OC(O)Ph	H	0.0
GHB-79	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)Ph	H	-12
GHB-80	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)Ph	H	-6
GHB-81	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O)Ph	H	0.0
GHB-82	N(CH ₃)CH ₂ (<i>α</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)Ph	H	-6
GHB-83	morpholine	C(O)Ph		(CH ₂) ₄	6
GHB-84	morpholine	C(O)CH ₂ Ph		(CH ₂) ₄	6
GHB-85	morpholine	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	0.0
GHB-107	N(CH ₃)(CH ₂) ₂ Ph	H	<i>n</i> -C ₄ H ₉	H	-29
GHB-108	N(<i>n</i> -C ₄ H ₉) ₂	H	<i>n</i> -C ₄ H ₉	H	-18
GHB-109	3-(CO ₂ Et)-piperidine	H	Ph	H	6
GHB-110	N(CH ₃)CH ₂ (<i>α</i> -C ₆ H ₁₁)	H	Ph	H	6
GHB-111	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OCH ₂ Ph	H	-18
GHB-112	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OCH ₂ Ph	H	-18
GHB-113	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OCH ₂ Ph	H	-6

^aThe ligand-induced anisotropy changes (Δr) were calculated using the equation $\Delta r = [(r_+ - r_-)/(r_+ + r_-)] \times 100$ where r_+ is the anisotropy of the positive control, r_- is the anisotropy of the negative control, and r is the anisotropy of the AM1A (0.1 μM) /tRNA (2.5 μM) complex in the presence of 1,4-disubstituted 1,2,3-triazoles (100 μM). Reported Δr values are average of replicate experiments. Error estimated to be ± 10 based on average standard deviation of r values.