Published in final edited form as:

Bioorg Med Chem. 2015 September 1; 23(17): 5702–5708. doi:10.1016/j.bmc.2015.07.018.

Factors that influence T box riboswitch efficacy and tRNA affinity

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Abstract

The T box riboswitch is an intriguing potential target for antibacterial drug discovery. Found primarily in Gram-positive bacteria, the riboswitch regulates gene expression by selectively responding to uncharged tRNA to control transcription readthrough. Polyamines and molecular crowding are known to specifically affect RNA function, but their effect on T box riboswitch efficacy and tRNA affinity have not been fully characterized. A fluorescence-monitored *in vitro* transcription assay was developed to readily quantify these molecular interactions and to provide a moderate-throughput functional assay for a comprehensive drug discovery screening cascade. The polyamine spermidine specifically enhanced T box riboswitch readthrough efficacy with an $EC_{50} = 0.58$ mM independent of tRNA binding. Molecular crowding, simulated by the addition of polyethylene glycol, had no effect on tRNA affinity for the riboswitch, but did reduce the efficacy of tRNA-induced readthrough. These results indicate that the T box riboswitch tRNA affinity and readthrough efficacy are intricately modulated by environmental factors.

Graphical abstract

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Keywords

RNA; Drug discovery; T box riboswitch; Spermidine; Molecular crowding

1. Introduction

The T box riboswitch regulates transcription of many amino-acid related bacterial genes (e.g., aminoacyl-tRNA synthetases) and is a novel target for the development of antibacterial therapeutic agents [1]. Located in the 5'-leader region of the mRNA transcript, the riboswitch structurally responds to cognate, uncharged tRNA (an indicator of amino acid starvation) to control transcription readthrough [1-3]. The anticodon of the tRNA base pairs with a specifier sequence in Stem I of the riboswitch [4,5] and forms contacts with the Stem I apical loop [6,7], while the tRNA acceptor end base pairs with nucleotides in the 7-nt bulge of the highly conserved antiterminator element which enables transcription readthrough by stabilizing the antiterminator [8,9]. In the absence of uncharged tRNA, a more stable structural element (the terminator) forms preferentially resulting in premature termination of transcription [1].

Antibacterial drug resistance is a significant global health concern making it essential to identify new antibacterial agents, ideally ones with a novel mechanism of action [10-12]. Targeting other riboswitches and noncoding RNA is an active, but relatively new area of drug discovery [13-18]. The T box riboswitch is an attractive potential target since it often regulates essential genes, is frequently found in multiple genes within an organism and, its default state is "off" where no transcription occurs if a sufficient quantity of native ligand (cognate uncharged tRNA) is not present or is unable to bind [1].

As part of a comprehensive drug discovery effort, we have been investigating the highly conserved antiterminator element as a specific RNA target within the T box riboswitch [19-26]. Using a fluorescence-based binding assay we determined that T box antiterminator model RNA binds aminoglycosides in a structure-specific manner primarily through

electrostatic interactions [27,28]. Synthetic 4,5-disubstituted oxazolidinones bind antiterminator model RNA with a structure-activity-relationship that involves hydrogen-bonding and other non-electrostatic interactions [29-31]. In a single-round *in vitro* transcription assay, the T box riboswitch function was also modulated by oxazolidinones [32]. The oxazolidinone analogs, 1,4-disubstituted 1,2,3-triazoles, bind antiterminator model RNA in a structure-specific manner [33-35] and inhibit tRNA binding to antiterminator model RNA [36].

The development of functionally relevant, medium-throughput primary and secondary screening assays has been essential to effectively identify ligands that bind T box antiterminator model RNA and disrupt its interaction with tRNA [37]. Follow-up functional studies using a single-round *in vitro* transcription assay have been used to assess select compounds [32], however, the multiple steps involved are not amenable to screening compound libraries, nor for readily screening multiple reaction conditions to precisely discern potentially subtle differences. Consequently, there is a significant need in the T box-targeted drug discovery cascade for a medium-throughput functional assay. In addition, the development of targeted therapeutic agents relies on a full understanding of the extent to which other factors affect the T box riboswitch mechanism. This work investigates the affinity and efficacy for tRNA-induced readthrough of the *Bacillus subtilis* *glyQS* T box riboswitch, using a fluorescence-monitored *in vitro* transcription assay (Figure 1), and the effect that molecular interactions have on these quantified descriptors in the presence of a co-factor, co-solutes and a 1,4-disubstituted 1,2,3-triazole potential inhibitor.

2. Materials and Methods

2.1 Nucleic Acids

The fluorescently labeled readthrough probe (RT_{prb}) was obtained from Trilink as 5' Cy5-CGCAccuccacuuuuuucuucaaaTGCG-Dabcyl 3' where the upper case letters represent deoxyribonucleotides and lower case letters 2'-*O*-methyl ribonucleotides. The design of RT_{prb} utilized a chimeric molecular beacon approach [38] and relied on the enhanced RNA binding of 2'-*O*-methyl nucleotides [39-41]. The Target Sequence RNA, used for testing the RT_{prb} fluorescence response, was obtained from Trilink as 5' UUAUGAAAGAAAAGUGGAGGUGC 3'.

The *B. subtilis* *glyQS* template dsDNA [2] was obtained from PCR amplification of a plasmid containing the template DNA using Pfu turbo DNA polymerase (Agilent Technologies) and purified by QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. The DNA templates for tRNA^{Gly} [42] and tRNA^{Tyr} [19] and discriminator base variants were amplified from plasmids and purified in a similar manner. The tRNA was prepared as previously described [43] using an AmpliScribe T7-Flash transcription kit (Epicentre) and purified by denaturing polyacrylamide gel electrophoresis (20%, 19:1 acrylamide/bis-acrylamide) followed by electroelution, then concentration using an Amicon Ultra-4 centrifugal filter unit with Ultracel-10 membrane (Millipore, MA) and dialysis in 1 mM MOPS, pH 6.5.

The Transcription Control template strand DNA 5'-
GGGTATTTAATTAAGCTTTTGCAAGGTTAGAATCATGTCTTGAATATGCCCTCCA
CTTTT
CTTTCATAAGTATAGTAAATGATCATATAAAAAGATGGACCAAATGTCAACTTT
TGTTTTTCGC
GCTTTGATTTTAAATACAATCCCGAATATTCTTCGTAATCTAGATTAAT-3' and the
complementary coding strand DNA were obtained from IDT and purified by denaturing
polyacrylamide gel electrophoresis (20%, 19:1 acrylamide/bis-acrylamide) followed by
electroelution. Together, the coding and template strands contain the *glyQS* promoter region
and transcription start site (nt 91-141 in *glyQS*), primer binding sites for DNA replication (nt
1-51 and nt 206-452 in *glyQS*) and the DNA template for the RNA target sequence of RT_{prb}
(nt 373-396 in *glyQS*). Mixing the template and coding strands together in a 1:1 ratio created
the dsDNA template for the Transcription Control reactions. The resulting dsDNA template
was amplified by PCR and used in the Transcription Control *in vitro* transcription reactions
as outlined below.

2.2 In vitro transcription

All transcription reaction mixtures (20 μ L each) were set up in a 384-well microplate. For
the T box riboswitch functional studies, each well contained a final concentration of 20 mM
Tris-HCl, 40 mM KCl, 4 mM MgCl₂, 1 mM DTT, 0.23 U/ μ L RiboGuard RNase inhibitor
(Epicentre), 100 nM RT_{prb}, 0.05 U/ μ L *E. coli* RNA polymerase Sigma-saturated
holoenzyme (Epicentre), 10 nM dsDNA template (*glyQS* or Transcription Control template
as specified). Stock solutions of ligands and co-solutes such as spermidine (125 mM in
H₂O), PEG-8000 (0.2 g/mL solution in H₂O, 20% wt/v) and tRNA (10 $\times$ final concentration)
were added to a final concentration as specified then NTPs were added to a final
concentration of 10 μ M CTP, 10 μ M GTP, 10 μ M UTP, 400 μ M ATP. This relative ratio of
NTPs has been used previously for multi-round T box transcription reactions [44] and
balances the need for high concentrations of the initial nucleotide to facilitate transcription
initiation [45] with the need for slower transcription elongation rates to minimize tRNA-
independent readthrough [46]. A final concentration of 5 mM spermidine was used in all
reactions except the spermidine dose-response experiments where the concentration of
spermidine was varied as indicated. For all the tRNA functional studies, n = 3, except for the
No NTP control reaction was n = 2 and the DMSO comparison studies were n = 4.

Prior to addition of the NTPs, the reaction mixture with all the rest of the ingredients was
mixed well by carefully pipetting up and down several times. The NTPs were then added to
initiate the transcription reaction before applying an optically-clear polyolefin film (VWR)
to prevent evaporation. No mixing was performed after adding NTPs to enable immediate
real-time monitoring of the fluorescence signal. Addition of NTPs was defined as the start
time of the *in vitro* transcription reaction. The transcription reaction mixtures were incubated
at 25°C for 3 h and a reading acquired every 5 minutes using a SpectraMax M5 microplate
reader with excitation = 642 nm, emission = 672 nm and the cutoff filter = 665 nm. The
averages of replicates and ranges of error noted by error bars (unless otherwise specified)
were determined in all experiments.

2.3 Analysis of real-time in vitro transcription data

For the Readthrough_{rate} determinations, the fluorescence readings from 30 to 60 minutes were fit to a linear equation using Prism (Graphpad) to determine the slope (RFU_{30-60min}/ t). For the Readthrough_{max} determinations, the maximum change in fluorescence was determined from RFU = RFU₁₈₀ - RFU₅ where RFU₅ is the initial fluorescence at 5 minutes and RFU₁₈₀ is the fluorescence at 180 minutes. For the tRNA^{Gly} (n = 3) and spermidine dose-response studies (n = 2), the Readthrough_{rate} transcription data were plotted against the log[tRNA^{Gly}] or log[spermidine] in Prism (Graphpad) and fit to a dose-responsive stimulation curve with variable slope to determine the EC₅₀ values. The Readthrough_{rate} was used for these dose-response studies since it minimized the effect of RT_{prb} background fluorescence observed at higher concentrations of tRNA^{Gly}. When [tRNA^{Gly}] = 1 μM (i.e., approximately two-orders of magnitude higher than the concentration used in the ligand screening assay) an initial non-specific enhancement in RT_{prb} fluorescence was observed before transcription reaction initiation (i.e., before addition of NTPs), but this background fluorescence dissipated over the course of the transcription reaction. With the Readthrough_{rate} approach, the slope (RFU_{30-60min}/ t) was measured at time points where the non-specific enhancement had fully dissipated or was minimal for the range of tRNA^{Gly} concentrations used in the dose-response experiments (0-1.6 μM). Finally, for ease of visual comparison in the spermidine dose-response experiments, the data were plotted as Normalized Readthrough_{rate} = Readthrough_{rate}/Readthrough_{rate0} where Readthrough_{rate0} is the rate of readthrough in the absence of spermidine in the corresponding control reaction.

The Z' factor for Readthrough_{max} was calculated using the equation $Z' = 1 - [3 \times (SD_{tRNA} + SD_0) / (\text{Readthrough}_{\text{max}+tRNA} - \text{Readthrough}_{\text{max}0})]$ where Readthrough_{max+tRNA} and SD_{tRNA} are the Readthrough_{max} in the presence of tRNA^{Gly} and its corresponding standard deviation, while Readthrough_{max0} and SD₀ are in the absence of tRNA^{Gly}. The Z' factor for Readthrough_{rate} was calculated using $Z' = 1 - [3 \times (SD_{tRNA} + SD_0) / (\text{Readthrough}_{\text{rate}+tRNA} - \text{Readthrough}_{\text{rate}0})]$ where Readthrough_{rate+tRNA} and SD_{tRNA} are the Readthrough_{rate} in the presence of tRNA^{Gly} and its corresponding standard deviation, while Readthrough_{rate0} and SD₀ are in the absence of tRNA^{Gly}.

2.4 Ligand Inhibition Assay

Triazole **1** was synthesized as previously described [33]. The *in vitro* transcription procedure described above was used to test for ligand-induced inhibition with the following changes: The final concentration of *E. coli* RNA polymerase holoenzyme (RNAP) was 0.02 U/μL. The concentration of tRNA^{Gly} was fixed at 15 nM and the ligand stock solution (in 50% v/v DMSO) was added prior to addition of NTPs to obtain the final ligand concentrations of 50 μM and a maximum of 5% v/v DMSO (n = 5). Control tRNA-induced *glyQS* transcription readthrough reactions without ligand (n = 8) also had a final concentration of 5% v/v DMSO as did the Transcription Control reaction with (n = 5) and without ligand (n = 8). The averages of replicates and standard deviation noted by error bars were determined in all experiments and an unpaired t-test analysis was performed to determine statistical significance with two-tailed P values as indicated.

For the apparent IC_{50} determination, the inhibitor ligand screening assay was used and tested with $[ligand] = 50 \mu M, 25 \mu M, 12.5 \mu M, 6.25 \mu M$ and $3.125 \mu M$. The $Readthrough_{max}$ with tRNA was determined at each ligand concentration (RFU_{tRNA+L} , $n = 2$) and then the percent of ligand-induced $Readthrough_{max}$ inhibition was determined as % inhibition = $[(RFU_{tRNA+L} - RFU_{tRNA}) / (RFU_{tRNA} - RFU_0)] \times 100$ where RFU_{tRNA} and RFU_0 are the average $Readthrough_{max}$ values in the absence of ligand for the control reactions with or without tRNA^{Gly} respectively ($n = 10$). The percent of $Readthrough_{max}$ inhibition (average with range noted by error bars) was then plotted against the logarithm of the ligand concentration and fit to a non-linear dose-response inhibition model with variable slope using Prism (GraphPad).

3. Results

3.1 Functionally relevant fluorescence monitored transcription readthrough assay

The fluorophore-quencher labeled probe (RT_{prb}) effectively monitored tRNA-induced transcription readthrough in a multi-round transcription assay of the *B. subtilis glyQS* T box riboswitch (Figure 2). In the absence of cognate tRNA, a basal level of transcription readthrough was observed. In contrast, addition of cognate tRNA^{Gly} (100 nM) resulted in a three-fold increase in transcription readthrough. This increase is cognate-tRNA specific since mismatched tRNA discriminator base (tRNA^{Gly}(U73A)), mismatched tRNA anticodon sequence (tRNA^{Tyr}(A73U)), and, fully mismatched tRNA (tRNA^{Tyr}) resulted in no enhancement over the basal level readthrough.

The transcription readthrough can be monitored as either $Readthrough_{max}$ (by measuring the maximal change in RT_{prb} fluorescence $RFU = RFU_{180min} - RFU_{5min}$) or $Readthrough_{rate}$ (by measuring the rate of RT_{prb} fluorescence increase over a range of the experimental time course, $RFU_{30-60min} / t$). The same cognate tRNA-dependent induction of transcription readthrough was observed for both monitoring approaches (Figure 2 and Supplementary data). Control experiments to verify RT_{prb} signal stability and establish calibration curves for RT_{prb} signal vs. target sequence concentration were conducted (Supplementary data). No signal decrease due to photobleaching or RT_{prb} degradation over time was observed and there was a linear correlation between the observed fluorescence of RT_{prb} and Target Sequence concentration up to at least $[Target] = 60$ nM. The average RFU_{max} observed at 3 h (~ 80 RFU) for RT_{prb} in the tRNA^{Gly}-induced *glyQS* transcription readthrough reaction indicates that approximately 28 nM of readthrough transcription product was produced (estimated from the calibration curve). This product concentration is almost three-fold larger than the concentration of DNA template used in the reaction (10 nM), consistent with a multi-round transcription reaction. Reagent limitation studies with the in vitro transcription readthrough indicate that complete consumption of NTPs is likely the primary limiting factor for the RT_{prb} fluorescent probe signal reaching a maximum after 3 h in the *glyQS* transcription reactions (Supplementary data).

3.2 tRNA^{Gly} dose-response characteristic of ligand-receptor interaction

The tRNA-induced *glyQS* transcription readthrough was measured over a multi-log concentration range of cognate tRNA^{Gly} to determine a dose-response relationship (Figure

2b). The observed sigmoidal curve is characteristic of a ligand-receptor interaction and was analyzed accordingly. In a dose-response analysis, the efficacy is defined as the maximal response induced by the natural ligand (i.e., tRNA^{Gly}) and the affinity (EC₅₀) is defined as the concentration of ligand that results in 50% of the maximal response [47]. With 0.05 U/μL RNAP, the observed tRNA^{Gly} affinity was EC₅₀ = 30 nM (95% CI = 23-40 nM, R² = 0.97). With 0.02 U/μL RNAP the observed affinity was EC₅₀ = 17 nM (95% CI = 11-26 nM, R² = 0.95).

3.3 Fluorescence-monitored assay suitable for medium-throughput assays

While both the Readthrough_{max} and Readthrough_{rate} monitoring approaches are potentially viable, the suitability of each monitoring method for medium-throughput screening was investigated. The screening window coefficient (Z'-factor) is a characteristic parameter used to quantify the quality of high throughput assays [48]. Negative Z'-factor values indicate an unacceptable assay and positive values approaching 1 indicate an excellent assay. For drug discovery studies, competitive inhibitor screening assays conditions ideally have the natural ligand (in this case tRNA^{Gly}) at a concentration that induces half the maximal response (the EC₅₀ value) in order to effectively observe competitive inhibitor-induced disruption. The tRNA^{Gly} concentrations investigated were at or near the observed EC₅₀ values. The effect of DMSO was also investigated because a small percentage of DMSO in the assay is unavoidable due to the limited water solubility of inhibitor candidates at high stock concentrations.

In the absence of DMSO, both Readthrough_{max} and Readthrough_{rate} monitoring approaches had acceptable Z'-factor values (Table 1). In contrast, in the presence of 5% DMSO, only the Readthrough_{max} approach had an acceptable Z'-factor value when using 0.02 U/μL of RNAP per reaction and [tRNA^{Gly}] = 15 nM (i.e., near the EC₅₀ value observed with 0.02 U/μL RNAP). In order to accommodate using DMSO stock solutions for less soluble ligands, some amount of DMSO in the transcription assay is unavoidable. Better Z'-factor values with 5% DMSO were observed for both Readthrough_{max} and Readthrough_{rate} with 0.05 U/μL of RNAP per reaction and [tRNA^{Gly}] = 40 nM (i.e., near the EC₅₀ value for 0.05 U/μL RNAP). However, the cumulative quantity of RNAP and tRNA^{Gly} required makes these assay conditions not optimal for a medium to high throughput screening in terms of cost and/or material preparation time. Consequently, the Readthrough_{max} monitoring approach with 0.02 U/μL RNAP and [tRNA^{Gly}] = 15 nM was used for the potential inhibitor study.

3.4 Transcription readthrough sensitive to spermidine and co-solvents

In the single-round in vitro transcription assays, a halted transcription elongation complex is generated at low levels of Mg²⁺ (e.g., 2 mM [45]), but then after initiation, the T box riboswitch requires increased levels of Mg²⁺ (15 mM) in order to maximize the level of tRNA-induced transcription readthrough [3]. For the development of an efficient, medium-throughput functional screening assay, this multi-step approach needed to be avoided. Previously, spermidine was identified as an additive that facilitated tRNA-induced transcription in radioactively-monitored multi-round *thrS* T box transcription [44]. Consequently, the affinity and efficacy effects of spermidine as a co-factor on *glyQS*

transcription readthrough in the fluorescence-monitored multi-round assay were investigated. As a control, the effect on overall transcription was also investigated using a Transcription Control DNA template, which contained the template for the same promoter region, first six 5' nucleotides in the *glyQS* leader RNA and the sequence targeted by RT_{prb}. Spermidine dose-response curves for the Transcription Control and *glyQS* transcription were compared (Figure 3). With 5 mM spermidine, a 40% increase in the maximum transcription of the target sequence was observed in the Transcription Control with a spermidine affinity of $EC_{50} = 0.82$ mM (95% CI = 0.74-0.91 mM, $R^2 = 0.95$). At higher concentrations of spermidine (i.e., 10 mM) a significant decrease in transcription for all conditions was observed. This is consistent with the known denaturing effect of polyamines at high concentrations [49]. Consequently, the corresponding data points were excluded from the dose-response curve fitting.

The presence of spermidine significantly enhanced the efficacy of *glyQS* transcription readthrough, especially tRNA^{Gly}-induced readthrough. With 5 mM spermidine, the basal level readthrough (without tRNA^{Gly}) was enhanced by 270% and tRNA^{Gly}-induced readthrough (with [tRNA^{Gly}] = 100 nM) was enhanced by 650%. The spermidine affinity for the basal and tRNA-induced readthrough were similar, $EC_{50} = 0.49$ mM (95% CI = 0.41-0.59 mM, $R^2 = 0.96$) and $EC_{50} = 0.58$ mM (95% CI = 0.49-0.68 mM, $R^2 = 0.98$) respectively.

Since molecular crowding has been shown to affect the structure and function of RNA [50], the effect of molecular crowding on the T box riboswitch was investigated by the addition of 5% (wt/v) polyethylene glycol (PEG). PEG has been used previously to mimic molecular crowding in the investigation of other functional RNAs [51]. PEG had no significant effect on the linear region of the calibration curve (Supplementary data) indicating that PEG does not disrupt the RT_{prb} binding to the target sequence. The presence of PEG reduced the Transcription Control rate significantly and reduced the Readthrough_{rate} for *glyQS* transcription with tRNA^{Gly} (Figure 4). Interestingly, there was no significant effect on the basal level transcription. In addition, there was no significant difference in tRNA^{Gly} affinity (EC_{50}) determined from the tRNA^{Gly} dose-response curve in the presence or absence of PEG. There was also no significant difference in spermidine affinity (EC_{50}) in the presence of 5% PEG (Supplementary data). In contrast, the tRNA^{Gly} efficacy was 54% smaller in the presence of PEG (Figure 4).

3.5 Monitoring ligand-induced inhibition

To explore the utility of the moderate-throughput assay for drug discovery functional studies of potential inhibitors, the effect of 1,4-disubstituted 1,2,3-triazole 1, known to bind T box antiterminator RNA [34], was investigated (Figure 5). Initial control studies were done to investigate the effect of 5% DMSO as a co-solvent since a small amount of DMSO from potential inhibitor stock solutions is unavoidable (due to limited aqueous solubility at high ligand concentrations). DMSO had no significant effect on the linear region of the calibration curve indicating that DMSO does not disrupt the RT_{prb} binding to the target sequence and while DMSO increased the observed transcription Readthrough_{rate} it was a non-specific effect with no significant difference in tRNA^{Gly} affinity (EC_{50}) determined

from the tRNA^{Gly} dose-response curve in the presence or absence of DMSO (Supplementary data).

Triazole **1** resulted in significant inhibition of tRNA-induced *glyQS* readthrough (apparent IC₅₀ = 8 μM) but also the basal-level *glyQS* readthrough and the Transcription Control (Figure 5, all P<0.0001). The triazole did not inhibit the fluorescence of RT_{prb} in the presence of Target Sequence, indicating that the observed reduction in signal for the other reactions is indeed due to inhibition of production of the target sequence and not a nonspecific inhibition of the molecular beacon signal. Overall, **1** appears to be inhibiting transcription with no significant specific effect on the riboswitch.

4. Discussion

The T box riboswitch is an intriguing potential target for the development of novel antibacterial agents [1]. In order to facilitate detailed investigations of factors that modulate T box riboswitch function and screen for potential inhibitors, a functionally relevant, medium to high throughput assay method was needed. Single-round transcription readthrough assays have been used to monitor riboswitch function [2,45]. However, the ligand-screening throughput for this approach is limited due to the multiple handling steps and separation of transcripts by gel electrophoresis. A medium to high throughput assay amenable to the quantitative analysis was needed to fully characterize subtle yet meaningful molecular interactions.

Our approach was to utilize a fluorophore-quencher transcription readthrough probe (RT_{prb}) targeting a sequence at the 3' end of the readthrough transcript of *B. subtilis glyQS* [2] to monitor multi-round transcription of the T box riboswitch (Figure 1). We utilized a chimeric molecular beacon probe design strategy [38] where the region complementary to the target sequence is composed of 2'-*O*-methyl nucleotides which enhances target binding affinity and kinetics [39,40] while the stem region is composed of deoxyribonucleotides since a duplex of 2'-*O*-methyl nucleotides would be too stable [40]. Transcripts that terminate prior to the target sequence are not directly measured using the RT_{prb}. Small molecule metabolite-sensing riboswitches have been monitored in a manner that simultaneously measured total transcription initiation and transcription readthrough using multiplex fluorescent probes [52]. However, for the T box riboswitch, the entire span of the leader region is involved in tRNA binding, and/or bridging tRNA binding regions [1]. Having a probe targeting the leader region before the termination site is not feasible with the native *glyQS* T box leader sequence since it could potentially perturb tRNA binding interactions. Consequently, the Transcription Control reaction, lacking the T box riboswitch, was used to monitor potential nonspecific effects on transcription.

The multi-round, fluorescence monitored T box transcription readthrough assay is functionally relevant and further facilitates investigation of molecular factors affecting the T Box riboswitch. Similar experimental buffer conditions have previously been used in a multi-round assay of the *B. subtilis thrS* T box riboswitch monitored using radiolabeled transcripts [44]. The cognate tRNA specificity observed (Figure 2) is consistent with that observed with single-round *glyQS* transcription readthrough assays [2]. In the absence of

cognate tRNA, some basal level readthrough is observed, consistent with previously reported single-round [3] and ^{32}P -monitored multiround assays [44]. The concentration of Mg^{2+} used in the assay (4 mM) is similar to the estimated value of free magnesium in bacteria $[\text{Mg}^{2+}]_{\text{free}} \approx 3 \text{ mM}$ [53,54] and significantly less than that used in the single-round assays. The observed EC_{50} (affinity) for tRNA^{Gly} (30 nM with 0.05 U/ μL RNAP) is consistent with the reported high affinity binding of tRNA^{Gly} to a truncated T box leader construct ($K_d = 150 \text{ nM}$) as measured by isothermal titration calorimetry [7] and is also consistent with high affinity binding between tRNA^{Gly} and the full *glyQS* leader RNA monitored by a filter binding assay [3]. In addition, while the presence of trace amounts of DMSO (from ligand stock solutions) is known to have small effects on RNA structure [55], only nonspecific effects were observed with 5% DMSO in the assay. Overall, this fluorescence-monitored, multi-round T box riboswitch functional assay complements the previously developed antiterminator-specific 1° and 2° screening assays [37] to create a more comprehensive T box antiterminator-targeted drug discovery screening cascade.

Spermidine was used in the fluorescence-monitored *in vitro* transcription assay in order to avoid the lower-throughput, multi-step procedure of the single-round *in vitro* T box transcription assays. Spermidine is known to facilitate multi-round transcription of the T box riboswitch *in vitro* but is likely only a substitute for a specific protein co-factor needed for tRNA-dependent transcription readthrough *in vivo* [44]. Nevertheless, investigating the effect of spermidine on the T box riboswitch *in vitro* provides insight into possible co-factor function and facilitates optimization of the drug discovery functional assay conditions. The *glyQS* leader affinity for spermidine in the presence of 100 nM tRNA^{Gly} ($\text{EC}_{50} = 0.58 \text{ mM}$) and in the absence of tRNA^{Gly} ($\text{EC}_{50} = 0.49 \text{ mM}$) was within the 95% confidence interval range of one another (Figure 3) indicating that spermidine binding to the *glyQS* leader is likely independent of tRNA binding. In addition, this binding may be specific to the riboswitch leader RNA since the spermidine EC_{50} for the Transcription Control (lacking the riboswitch) was higher ($\text{EC}_{50} = 0.82 \text{ mM}$) than the EC_{50} values for *glyQS* (both with or without tRNA^{Gly}). While the difference is not large, it is of significance since the respective 95% confidence intervals do not overlap. In addition, spermidine enhanced the overall efficacy of *glyQS* transcription (maximum readthrough) to a greater extent (650% with tRNA^{Gly}, 270% without tRNA^{Gly}) than it enhanced the transcription of the target sequence in the Transcription Control experiment (40%). Overall, these differences in spermidine affinity and riboswitch readthrough efficacy compared to those observed with the Transcription Control indicate there may be specific interactions between spermidine and the T box leader region that affect its function. Biogenic polyamines, such as spermidine, have been shown to have very specific structural and functionally relevant interactions with RNA [56,57].

Spermidine also appears to reduce the Mg^{2+} requirement for *glyQS* transcription readthrough. In the single-round transcription assay, tRNA^{Gly} bound the *glyQS* leader RNA equally well at 5 mM and 15 mM Mg^{2+} , but no significant transcription readthrough was observed at 5 mM Mg^{2+} and maximal readthrough required 15 mM Mg^{2+} [3]. In contrast, in the *glyQS* multi-round transcription assay, significant tRNA-induced readthrough is observed at much lower levels of Mg^{2+} (4 mM Mg^{2+}) with 5 mM spermidine present. Since

spermidine is known to bind and stabilize bulged RNA [56], it is possible that spermidine may be binding to the bulge region of the T box antiterminator RNA and facilitating transcription readthrough.

Molecular crowding, mimicked with inert co-solutes like PEG, is known to enhance the function of other noncoding RNAs, reduce the Mg^{2+} requirement [51] and favor more compactly folded RNA structures [50], but its effect on the T box riboswitch has not been investigated. Addition of 5% PEG nonspecifically reduced the observed rate of production of the target sequence both in the Transcription Control and in the tRNA^{Gly}-induced *glyQS* readthrough (Figure 4). There also was no significant change in tRNA^{Gly} affinity (EC_{50}). Interestingly, however, the tRNA^{Gly} efficacy for inducing readthrough was significantly decreased in the presence of PEG whereas there was little effect on the basal level readthrough (without tRNA^{Gly}). This indicates that molecular crowding may be facilitating basal level readthrough since the nonspecific transcription inhibition of PEG was not observed in the basal level readthrough reaction.

Previous T box riboswitch targeted drug discovery studies used a radiolabel-monitored single-round *glyQS* transcription readthrough assay for functional studies [32] but throughput constraints limited the number of experiments that could be run simultaneously. With the medium throughput fluorescence-monitored functional assay, multiple replicates of inhibition reactions and associated controls can be run simultaneously, thus facilitating the discernment of potentially subtle differences. The potential inhibitory effect of 1, 4-disubstituted 1, 2, 3-triazole **1**, which is known to bind the T box antiterminator RNA element and disrupt its interaction with tRNA [34,36], was investigated. Triazole **1** inhibited overall transcription, but with no significant additional inhibitory effect on the T box riboswitch (Figure 5).

5. Conclusion

The results reported in this study indicate that molecular interactions with co-factors such as spermidine and molecular crowding can influence T box riboswitch efficacy in a manner that is not necessarily dependent on also affecting tRNA affinity. These findings have significant implications for the importance that molecular recognition can play in T box riboswitch function and for future drug discovery efforts targeting this unique regulatory mechanism. In addition, the assay developed provides a vital moderate-throughput functional assay for a comprehensive drug discovery screening cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (GM073188 to JH, and C06 RR-014575-01 to Ohio University for the research facility). We wish to thank Prof. T.M. Henkin for the *glyQS* and tRNA^{Gly} template DNA for the *in vitro* T box transcription studies and G. Aquaah-Harrison for compound synthesis.

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Figure 1. Schematic of *glyQS* T box riboswitch and fluorescence-monitored transcription readthrough

a) In the absence of tRNA^{Gly} transcription terminates prior to the RNA sequence targeted by the fluorescent probe RT_{prb}. The hairpin form of RT_{prb} has minimal background fluorescence due to Cy5 fluorescence being quenched by Dabcyl. b) Transcription readthrough induced by tRNA^{Gly} produces the full-length transcript, including the target sequence of RT_{prb}. RT_{prb} binds the target sequence, unfolding the hairpin structure such that Dabcyl no longer quenches Cy5 and fluorescence increases.

Figure 2. Multi-round assay for tRNA^{Gly}-specific induction of *glyQS* transcription readthrough
a) Comparison of Readthrough_{max} of cognate tRNA^{Gly} (wt, acceptor end 5'-UCCA-3') with tRNA^{Gly}(U73A), tRNA^{Tyr}(A73U) and tRNA^{Tyr}(wt, acceptor end 5'-ACCA-3'). For all reactions with tRNA, [tRNA] = 100 nM. b) Dose-response curve of tRNA^{Gly} readthrough induction.

Figure 3. Spermidine effect on efficacy of tRNA^{Gly}-induced *glyQS* readthrough

a) Structure of spermidine. b) Spermidine dose-response effect on *glyQS* transcription readthrough. In the absence of tRNA^{Gly} (open circle), spermidine EC₅₀ = 0.49 mM (95% CI = 0.41-0.59 mM, R² = 0.96). In the presence of tRNA^{Gly} (filled circle), spermidine EC₅₀ = 0.58 mM (95% CI = 0.49-0.68 mM, R² = 0.98). The Transcription Control data (triangle) reflect the overall transcription reaction since the Transcription Control DNA template lacks the T box riboswitch. For the Transcription control, the spermidine EC₅₀ = 0.82 mM (95% CI = 0.74-0.91 mM, R² = 0.95). The data points at [spermidine] = 10 mM were excluded from EC₅₀ calculations as discussed in Results.

Figure 4. Molecular crowding effect on tRNA^{Gly}-induced *glyQS* readthrough

a) Transcription Control (striped bars), *glyQS* readthrough in the presence of 100 nM tRNA^{Gly} (shaded bars), and *glyQS* readthrough in the absence of tRNA^{Gly} (basal level transcription, unfilled bars) were performed in the presence or absence of 5% (wt/v) PEG-8000. The Transcription Control data reflect the overall transcription reaction since the Transcription Control DNA template lacks the T box riboswitch. b) Effect of molecular crowding on the affinity (EC₅₀) of tRNA^{Gly} to induce *glyQS* readthrough. For tRNA^{Gly} EC₅₀ = 30 nM in the absence of PEG (open circle, 95% CI = 22-39 nM, R² = 0.97) and EC₅₀ = 26 nM in the presence of 5% PEG (filled circle, 95% CI = 13-50 nM, R² = 0.84).

Figure 5. Effect of 1, 4-disubstituted 1, 2, 3-triazole on *glyQS* transcription readthrough
a) Dose-response of compound **1** on *glyQS* transcription readthrough in the presence of 15 nM tRNA^{Gly} . Apparent $\text{IC}_{50} = 8 \mu\text{M}$ (95% CI = 4-15 μM , $R^2 = 0.86$). Inset: Structure of triazole **1**. b) $\text{Readthrough}_{\text{max}}$ without (open bars) and with **1** (50 μM , shaded bars) for *glyQS* basal level transcription, *glyQS* with tRNA^{Gly} (15 nM), and, the Transcription Control reaction. For each reaction condition, the percent of ligand-induced inhibition is indicated. The Transcription Control data reflect the effect of **1** on the overall transcription reaction since the Transcription Control DNA template lacks the T box riboswitch. Also shown is the effect of **1** on RT_{prb} fluorescence in the presence of Target Sequence RNA (20 nM).

Table 1
Z' factor analysis of fluorescence monitoring methods

Condition			Z' factor			
[RNAP]	[tRNA ^{Gly}]	[DMSO] (v/v)	Readthrough _{max}	RFU (180 min)	Readthrough _{rate}	RFU _{30-60min} / t
0.02 U/μL	15 nM	5%		0.10		-0.69
0.02 U/μL	15 nM	0%		0.24		0.14
0.05 U/μL	40 nM	5%		0.26		0.12
0.05 U/μL	100 nM	0%		0.59		0.57