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LIGAND-INDUCED CHANGES IN T BOX ANTITERMINATOR RNA STABILITY

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Abstract

The T box antiterminator RNA element is an important component of the T box riboswitch that controls the transcription of vital genes in many Gram-positive bacteria. A series of 1,4-disubstituted 1,2,3-triazoles was screened in a fluorescence-monitored thermal denaturation assay to identify ligands that altered the stability of antiterminator model RNA. Several ligands were identified that significantly increased or decreased the melting temperature (T_m) of the RNA. The results indicate that this series of triazole ligands can alter the stability of antiterminator model RNA in a structure-dependent manner.

Keywords

antiterminator; T_m ; riboswitch; tRNA; ligand screening

Introduction

Riboswitches are an intriguing and challenging target for drug discovery. These non-coding RNA regions are found in a wide variety of bacteria, including pathogenic bacteria, and respond to metabolic effector molecules to regulate transcription and translation of important bacterial genes (1, 2). The T box transcription antitermination riboswitch binds and structurally responds to non-aminoacylated (uncharged) cognate tRNA to regulate transcription of amino acid biosynthesis, transport and metabolism genes (3). The riboswitch is located within the 5'-untranslated region (5'-UTR) of the mRNA and forms specific base pairs with the tRNA both at the anticodon and at the acceptor end (Figure 1) (4, 5). The former base pairing confers the cognate specificity while the latter base pairing plays a direct role in the structural response of the riboswitch to the uncharged cognate tRNA (3).

There are multiple conserved structural and sequence elements in the 5'-UTR of genes regulated by the T box riboswitch mechanism (6–8). It is the base pairing of the uncharged tRNA acceptor end nucleotides with four nucleotides in the bulge of an antiterminator element in the 5'-UTR, however, that turns the riboswitch “on” by stabilizing the antiterminator (9). The antiterminator element alone is thermodynamically less stable than an alternative structural element called the terminator. Consequently, without uncharged cognate tRNA present, the terminator forms preferentially and transcription is terminated shortly after the terminator is transcribed and before the protein coding region of the mRNA is reached (Figure 1b) (3). Since the antiterminator and terminator elements share common nucleotides, only one structural element can exist at a time. During transcription, the

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Supplementary Material

UV-monitored thermal denaturation data is provided in the Supplementary Material.

riboswitch binds uncharged cognate tRNA (if present in sufficient quantities) and the tRNA stabilizes the antiterminator before the full terminator sequences are transcribed (9, 10). In this manner, the riboswitch can respond to metabolic needs by switching “on” transcription of the entire mRNA in response to the metabolic signal of high levels of uncharged cognate tRNA.

The T box antiterminator RNA is an ideal target for drug discovery given the importance of the genes regulated by the T box riboswitch and the high sequence and secondary structure conservation of the antiterminator element (6–8). As a constitutively “off” riboswitch (i.e., one that is “off” unless the metabolic effector molecule binds), the T box riboswitch presents a unique challenge from a drug discovery perspective. The challenge is to develop ligands with high specificity and affinity that do not overly stabilize the antiterminator, or turn “on” the riboswitch, in a tRNA-independent manner. The goal is to find small molecules that disrupt tRNA binding without providing excessive stabilization to the antiterminator that might result in the ligand-antiterminator complex precluding the formation of the terminator element of the riboswitch. We have previously reported ligand classes that bind T box antiterminator RNA in a specific manner (11–16). As part of a comprehensive drug discovery project focused on targeting the T box antiterminator RNA, we report in this paper 1,4-disubstituted 1,2,3-triazole ligands that affect antiterminator stability as determined using a novel, fluorescence-monitored thermal denaturation assay (Figure 2).

Methods and Materials

Synthesis and RNA Preparation

All 1,4-disubstituted 1,2,3-triazoles were prepared as previously described (14). Double-labeled T box antiterminator RNA 5'-Carboxyrhodamine-AM1A-3'-BlackHoleQuencher2 (5'-ROX-AM1A-BHQ2, Figure 2b) was purchased from Trilink BioTechnologies, Inc. in a PAGE and RP-HPLC purified form and dialyzed in 10 mM NaH₂PO₄ pH 6.5, 0.01 mM EDTA prior to use.

Melting Temperature (T_m) assay development

A fluorescence-monitored thermal denaturation assay was developed to monitor T_m changes in a multiplex assay. To evaluate the relevance of the method, the T_m of 5'-ROX-AM1A-BHQ2 was measured at different NaCl concentrations. The 5'-ROX-AM1A-BHQ2 was renatured by heating to 90 °C for 1.5 min followed by slow cooling to room temperature. The T_m experiments consisted of 100 nM 5'-ROX-AM1A-BHQ2 and various NaCl concentrations (0, 10, 20 and 50 mM) in 10 mM NaH₂PO₄ pH 6.5, 0.01 mM EDTA buffer. The samples were incubated in a Statagene Mx3000P qPCR instrument at 30 °C for 15 min at which point, the temperature was increased 1 degree and maintained for 3 minutes for each temperature step before recording the fluorescence (λ_{ex} = 585 nm, λ_{obs} = 610 nm). A total of 61 temperature steps were completed to reach the final temperature of 90 °C. The T_m was determined from the first derivative of the data calculated using OD Deriv (17). For DMSO control experiments, the conditions were the same as described with the addition of 5% (v/v) DMSO in 10 mM NaH₂PO₄, 0.01 mM EDTA buffer. For comparison studies, UV-monitored thermal denaturation data was obtained as previously described (18).

T_m ligand screening

The fluorescence-monitored single ligand concentration T_m screening was measured using a Statagene Mx3000P qPCR instrument. Each experiment contained a final concentration of 100 nM 5'-ROX-AM1A-BHQ2 and 100 μ M ligand in 10 mM NaH₂PO₄, pH 6.5, 0.01 mM EDTA, 5% (v/v) DMSO buffer. Fluorescence-monitored thermal denaturation data of duplicate experiments were acquired as described above and the T_m was determined. The

average AM1A melting temperature changes (ΔT_m) were calculated using the equation $\Delta T_m = T_m - T_{m0}$, where the T_{m0} is the AM1A melting temperature in the absence of ligand and T_m is the melting temperature in the presence of the ligand.

Results and Discussion

Multiplex thermal stability assay

Molecular beacons have been used in a variety of molecular biology applications, including real-time PCR, monitoring nucleic acid-protein interactions and in vivo RNA detection (19). The fluorescence-monitored thermal denaturation profile of molecular beacons has been used to monitor the stability and unfolding of nucleic acid structures (20) and to identify small molecules that could stabilize stem-loop RNA structures (21). A molecular beacon thermal denaturation profile is obtained by measuring the fluorescence intensity as a function of temperature. Due to the addition of fluorescent groups at the end of the nucleic acid stem region, the fluorescently measured T_m is often higher than the T_m measured by UV-monitored thermal denaturation. Different fluorophores and quenchers differentially contribute to the thermodynamic stability of the molecular beacons (22).

In this study, T box antiterminator model RNA AM1A was double-labeled with Carboxy-X-Rhodamine (ROX) at the 5' end and Black Hole Quencher 2 (BHQ2) at the 3' end to construct molecular beacon 5' ROX-AM1A-BHQ2 (Figure 2b). ROX was selected because its quantum yield does not change significantly with increasing temperature (23). In order to achieve the maximum quenching efficiency, the fluorophore-quencher pair should have adequate spectral overlap. Thus, BHQ2 was selected due to the good spectral overlap between the BHQ2 absorbance spectrum and the ROX emission spectrum (23).

The fluorescence-monitored T_m assay was based on the premise that when the AM1A exists in its native secondary structure, the A1 helix conformation brings ROX and BHQ2 in close proximity where the fluorescence of ROX is effectively quenched. As the temperature increases, the nucleic acid starts to denature from the native secondary structure to a random linear polynucleotide (24). During this process ROX and BHQ2 are separated from each other, leading to the observed increase in fluorescence (Figure 2a).

The validity of monitoring ligand-induced stabilization using the fluorescently labeled AM1A was evaluated by comparison to unlabeled AM1A and by monitoring the effect of conditions known to affect RNA stability. Results are shown in Figure 3 and summarized in Table 1. The fluorescence-monitored T_m observed for ROX-AM1A-BHQ2 (71°C) was higher than that observed by UV-monitored thermal denaturation (24) of unlabeled AM1A in the same buffer ($T_m = 51^\circ\text{C}$, Supplementary Material). This is likely due to the end labeled fluorophore and quencher pair stacking on the first base pair of the double helix. This effect has been observed in similar nucleic acid fluorophore/quencher constructs and contributes to the overall structural stability of the nucleic acid (25).

Monovalent cations are known to stabilize RNA secondary structures and increase the observed T_m (26). As expected, an increase in the concentration of Na^+ resulted in an increase in the T_m observed by the fluorescence-monitored thermal denaturation method (Figure 3a and Table 1). The Na^+ -dependent shift in T_m for unlabeled AM1A is comparable (Supplementary Material), indicating that conjugation with the fluorophore and quencher does not affect the dependence of AM1A stability on buffer ionic conditions. Overall, the data indicate that the stability of the ROX-AM1A-BHQ2 is suitably responsive to environmental conditions and that the fluorescence monitored ΔT_m can be utilized in screening for ligand-induced stabilization.

Since DMSO is known to denature nucleic acids at high concentrations (27), the effect of 5% (v/v) DMSO on the T_m was tested to determine if the ligand stock solution solvent would affect the T_m results. No significant change in the T_m was observed when DMSO was present (Figure 3b and Table 1) indicating that the relatively low final concentration of DMSO in the assay buffer does not interfere with the results.

Previous studies have indicated that Mg^{2+} facilitates both complete tRNA binding to antiterminator model RNA (28) and in vitro transcription antitermination (4). Consequently, we wished to investigate the effect of Mg^{2+} on the fluorescently monitored thermal denaturation. However, at 5 mM Mg^{2+} , the minimal concentration used in tRNA-antiterminator binding studies (29), the ROX-AM1A-BHQ2 was so stabilized that a T_m could not be determined due to insufficient denaturation even at the highest temperature (Supplementary Material). The fluorophore/quencher labels stabilize the antiterminator model RNA to such an extent that it is a challenge to determine the T_m even with just the addition of monovalent cations (e.g., 50 mM NaCl, figure 3a). While this precludes the use of the fluorescently monitored T_m assay in studying the tRNA-antiterminator complex, the low salt conditions of the assay are still relevant for looking at small molecule-RNA interactions. The structure of the antiterminator model RNA is not significantly dependent on Mg^{2+} nor salt until 100 mM NaCl and even then, the excess salt only results in selective stabilization of one of multiple conformers that is already present at lower salt concentrations (30).

Ligand binding site implications

The ligand-induced ΔT_m of 1,4-disubstituted 1,2,3-triazoles was determined at 100 μ M, the same concentration previously used to screen for antiterminator binding (14). The majority of the compounds either had no effect on the T_m at the concentration tested or led to a slight increase in the T_m (Table 2). The compounds that most stabilized ROX-AM1A-BHQ2 (positive ΔT_m) were GHB-7, GHB-74, GHB-75, GHB-77, GHB-109. The compound that most destabilized the antiterminator was GHB-110. This is consistent with other ligand-induced ΔT_m RNA studies. Typically, if there is an effect, ligands that bind RNA tend to stabilize the RNA secondary structure, as determined by UV-monitored thermal denaturation. There are instances, however, where ligands have been shown to destabilize the secondary structure, resulting in a reduction in the observed T_m (31). Interestingly, there is evidence for very structure-specific ligand-induced T_m effects since the ΔT_m for GHB-7 was 3.2°C while GHB-26 had no significant effect on the T_m ($\Delta T_m=0.2^\circ\text{C}$). These two compounds differ only by a methylene group within NR_2 suggesting that the exact length of the alkyl phenyl chain (and thereby placement of the functional group when bound to AM1A) may play a role in ligand-specific binding and stabilization of the RNA.

Conclusion & Future Directions

The results from this study indicate that the fluorescence-monitored thermal denaturation assay is a useful method for screening ligands to determine whether or not they stabilize the T box antiterminator RNA element. More importantly, through the use of this assay, several 1,4-disubstituted 1,2,3-triazoles were identified that significantly altered the observed stability of the antiterminator model RNA AM1A in a structure-dependent manner. A ligand-induced ΔT_m does not, a priori, indicate that the ligand will be effective at disrupting the tRNA-antiterminator RNA complex, however, it is very intriguing that GHB-7, one of the compounds with the greatest shift in T_m , is also one of the compounds that resulted in the greatest disruption of the tRNA-antiterminator complex in a fluorescence anisotropy assay (32). Further studies are in progress to characterize the interactions of the lead triazole compounds with AM1A in order to determine what specific structural features lead to

stabilization or destabilization of the antiterminator model RNA. This information will be vital for the development of potential antibacterial agents that target the T box riboswitch.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Smith AM, Fuchs RT, Grundy FJ, Henkin TM. Riboswitch RNAs: Regulation of gene expression by direct monitoring of a physiological signal. *RNA Biol.* 2010; 7:104–10. [PubMed: 20061810]
2. Zhang J, Lau MW, Ferre-D'Amare AR. Ribozymes and riboswitches: modulation of RNA function by small molecules. *Biochemistry.* 2010; 49:9123–32. [PubMed: 20931966]
3. Green NJ, Grundy FJ, Henkin TM. The T box mechanism: tRNA as a regulatory molecule. *FEBS Lett.* 2010; 584:318–24. [PubMed: 19932103]
4. Yousef MR, Grundy FJ, Henkin TM. Structural transitions induced by the interaction between tRNA^{Gly} and the *Bacillus subtilis* *glyQS* T box leader RNA. *J Mol Biol.* 2005; 349:278–87.
5. Nelson A, Henkin TM, Agris PF. tRNA regulation of gene expression: interactions of an mRNA 5'-UTR with a regulatory tRNA. *RNA.* 2006; 12:1254–61. [PubMed: 16741230]
6. Gutierrez-Preciado A, Henkin TM, Grundy FJ, Yanofsky C, Merino E. Biochemical features and functional implications of the RNA-based T box regulatory mechanism. *Microbiol Mol Biol Rev.* 2009; 73:36–61. [PubMed: 19258532]
7. Vitreschak AG, Mironov AA, Lyubetsky VA, Gelfand MS. Comparative genomic analysis of T-box regulatory systems in bacteria. *RNA.* 2008; 14:717–35. [PubMed: 18359782]
8. Wels M, Kormelink TG, Kleerebezem M, Siezen RJ, Francke C. An in silico analysis of T-box regulated genes and T-box evolution in prokaryotes, with emphasis on prediction of substrate specificity of transporters. *BMC Genomics.* 2008; 9:330–45. [PubMed: 18625071]
9. Grundy FJ, Yousef MR, Henkin TM. Monitoring uncharged tRNA during transcription of the *Bacillus subtilis* *glyQS* gene. *J Mol Biol.* 2005; 346:73–81. [PubMed: 15663928]
10. Grundy FJ, Henkin TM. Kinetic analysis of tRNA-directed transcription antitermination of the *Bacillus subtilis* *glyQS* gene in vitro. *J Bacteriol.* 2004; 186:5392–9. [PubMed: 15292140]
11. Means JA, Hines JV. Fluorescence resonance energy transfer studies of aminoglycoside binding to a T box antiterminator RNA. *Bioorg Med Chem Lett.* 2005; 15:2169–72. [PubMed: 15808490]
12. Means JA, Katz SJ, Nayek A, Anupam R, Hines JV, Bergmeier SC. Structure activity studies of oxazolidinone analogs as RNA-binding agents. *Bioorg Med Chem Lett.* 2006; 16:3600–4. [PubMed: 16603349]
13. Anupam R, Bergmeier SC, Green NJ, Grundy FJ, Henkin TM, Means JA, et al. 4,5-Disubstituted Oxazolidinones: High affinity molecular effectors of RNA function. *Bioorg Med Chem Lett.* 2008; 18:3541–4. [PubMed: 18502126]
14. Acquaah-Harrison G, Zhou S, Hines JV, Bergmeier SC. Library of 1,4-disubstituted 1,2,3-triazole analogs of oxazolidinone RNA-binding agents. *J Comb Chem.* 2010; 12:491–6. [PubMed: 20557032]
15. I, Maciagiewicz; S, Zhou; Bergmeier, SC.; Hines, JV. Structure activity studies of RNA-binding oxazolidinone derivatives. *Bioorg Med Chem Letters.* 2011:4524–7.
16. Orac, CM.; Zhou, S.; Means, JA.; Boehme, D.; Bergmeier, SC.; Hines, JV. Synthesis and stereospecificity of 4,5-disubstituted oxazolidinone ligands binding to T-box riboswitch RNA. *J Med Chem.* 2011. In press <http://dx.doi.org/10.1021/jm2006904>
17. Draper DE, Gluck TC. Melting studies of RNA unfolding and RNA-ligand interactions. *Methods Enzymol.* 1995; 259:281–305. [PubMed: 8538459]

18. Gerdeman MS, Henkin TM, Hines JV. *In vitro* structure-function studies of the *Bacillus subtilis* *tyrS* mRNA antiterminator: Evidence for factor independent tRNA acceptor stem binding specificity. *Nucleic Acids Res.* 2002; 30:1065–72. [PubMed: 11842119]
19. Goel G, Kumar A, Puniya AK, Chen W, Singh K. Molecular beacon: a multitask probe. *J Appl Microbiol.* 2005; 99:435–42. [PubMed: 16108784]
20. Darby RA, Sollogoub M, McKeen C, Brown L, Risitano A, Brown N, et al. High throughput measurement of duplex, triplex and quadruplex melting curves using molecular beacons and a LightCycler. *Nucleic Acids Res.* 2002; 30:e39. [PubMed: 11972354]
21. Donahue CP, Ni J, Rozners E, Glicksman MA, Wolfe MS. Identifications of tau stem loop RNA stabilizers. *J Biomol Screen.* 2007; 12:789–99. [PubMed: 17525136]
22. Owczarzy R, Moreira BG, You Y. Effects of fluorescent dyes, quenchers, and dangling ends on the thermodynamic stability of DNA duplexes. *Biophysical Journal.* 2004; 86:310A-A.
23. Marras SAE. Interactive fluorophore and quencher pairs for labeling fluorescent nucleic acid hybridization probes. *Molecular Biotech.* 2008; 38:247–55.
24. Puglisi JD, Tinoco I Jr. Absorbance melting curves of RNA. *Methods Enzymol.* 1989; 180:304–25. [PubMed: 2482421]
25. Moreira BG, You Y, Behlke MA, Owczarzy R. Effects of fluorescent dyes, quenchers, and dangling ends on DNA duplex stability. *Biochem Biophys Res Commun.* 2005; 327:473–84. [PubMed: 15629139]
26. Owczarzy R, You Y, Moreira BG, Manthey JA, Huang LY, Behlke MA, et al. Effects of sodium ions on DNA duplex oligomers: improved predictions of melting temperatures. *Biochemistry.* 2004; 43:3537–54. [PubMed: 15035624]
27. Strauss JH Jr, Kelly RB, Sinsheimer RL. Denaturation of RNA with dimethyl sulfoxide. *Biopolymers.* 1968; 6:793–801. [PubMed: 5654613]
28. Means JA, Simson CM, Zhou S, Rachford AA, Rack J, Hines JV. Fluorescence probing of T box antiterminator RNA: Insights into riboswitch discernment of the tRNA discriminator base. *Biochem Biophys Res Commun.* 2009; 389:616–21. [PubMed: 19755116]
29. Means JA, Wolf S, Agyeman A, Burton JS, Simson CM, Hines JV. T box riboswitch antiterminator affinity modulated by tRNA structural elements. *Chem Biol Drug Design.* 2007; 69:139–45.
30. Jack KD, Means JA, Hines JV. Characterizing riboswitch function: Identification of Mg²⁺ binding site in T box antiterminator RNA. *Biochem Biophys Res Commun.* 2008; 370:306–10. [PubMed: 18371302]
31. Fernandez-Saiz M, Schneider H-J, Sartorius J, Wilson WD. A cationic cyclophane that forms a base-pair open complex with RNA duplexes. *J Am Chem Soc.* 1996; 118:4739–45.
32. Zhou S, Acquaah-Harrison G, Bergmeier SC, Hines JV. Anisotropy studies of tRNA - T box antiterminator RNA complex in the presence of 1,4-disubstituted 1,2,3-triazoles. *Bioorg Med Chem Lett.* 2011 In press. 10.1016/j.bmcl.2011.09.095

Figure 1. T box riboswitch

Schematic of T box riboswitch mechanism for a) transcription termination in the presence of excess aminoacylated cognate tRNA and b) transcription antitermination in the presence of excess non-aminoacylated cognate tRNA.

Figure 2. Fluorescence monitored thermal denaturation screening assay

a) Schematic of fluorescence-monitored thermal denaturation assay. Upon thermal denaturation, the fluorophore switches from a quenched (open red circle) to unquenched (filled red circle) state. b) Antiterminator model RNA AM1A (labeled with Carboxyrhodamine (ROX) at the 5'-end and with Black-hole-quencher 2 (BHQ2) at the 3'-end). c) 1,4-disubstituted 1,2,3-triazole ligand

Figure 3. T_m model study of fluorescently labeled antiterminator model RNA AM1A
Fluorescence-monitored thermal denaturation of ROX-AM1A-BHQ2 in the presence of a) differing concentrations of Na^+ and b) 5% (v/v) DMSO where dF/dT is the change in fluorescence intensity at 610 nm as a function of change in temperature. The T_m is the temperature at maximal dF/dT .

Table 1

T_m (°C) of ROX-AM1A-BHQ2 determined using fluorescence-monitored thermal denaturation method.^a

[NaCl] (mM)	DMSO (v/v)	T_m (°C)
0	-	71.3
10	-	73.5
20	-	75.5
50	-	80.4
0	5%	71.2

^a T_m measured in 10 mM NaHPO₄, pH 6.5, 0.01 mM EDTA with 100 nM ROX-AM1A-BHQ2

Table 2

Ligand-induced ΔT_m ($^{\circ}\text{C}$) of ROX-AM1A-BHQ2^a

Ligand	NR ₂	R ¹	R ²	R ³	ΔT_m^b
GHB-7	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O)NHCH ₂ Ph	H	3.2
GHB-9	N(CH ₃)(CH ₂) ₃ Ph	H	<i>n</i> -C ₄ H ₉	H	0.7
GHB-10	4-Ph-piperazine	H	(CH ₂) ₄		-0.5
GHB-11	3-(CO ₂ Et)-piperidine	H	<i>n</i> -C ₄ H ₉	H	-0.2
GHB-12	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	<i>n</i> -C ₄ H ₉	H	-0.2
GHB-13	4-Ph-piperazine	H	<i>n</i> -C ₄ H ₉	H	-0.2
GHB-14	3-(CO ₂ Et)-piperidine	H	(CH ₂) ₄		-0.3
GHB-15	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	(CH ₂) ₄		-0.7
GHB-16	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	<i>n</i> -C ₄ H ₉	H	-0.2 ^c
GHB-17	N(CH ₃)(CH ₂) ₂ Ph	H	(CH ₂) ₄		-0.3
GHB-18	N(<i>n</i> -C ₄ H ₉) ₂	H	(CH ₂) ₄		0.4 ^c
GHB-19	4-Ph-piperidine	H	<i>n</i> -C ₄ H ₉	H	-0.2
GHB-20	4-Ph-piperidine	H	(CH ₂) ₄		0.3
GHB-21	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	(CH ₂) ₄		-0.8
GHB-22	4-Ph-piperazine	H	Ph	H	0.9
GHB-23	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	Ph	H	-0.7
GHB-24	4-Ph-piperazine	H	CH ₂ OCH ₂ Ph	H	0.4
GHB-25	3-(CO ₂ Et)-piperidine	H	CH ₂ OCH ₂ Ph	H	0.6
GHB-26	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OC(O)NHCH ₂ Ph	H	-0.2
GHB-27	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)NHCH ₂ Ph	H	-0.2
GHB-28	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)NHCH ₂ Ph	H	-0.2
GHB-29	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O)NHCH ₂ Ph	H	0.7
GHB-30	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)NHCH ₂ Ph	H	-0.3
GHB-31	4-Ph-piperidine	C(O)Ph	(CH ₂) ₄		-0.3
GHB-32	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)Ph	(CH ₂) ₄		-0.2
GHB-33	N(CH ₃)(CH ₂) ₃ Ph	C(O)CH ₂ OPh	(CH ₂) ₄		-0.2

Ligand	NR ₂	R ¹	R ²	R ³	ΔT_m^b
GHB-34	4-Ph-piperidine	C(O)CH ₂ OPh	(CH ₂) ₄		0.3
GHB-35	4-Ph-piperazine	C(O)CH ₂ OPh	(CH ₂) ₄		-0.2
GHB-36	N(CH ₃)(CH ₂) ₂ Ph	C(O)CH ₂ OPh	(CH ₂) ₄		0.4
GHB-37	N(<i>n</i> -C ₄ H ₉) ₂	C(O)CH ₂ OPh	(CH ₂) ₄		0.4
GHB-38	3-(CO ₂ Et)-piperidine	C(O)CH ₂ OPh	(CH ₂) ₄		0.8
GHB-39	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)CH ₂ OPh	(CH ₂) ₄		0.7
GHB-40	4-Ph-piperazine	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-0.3
GHB-41	4-Ph-piperazine	H	CH ₂ OC(O)Ph	H	-0.3
GHB-42	morpholine	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-0.2
GHB-43	morpholine	H	CH ₂ OC(O)Ph	H	-0.1
GHB-44	N(<i>n</i> -C ₄ H ₉) ₂	C(O)CH ₂ Ph	(CH ₂) ₄		0.3
GHB-45	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	C(O)CH ₂ Ph	(CH ₂) ₄		0.4
GHB-46	morpholine	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	0.4
GHB-47	morpholine	C(O)CH ₂ OPh	(CH ₂) ₄		0.9
GHB-48	3-(CO ₂ Et)-piperidine	C(O)CH ₂ Ph	(CH ₂) ₄		0.7
GHB-49	3-(CO ₂ Et)-piperidine	C(O)Ph	(CH ₂) ₄		-0.3
GHB-50	N(CH ₃)CH ₂ Ph	H	Ph	H	-0.1
GHB-51	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	C(O)Ph	(CH ₂) ₄		0.9
GHB-57	4-Ph-piperazine	H	CH ₂ OC(O)NHCH ₂ Ph	H	0.7
GHB-58	4-Ph-piperazine	C(O)Ph	(CH ₂) ₄		-0.3
GHB-59	4-Ph-piperazine	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	-0.3
GHB-60	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	C(O)CH ₂ OPh	(CH ₂) ₄		-0.3
GHB-61	4-Ph-piperidine	H	CH ₂ OC(O)NHCH ₂ Ph	H	0.2
GHB-62	morpholine	H	(CH ₂) ₄		0.3
GHB-63	morpholine	H	<i>n</i> -C ₄ H ₉	H	-0.1
GHB-64	morpholine	H	Ph	H	-0.1
GHB-65	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OCH ₂ Ph	H	0.4
GHB-66	morpholine	H	CH ₂ OCH ₂ Ph	H	0.8
GHB-67	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	0.7

Ligand	NR ₂	R ¹	R ²	R ³	ΔT_m^b
GHB-68	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-0.3
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	-0.3
GHB-70	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)NH(<i>n</i> -C ₄ H ₉)	H	-0.3
GHB-71	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	0.7
GHB-72	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	0.7
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	0.7
GHB-74	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OC(O) <i>c</i> -C ₆ H ₁₁	H	1.6
GHB-75	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	4.1 ^c
GHB-76	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	0.3
GHB-77	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OC(O)Ph	H	1.6
GHB-78	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OC(O)Ph	H	0.7
GHB-79	N(<i>n</i> -C ₄ H ₉) ₂	H	CH ₂ OC(O)Ph	H	-0.2
GHB-80	3-(CO ₂ Et)-piperidine	H	CH ₂ OC(O)Ph	H	0.7
GHB-81	N(CH ₃)(CH ₂) ₂ CH(CH ₃) ₂	H	CH ₂ OC(O)Ph	H	0.7
GHB-82	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	CH ₂ OC(O)Ph	H	0.7
GHB-83	morpholine	C(O)Ph	(CH ₂) ₄		0.2
GHB-84	morpholine	C(O)CH ₂ Ph	(CH ₂) ₄		-0.4
GHB-107	morpholine	H	CH ₂ OC(O) <i>n</i> -C ₇ H ₁₅	H	0.1
GHB-108	N(CH ₃)(CH ₂) ₂ Ph	H	<i>n</i> -C ₄ H ₉	H	-0.2 ^d
GHB-109	N(<i>n</i> -C ₄ H ₉) ₂	H	<i>n</i> -C ₄ H ₉	H	1.1
GHB-110	3-(CO ₂ Et)-piperidine	H	Ph	H	-0.9
GHB-111	N(CH ₃)CH ₂ (<i>c</i> -C ₆ H ₁₁)	H	Ph	H	0.1
GHB-112	N(CH ₃)(CH ₂) ₃ Ph	H	CH ₂ OCH ₂ Ph	H	0.1
GHB-113	N(CH ₃)(CH ₂) ₂ Ph	H	CH ₂ OCH ₂ Ph	H	0.1

^a All compounds (100 μM) were assayed in 10 mM NaH₂PO₄, pH 6.5, 0.01 mM EDTA, 5% (v/v) DMSO with 100 nM ROX-AM1A-BHQ2. λ_{ex} = 585 nm, λ_{em} = 610 nm, heating rate = 0.33 °C/min.

^b Average ΔT_m values where ΔT_m = T_m - T_{m0} and standard deviation of duplicate experiments 0.5°C unless otherwise noted.

^c Standard deviation of duplicate experiments 0.6°C

p Standard deviation of duplicate experiments 0.8°C