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Synthesis and Testing of a Focused Phenothiazine Library for Binding to HIV-1 TAR RNA

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Summary

We have synthesized a series of phenothiazine derivatives, which were used to test the structure-activity relationship of binding to HIV-1 TAR RNA. Variations from our initial compound, 2-acetylphenothiazine, focused on two moieties: ring substitutions and n-alkyl substitutions. Binding characteristics were ascertained via NMR, principally by saturation transfer difference spectra of the ligand and imino proton resonance shifts of the RNA. Both ring and alkyl substitutions manifested NMR changes upon binding. In general, the active site, while somewhat flexible, has regions that can be capitalized for increased binding through van der Waals interactions and others that can be optimized for solubility in subsequent stages of development. However, binding can be nontrivially enhanced several-fold through optimization of van der Waals and hydrophilic sites of the scaffold.

Introduction

Synthesis and evaluation of focused libraries of small molecules for binding to specific targets are crucial for generating leads in the drug discovery process. During the knowledge-based drug design process, repeated cycles of synthesis and testing of smaller libraries while optimizing for a desired specificity or affinity is an efficient way to generate high-quality lead compounds [1, 2]. Although RNA is a potentially valuable drug target, few compounds specifically targeting RNA are currently on the market; some antibiotics in current clinical use provide a precedent for targeting RNA since they bind to conserved microbial rRNA structural motifs. One advantage of targeting RNA compared to developing drugs against protein receptors is that it can open new fronts in the fight against diseases [3].

According to World Health Organization estimates, 42 million people are living with HIV/AIDS worldwide. With growing resistance of the retrovirus HIV-1 (human immunodeficiency virus, type 1) to current drugs, there is need for research on other HIV-1 targets. The HIV-1 genomic RNA itself presents possibilities. One of the more prominent RNA targets is the HIV-1 TAR RNA motif, which plays an important role in the life cycle of HIV [4, 5]. Use of a selective inhibitor that blocks the interaction of TAR and the virus-encoded protein Tat, which regulates RNA transcriptase processivity, is one possible way of keeping the virus from proliferating [6]. A number of recent studies have identified small-molecule ligands that bind to TAR RNA, thus inhibiting Tat binding or Tat transactivation [7–10].

We have previously identified a series of "hits," i.e., compounds that specifically bind HIV-1 TAR RNA, by using a sequential combination of virtual screening followed by limited experimental screening [7]. Some of these hits belong to a class of compounds called phenothiazines, which have been used as antipsychotic agents for many years. Besides its proven high bioavailability and low toxicity, relative to other RNA binding drugs, phenothiazine is an attractive scaffold for potential lead development due to its low molecular weight and single positive charge, which afford ample room to build in enhanced binding affinity.

By testing a small set of commercially available phenothiazines against the TAR RNA structure, we were able to establish that this class of compounds is an interesting scaffold for further derivatization [11]. NMR is especially useful at this stage of the optimization process because the tested compounds do not yet have a high affinity, thus requiring methods that are able to identify and classify weak interactions. NMR is also quite robust in that no false positives or negatives are found in NMR screening, where a hit is defined as a small molecule interacting specifically with the active site of the biomolecule. NMR also allows us to determine the affinity and specificity of the small molecules tested by monitoring imino proton chemical shifts of the RNA target [12, 13]. Differential line broadening (DLB) has been used for many years to identify ligand moieties involved in binding interactions [14]. Similarly, saturation transfer difference (STD) NMR has proven to be efficient in identifying and characterizing weakly binding compounds even to small RNA constructs [15].

The initial testing of commercial phenothiazines showed that substitutions around the phenothiazine ring and the presence of a basic amine functionality were important structural features for binding activity [11]. An NMR structure of acetylpromazine complexed with the HIV-1 TAR RNA construct has also been solved in our lab [16], and we used this structure to illuminate the SAR data obtained (Figure 1). The same 27 nt RNA construct used in structural elucidation of the acetylpromazine TAR RNA complex is utilized for the binding studies reported here. The objective was to generate derivatives of the known ligand basic phenothiazine scaffold in order to ascertain some aspects of a structure-activity relationship. To identify the crucial binding interactions of the original scaffold, two libraries were designed: the first one to probe substitutions of the phenothiazine ring system, and the second one to probe aliphatic side chain substitutions.

Results and Discussion

Chemistry

We set out to evaluate structure-activity relationships for binding of a series of compounds with the

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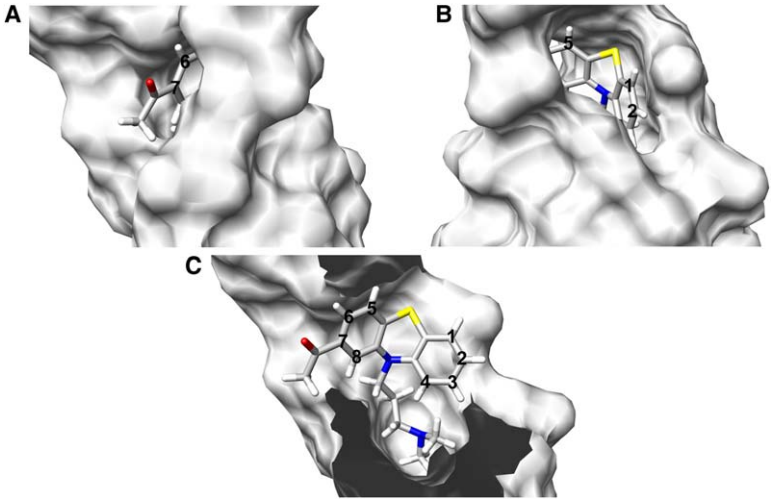


Figure 1. Binding Site of TAR RNA with Acetylpromazine Bound

See Compound 6 in Figure 3. The NMR structure has PDB code 1LVJ [16].

(A) Perspective of the phenothiazine scaffold from the minor groove.

(B) Perspective of the phenothiazine scaffold from the major groove.

(C) Perspective of the entire compound. The second half of RNA was removed for visualization purposes.

phenothiazine scaffold to TAR RNA. A concomitant goal was to explore the ability to moderate the potency of the commercially available compounds already tested. We dissected the scaffold into two parts: substitutions of the phenothiazine rings and substitution of the n-alkyl linker variations. We proceeded to synthesize chemical libraries with modifications in each of those moieties.

The phenothiazine ring system was constructed from diarylamine starting materials by an iodine-catalyzed reaction with sulfur [17]. We performed many variations of this reaction in an attempt to improve the generally low yields and found significant improvement by conducting the cyclization with microwave irradiation. While microwave conditions have been briefly described before for the synthesis of phenothiazines, they relied upon the use of strong acid or Lewis acid catalysts or the use of dry conditions [18, 19]. Under our optimized conditions, the reaction is carried out in water in a sealed reaction vessel and is heated to 190°C for 20 min under microwave irradiation (Figure 2A, Compounds 1 and 2). This method was very convenient, since the hydrophobic product immediately precipitated upon cooling and could easily be purified by filtration. This new, to our knowledge, method for the synthesis of phenothiazines was successfully used to synthesize a set of new substituted 10*H*-phenothiazines (Figure 2A, Compound 2). The 10*H*-phenothiazines were then alkylated by using sodium hydride and 1-chloro-3-iodopropane (or appropriate chloroiodoalkane) to give the chloroalkylphenothiazine (Figure 2A, Compound 3). This intermediate could then be aminated with dimethylamine (or another secondary amine) in a sealed vessel with microwave irradiation to afford the ring-substituted phenothiazine library (Figure 2A, Compound 4). The subset of these compounds that were soluble and stable enough to be tested for binding by NMR is reported in Figure 3.

For evaluation of side chain substituents, we decided to fix the ring scaffold as 2-acetylphenothiazine and 2-chlorophenothiazine and to vary the length of the alkyl linkers and the substituents on the basic amine group (Figure 2B). We again alkylated the 10*H*-phenothiazine ring by using sodium hydride and four chloroiodo alkyl chains, the length of which varied from 3 to 6 methylene units (Figure 2B, Compounds 5–7). We found that the resulting alkyl chloride intermediate was not sufficiently reactive to yield product with a diverse set of amines, so we used the Finkelstein reaction to convert the chloroalkane to an iodoalkane (Figure 2B, Compound 8). The iodoalkane intermediate then could be reacted with a diverse set of primary and secondary amines to afford the completed side chain variation library (Figure 2B, Compound 9). In general, these reactions proceeded quite cleanly, with the major side reaction being the elimination of the iodide in longer linkers. For this set, those compounds soluble and stable enough to be tested by NMR are reported in Figure 4.

Library Screening

STD NMR experiments and differential line broadening (DLB) of proton resonances on the ligand enabled assessment of ligand binding to the RNA target [12, 14, 15]. RNA imino proton chemical shifts were monitored to identify and characterize the location of small-molecule binding to the 27 nt construct of HIV-1 TAR RNA. Only stable, water-soluble compounds were utilized for these studies.

The affinities of the compounds for the RNA construct were divided into three categories by a combination of DLB and STD effects. For binders deemed more promising by ligand NMR results, RNA imino proton chemical shift measurements were also carried out. We were able to classify 24 compounds according to their RNA binding interactions manifest in resonance line broadening and STD spectral intensities. Figure 5 shows reference and STD spectra of two selected compounds from the libraries. Compound 17 exhibited strong line broadening effects and substantial STD enhancements (Figure 5A). Compounds with these characteristics were categorized as strong binders. On the other hand, Compound 23 shows only weak broadening of the aromatic protons, at similar ligand and RNA concentrations as for Compound 17 (Figure 5B). Compounds showing these characteristics were categorized as weak binders.

Nine compounds were examined to determine the effects of aromatic ring substitutions on binding affinity (Figure 3). For analysis, we divided the substituents by location on the ring system (see Figure 1 for numbering).

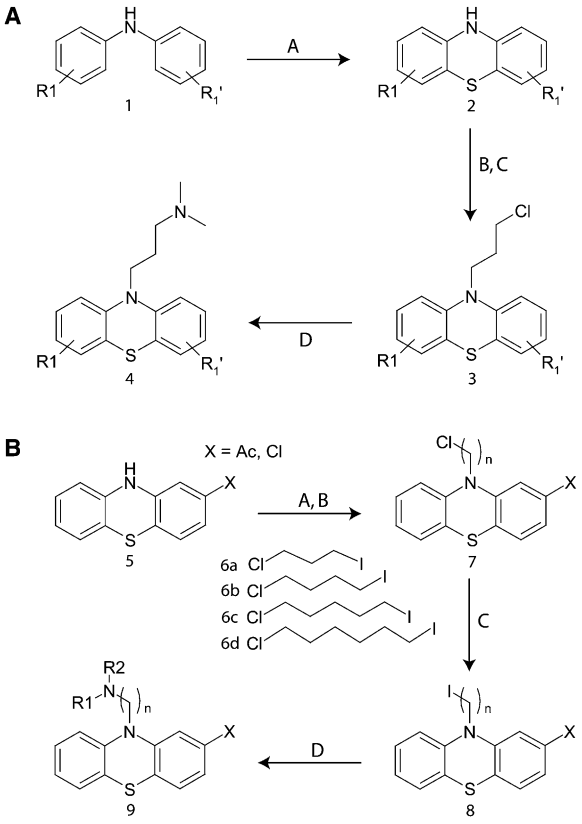


Figure 2. Synthesis of a 10*H*-Phenothiazine Library

For more information, see Experimental Procedures ("Synthesis of Compounds") and Results and Discussion ("Chemistry").

(A) Ring variant syntheses. The conditions for syntheses are: (A) 2 eq. S_8, catalyst I_2, water, 50 W microwave, 190°C, 20 min; (B) 1.2 eq. NaH, DMF, 0°C, 1 hr; (C) 5 eq. $Cl(CH_2)_3I$ (slow reverse addition), RT, 1 hr; (D) 4 eq. dimethylamine (8{1}), 2M THF, DMF, 30 W microwave, 100°C, 40 min.

(B) Side chain variations. The conditions for syntheses are: (A) 2.1 eq. NaH, THF, DMSO; (B) 3 eq. $Cl(CH2)_nI$, (6a–6d), reverse add., THF, DMSO, RT, 2 hr; (C) 5 eq. KI, 2-methyl-4-propanone, 130°C, reflux, 40 hr; (D) 2 eq. HNR1R2, DMF, 60°C, 20 hr.

Note, however, that the symmetry of the phenothiazine ring system requires us to consider whether a substituent is exerting its influence on the minor groove or major groove side of the complex. Bond rotation around the linker-N-to-alkyl-C bond will enable almost the same conformation in the complex. As shown in Figure 1, we have found that a hydrophilic group at position 7 (or 3) will be exposed to solvent on the minor groove side of the complex. That being said, TAR RNA has been shown to be extremely flexible in solution, and portions of the active site can potentially distort to accommodate particular substituents [10, 16, 20–22].

In Figure 3A, we examine the effect of adding methyl groups. In Compound 2, the methyl group is in position 2 on the ring scaffold. If a substituent in this position is oriented toward the major groove side of the binding pocket, as in position 6, it will sustain some amount of van der Waals (VDW) clash. Therefore, we expect that the methyl orientation is toward the minor groove, making room for the single methyl substituent. The double methyl substituent supports this hypothesis with reduced binding affinity, which we attribute to VDW clash from the second methyl group, which must be on the major groove side.

In Figures 3B–3D, we have ranked the compounds by hydrophicility. In Figure 3B, substituents in position 1 are solvent exposed but could also pack with the top portion of the binding site. This orientation rationalizes the improved binding for the more hydrophilic hydroxyl substituent as well as the additional increase in affinity from VDW interactions with the methoxy substituent. The carbon at position 7 in Figure 3C is completely solvent exposed. Therefore, we propose that increasing hydrophilicity of the substituent in this position improves binding in the series. Finally, the carbon 3 position is completely buried. Therefore, the more hydrophobic compounds in Figure 3D still have reasonable binding. The methoxyl substituent has reduced binding in the series due to VDW clashes in the major groove orientation.

For the side chain-substituted library, the ratio of the STD intensities of the aromatic region resonances versus the aliphatic region resonances and line broadening were used to rank the small library of compounds. From analysis of the STD spectra of the originally discovered ligand acetylpromazine, we knew that the aromatic rings of the heterotricyclic structure have a closer interaction with the RNA protons than the aliphatic side chain [11]. This is not apparent in the NMR structure of acetylpromazine in complex with TAR RNA, where the side chain and ring system of the ligand appear to be equally interactive with the RNA [16]. As discussed previously [11], we believe that this apparent discrepancy may be due to the fact that we are pinning down the relatively flexible side chain in the structure calculation by intermolecular NOEs, whereas that particular conformation with the side chain pinned down in the minor groove may represent less than half of the population of the ligand in the bound form. The weaker STD intensities of the side chain implied that optimization of that region of the ligand would be beneficial. Phenothiazine derivatives with stronger STD intensities in the aliphatic side chain may reflect a larger population of bound ligand with the side chain immobilized in the minor groove.

For the side chain variation series, the relative STD enhancements of the aromatic protons were larger than those of the side chains, providing strong evidence that it is the phenothiazine ring that provides much of the binding energy. However, in the substituent comparison, the phenothiazine ring system remained the same within each series. Therefore, it was possible to correlate stronger aliphatic STD enhancements with higher affinity of the ligands because the phenothiazine remained largely unchanged. In addition, stronger relative STD signals of the aliphatic groups were reflected in stronger DLB effects. Detailed epitope mapping of the ligands was not performed due to significant overlap in some cases, especially of the aromatic protons.

The qualitative binding affinities for the side chain variations can be found in Figure 4. In the solved structure, the minor groove portion of the lower half of the binding site is largely comprised of an unpaired cytosine from the loop region of the RNA. This cytosine closes over the ligand situated at the binding site; in fact, the ligand cannot get into the structure determined without the

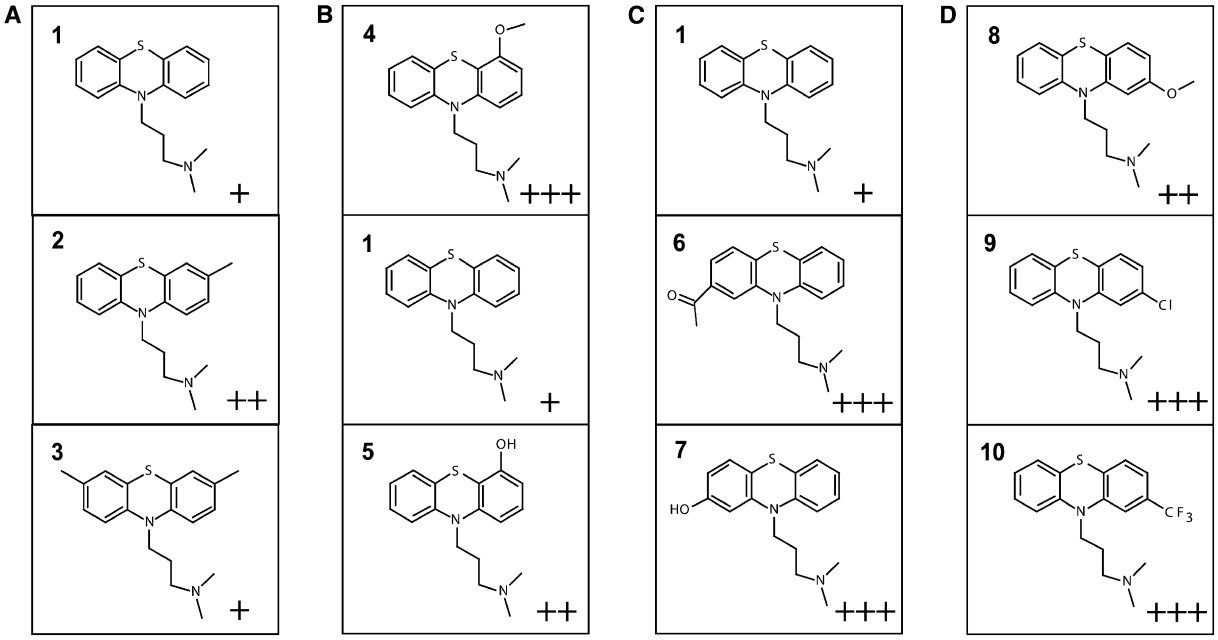


Figure 3. Binding between Phenothiazine Ring Derivatives and TAR RNA

(A–D) Binding was classified into three binding categories: affinities of ~0.1–1 mM (+++), affinities of ~1–5 mM (++), and affinities weaker than ~5 mM (+). For all panels, the compounds are at a concentration of 500 μM and the RNA is at a concentration of 50 μM. Compounds are separated from left to right by the location of substituents on the rings and are roughly ordered from top to bottom by increasing hydrophilicity.

bulge cytosine being moved aside. While this cytosine is within hydrogen bonding distance of the ribose of the following nucleotide, we hypothesize that this portion of the binding site is somewhat malleable and thus able to accommodate larger groups on the side chain region. For the acetylphenothiazine scaffold derivatives in Figure 4A, we suggest that the cyclohexyl structure is intrinsically stable enough to deform the active site when combined with additional VDW interactions. Compound 14, however, has a bit too much bulk for the active site as well as significant entropy loss upon binding, resulting in a decrease in binding. For the remaining compounds in

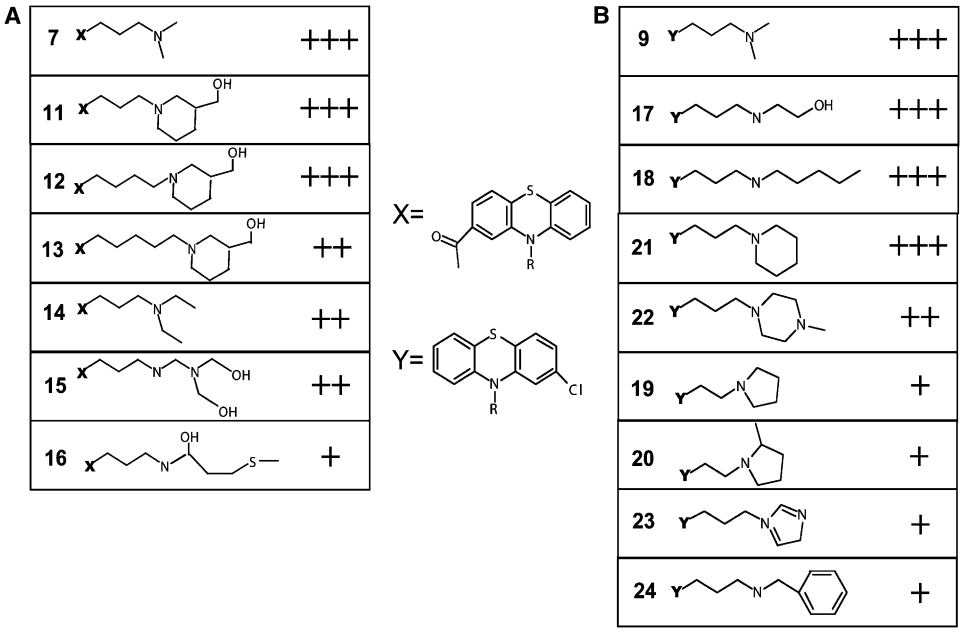


Figure 4. Phenothiazine Scaffolds with Side Chain Substitutions Tested for Binding to TAR RNA

(A and B) As explained in the Results and Discussion ("Library Screening"), the chlorine substituent causes the ring system to flip in orientation from the structure in Figure 1. As a result, affinity comparisons can only be made within a particular column. Binding categories and experimental conditions same as for Figure 3.

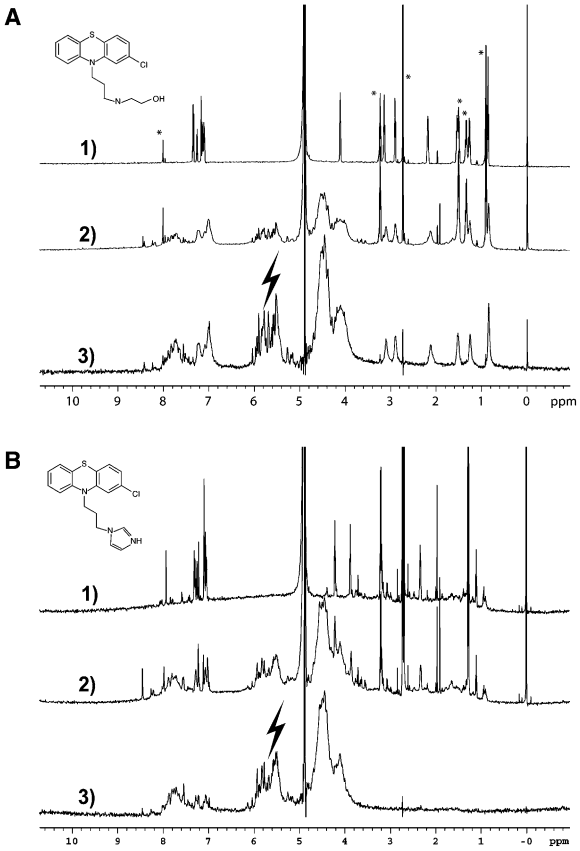


Figure 5. Representative NMR Spectra of Phenothiazine Compounds from Figure 4

For all spectra, the compound is at a concentration of 500 μM and the RNA is at a concentration of 50 μM.

(A) Compound 17: (1) Reference spectrum of compound alone; an asterisk indicates impurities. (2) Reference spectrum of compound in the presence of TAR RNA showing considerable line broadening induced by the oligonucleotide. (3) STD NMR spectrum of the compound in the presence of TAR RNA. The RNA is irradiated at 5.8 ppm, as indicated in the figure by the arrow. Signals of Compound 17 indicating the binding interaction are clearly visible, while those of the impurities indicated by asterisks are subtracted. RNA signals are also visible because the molecular size is not large enough to eliminate the background signals by a relaxation filter.

(B) Compound 23: (1) Reference spectrum of compound alone. (2) Reference spectrum of the compound in the presence of TAR RNA showing a small amount of line broadening localized to the aromatic ring protons. (3) STD NMR spectrum of the compound in the presence of TAR RNA. The RNA is irradiated at 5.8 ppm, as indicated in the figure by the arrow. In this case, signals for the aromatic protons manifest an effect, while the side chain and the imidazole group are subtracted.

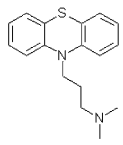
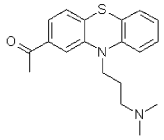
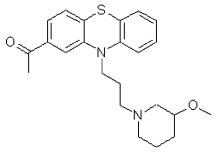
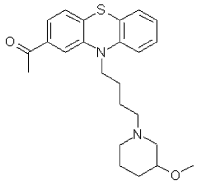
Compound	Dissociation Constants
	5 mM
	0.27 mM
	0.18 mM
	0.14 mM

Figure 6. Binding Affinities as Determined by Chemical Shift Monitoring of TAR RNA Imino Proton Resonances for Selected Compounds

the list, the substituents have enough bulk to cause little VDW clash, but too much internal flexibility to distort the active site. In addition, because these compounds are so flexible, the entropic penalty to bind these molecules must be higher than the enthalpic gain.

For the choro-substituted phenothiazines in Figure 4B, the side chain will be in a slightly different orientation in response to the flipped scaffold (relative to the acetylphenothiazine series); thus, this series cannot be directly compared to that in Figure 4A. However, many of the same conclusions can be drawn. For Compounds 17 and 18, the single substituents have a thinner diameter than the original dimethyl substituents, which we hypothesize enables these flexible chains to adapt to the minor groove. The two cyclohexyl compounds most likely have the same effect as what is shown in Figure 4A; they are intrinsically stable enough to deform the active site while compensating with additional VDW packing. The cyclopentyl rings have a shorter chain length than any other substituent in the series. Because the active site does not widen until a bit further down, these substituents are likely disrupting the upper portion of the active site. Finally, for both of the aromatic compounds, the stacking interactions with bases are not possible without significant rearrangement of the TAR structure, resulting in entropic loss of binding. In this case, the substituents would be solvent exposed, thus explaining their reduced binding.

For compounds that showed large STD intensities and exchange broadening effects, the RNA imino proton resonances were monitored. Imino proton chemical shift changes upon addition of ligand provide information about the binding site on the RNA and also permit K_D values to be determined (Figure 6). For Compound 11, the most potent of the tested molecules, the calculated K_D value was 140 μM, or about twice that of the original ligand, acetylpromazine [11]. As shown for Compound 11 with the 3-hydroxymethylpiperidine ring (see Figure 7), the imino resonance that shifts most upon

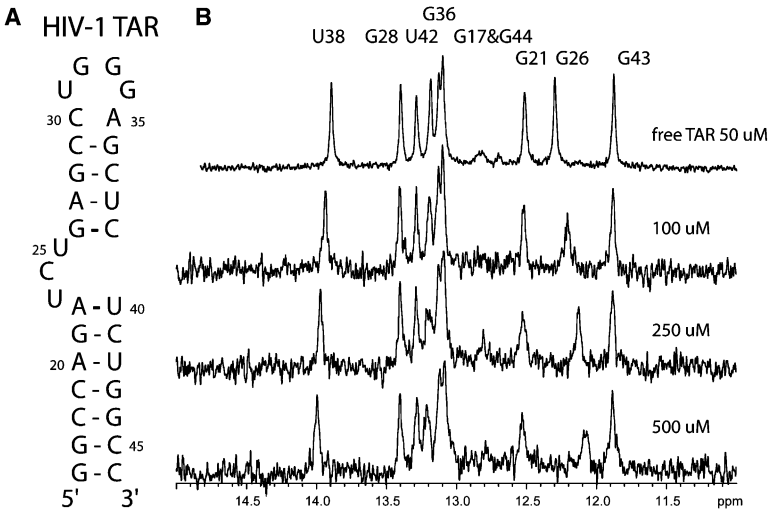


Figure 7. Representative Imino Proton NMR Spectra of RNA in the Presence of Compound 11 from Figure 4

For all spectra, the RNA is at a concentration of 50 μM.

(A) Sequence of the TAR RNA construct used for testing the phenothiazine derivatives.

(B) Chemical shift monitoring of TAR RNA imino proton resonances upon addition of Compound 11 in increasing concentrations, from 0 to 500 μM. The chemical shift difference is plotted against the compound concentration to determine the dissociation constant of the TAR-ligand complex.

addition of the ligand is that of G26 of the HIV-1 TAR RNA. The imino proton resonance of G26 exhibits a large upfield shift due to ring current effects from the stacked ligand benzene I. As with Compound 11, the other ligands exhibited imino chemical shifts only for residues in the vicinity of the TAR bulge. It should also be noted that there was no evidence that more than one of the tested ligands binds to the RNA at the same time, as no significant imino chemical shifts were observed outside of the binding site for any of the tested compounds. From previous studies, which entailed several different kinds of multinuclear and multidimensional NMR spectra, we also found that only residues in the vicinity of the bulge experienced changes upon ligand binding, and that there were no changes evident for other residues upon addition of a large excess of ligand [7, 16].

We would like to emphasize that a well-defined SAR cannot be expected from compounds with binding affinities in this range. However, the SAR performed for the focused library of phenothiazines suggests a few avenues for future developments. A side chain with 3–4 methylenes between the ring and the heteroatom seems optimal (Figure 4). Consistent with the NMR structure, a flexible linear chain will snake nicely along the minor groove. In addition to VDW interactions, heteroatoms or functional groups with hydrogen bonding capabilities may improve binding. Some rigidity in the side chain will decrease entropic loss upon binding, but the orientation and nature of further derivitization will become more critical. Ring substituents may further enhance binding, and extension beyond the methoxyl group in position 1 should gain additional VDW interactions. The results of Figure 3 also imply that it might be of value to have a phenothiazine with a hydrophilic substituent in position 7 and a small hydrophobic substituent in position 3. These implications from the SAR are entirely consonant with the structure.

Significance

The synthesis of the focused library of phenothiazine derivatives—a class of nontoxic, bioavailable RNA binders—and analysis of the SAR for binding to HIV-1 TAR RNA reported here are important extensions of earlier work. The study explores structural features affecting phenothiazine interactions with TAR RNA. The observation that both moieties varied here—ring substituents and the n-alkyl substitutions—affect binding agrees with our published structure of TAR with acetylpromazine. In addition, the results described above suggest that the binding site, while flexible, has regions that restrict growth. There are also several areas of the binding site that are solvent exposed, which can be exploited in later stages of development.

The most significant factor in the data is that compounds possessing a four-atom linker between the tricycle and the distal base are among the tightest binders. In the pharmacological literature, neuroleptic activity is strongly associated with a three-atom linker, and antihistamine activity is associated with a two- or three-carbon linker. Obviously, any RNA binding compound would require the ability to dissociate these undesirable activities from therapeutic effects. Compounds with longer linkers provide an attractive potential route toward that goal.

In future work, knowledge gained in this study can guide further enhancement of specificity. The phenothiazines studied have a single positive charge and relatively low molecular weight. In addition to modifications studied here, increased affinity may be gained by combining the appropriate substituted phenothiazine with other groups known to enhance RNA binding (e.g., amino sugars, guanidino groups, and intercalating groups). However, as shown here, binding affinity can be improved through optimization of VDW and hydrophilic sites of the scaffold. This hypothesis is supported by the increase in binding from the naked promazine scaffold to the final product from this study, which have binding affinities of 5 mM and 140 μM, respectively.

Experimental Procedures

NMR Experiments

Unlabeled RNA samples were prepared and purified as previously described [11]. NMR experiments were performed on a Varian Inova

600 MHz spectrometer. Typically, a spectrum of the compound alone at a 250 or 500 μM concentration was acquired, and then RNA was added from a concentrated stock solution to produce a 25 or 50 μM final RNA concentration. Reference and STD spectra were compared. STD NMR spectra were acquired by internal subtraction via phase cycling. On-resonance irradiation was set to 5.8 ppm, and off-resonance irradiation was set to ~30 ppm where no RNA resonances are present. Typically, 256 scans were acquired for the STD experiments. Presaturation of RNA resonances was achieved by an appropriate number of band-selective G4 Gaussian cascade pulses to give a saturation time of 2 s. The temperature was set to 15°C in all STD experiments. 1D-NMR imino proton spectra were acquired by using hard-pulse WATERGATE water suppression, and the excitation profile was optimized for maximum intensity at 13 ppm.

For the qualitative ranking of compound binding affinities, spectra of resonances from the ligand alone were compared with the same resonances in STD spectra acquired with ligand in the presence of RNA (see Figure 5 for representative examples). A classification of binding was made according to observed STD intensities and line broadening effects (Figures 3 and 4).

For a select subset of derivatives, dissociation constant (K_D) values were determined by titrating the RNA with ligand and monitoring the RNA imino proton chemical shift differences (Figure 6). Typically, five titration points were taken for each compound for which we determined the K_D. In a previous study, the first compound of this series, acetylpromazine, was found to have a binding affinity of 270 μM [11]. The qualitative study described above found the remaining compounds to be in the same binding range of ~0.1–1.0 mM. Substantial line broadening observed for some of the tested compounds stems mostly from the broadening of resonances due to chemical exchange of the ligand either between free and bound species or, more likely, from multiple conformations on the RNA; the latter may result from the symmetry of the ligand binding in a similar fashion, but with the ring flipping 180°. Except for the parent compound listed for comparison, for the compounds whose K_D values were determined (Figure 6), the symmetry was broken such that one orientation of binding was strongly preferred; this was clearly evident in the quality of the various spectra (primarily lineshapes) acquired for these complexes compared with the others. For these three compounds, it appears that fast-exchange conditions were obtained. As noted already, our NMR results indicate that these ligands bind to only a single site on our TAR RNA construct. The equation for single-site binding under the fast-exchange regime,

$$K_D = C_L\left(\frac{\delta - \delta_F}{\delta_B - \delta}\right), \quad (1)$$

was used to obtain K_Ds as chemical shifts were observed for the complex (δ), as a function of the concentration of the ligand (C_L), relative to the chemical shifts for free RNA (δ_F) and ligand bound RNA (δ_B) [23].

Synthesis of Compounds

All reagents and starting materials were purchased from commercial sources and were used without further purification; solvents were anhydrous HPLC grade. All parallel synthesis steps were carried out in polypropylene fritted reaction tubes with the Bohdan Miniblock 48-position reaction blocks. Microwave reactions were done in a CEM Discover microwave reactor system. The reaction schemes described below can be seen in Figure 2.

General Procedure for the Preparation of Substituted 10H-Phenothiazines

Each diphenylamine (Figure 2A, Compound 1) (2.0 mmol) was sealed in a microwave reaction vessel with sulfur (128 mg, 4.0 mmol), a crystal of iodine, and 2.5 ml doubly distilled water. The vessels were heated to 190°C for 20 min and then cooled to room temperature. The crude mixture was extracted with 10 ml ethyl acetate and then purified by column chromatography (silica gel; 90% ethyl acetate, 10% hexanes) to give the substituted 10*H*-phenothiazine product (Figure 2A, Compound 2) (5%–45% yield).

General Procedure for the Alkylation of 10H-Phenothiazines

Sodium hydride (0.06 mmol) was added slowly at 0°C to each stirring solution of 10*H*-phenothiazine (Figure 2A, Compound 2) (0.05 mmol) dissolved in DMF (10 ml). The reaction was stirred for 30 min, and was then allowed to warm to room temperature. This solution was then added dropwise into a stirring solution of 1-chloro-3-iodopropane (27 μl, 0.25 mmol) in DMF and was stirred for 1 hr. The reaction mixture was extracted from a brine solution with ethyl acetate (25 ml, 3×) and was then evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel; 90% ethyl acetate, 10% hexanes) to give the substituted 10*H*-phenothiazine product (Figure 2A, Compound 3) (35%–85% yield).

General Procedure for the Amination of 10-(3-Iodopropyl)-Phenothiazines

Dimethylamine (4 eq., 40 μl of a 2 M solution in THF) was added to each member of the substituted 10*H*-phenothiazine product (Figure 2A, Compound 3) (0.02 mmol) dissolved in 1 ml DMF. The mixture was sealed into a microwave reaction vessel and was heated at 100°C for 40 min. The crude reaction mixture was then purified by silica gel prep-TLC by using a 94.9:5:0.1 ratio of dichloromethane:methanol:triethylamine. Pure products (Figure 2A, Compound 4) were isolated in 60%–85% yields.

General Procedure for the Alkylation of (2-Chloro or 2-Acetyl)-10H-Phenothiazines

Sodium hydride (21 mmol, 60% dispersion in mineral oil) was slowly added to a mixture of 2-chloro- or 2-acetyl- 10*H*-phenothiazine (Figure 2B, Compound 5) (10 mmol) stirring in a 1:1 solution of DMSO:THF at 0°C. The mixture was allowed to warm to 25°C and was then added to a stirring solution of iodochloroalkanes (Figure 2B, Compounds 6a–6d) (30 mmol) in N,N-dimethylformamide (20 ml). The reaction mixture was stirred at room temperature for 2 hr, and 100 ml brine was added to stop the reaction. The crude product was extracted into ethyl acetate (3×, 20 ml) and then purified by column chromatography (silica gel; 90% ethyl acetate, 10% hexanes) to give the title compounds (Figure 2B, Compound 7) in 40%–80% yield.

General Procedure for the Iodo Halogen Exchange Reaction

A mixture of potassium iodide (5 re, 5 mmol) and each member of the alkylated (2-chloro or 2-acetyl-)10*H*-phenothiazines (Figure 2B, Compound 7) (1 mmol) was heated until reflux in 2-methyl-4-propanone until the reaction had reached completion as determined by NMR (about 3 days). The solvent was removed under vacuum, and the product (Figure 2B, Compound 8) (95% yield) was used without any further purification.

General Procedure for the Amination Diversity Step

Each iodoalkane intermediate (Figure 2B, Compound 8) (0.33 mmol) was dissolved with N,N-dimethylformamide (5 ml) and divided into five portions (0.06 mmol each). Each portion was mixed with 0.13 mmol *n*-butylamine, benzylamine, 2-aminoethanol, piperidine, and imidazole. The crude reaction mixtures were worked up by extracting into methylene chloride (40 ml) from a 10% NaOH solution (40 ml). Solvent was removed under vacuum, and each compound was then purified by silica gel prep-TLC by using a 94.9:5:0.1 ratio of dichloromethane:methanol:triethylamine. Pure products (Figure 2B, Compound 9) were isolated in 60%–85% yields.

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