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Design, Synthesis and Evaluation of RNA-binding [2.2.1] Bicyclic Scaffolds: Application to HIV-1 Tat/TAR Inhibition

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Abstract

In this paper, a [2.2.1] bicyclic core is applied as a peptidomimetic scaffold capable of reproducing the structural relationship between the critical residues of the Tat protein, in an effort to create small molecules capable of binding to TAR RNA and inhibiting the Tat/TAR interaction. A systematic twenty-member panel mimicking an Arginine-Aspartic acid (R-D) moiety was synthesized and the ability of the individual members to inhibit the Tat/TAR interaction was evaluated. A gel-shift mobility assay was used to establish that several bicyclic agents inhibited the RNA-protein interaction with a micromolar level of activity.

Keywords

HIV TAR RNA, RNA-small Molecule, Cycloaddition, Inhibitor

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1. Introduction

RNA is one of the oldest therapeutic targets, and remains an important area of study. It has been known for many years that aminoglycoside antibiotics can alter the function of RNA. Perhaps the earliest example of their effect was the retardation of translation, which was shown to be caused by binding of these molecules to the 16S ribosomal RNA [1]. Later, it was discovered that some classes of aminoglycosides can also interfere with catalytic RNAs, such as Group I introns [2] as well as hammerhead [3] and hepatitis delta virus ribozymes [4].

The design of RNA-binding small molecules is a rapidly growing area of research, particularly because of the prevalent role that RNA-protein interactions play in many cellular processes [5-7]. As a macromolecule, RNA also exhibits an enormous amount of structural diversity. It is composed of several small units of secondary structure, such as double-helical stretches, mismatches, bulges, internal loops, and pseudoknots [8]. A great deal of sequence- and structure-specific contacts can be made between these architectural subunits and proteins, metal ions, and small molecule ligands. One particularly interesting application of these modular properties of RNA is in the design of drugs targeting specific RNA fragments. This is especially promising for viruses that employ RNA as the carrier of genetic information. Furthermore, recent advances in siRNA and guide RNAs for CRISPR/Cas9 therapeutics have made RNA-based therapeutics particularly attractive [9].

Conventional strategies for combating retroviruses are based on interference with viral proteins, thus altering their function. However, these organisms can mutate rapidly, and natural selection quickly results in new strains that are resistant to the specific molecular agents. In theory, this would happen much slower for RNA-targeting drugs because unlike DNA, there is no cellular repair mechanism for these nucleic acids [10]. Moreover, the great diversity of higher

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order structures present in RNA improves the likelihood of designing an inhibitor with dramatic specificity. Despite these advantages, rational drug design has been only moderately successful in targeting RNA molecules, requiring strategies that are fundamentally different from conventional protein-targeted drug design [11-16]. Being chemically quite distinct from protein, the most beneficial functionality for RNA affinity, namely basic and/or charged residues, is not the most beneficial for enhancing drug-like properties, such as the ability for a molecule to traverse eukaryotic cell membranes. Various approaches include designing peptidomimetics using peptoids and D-amino acids [17, 18], RNA-ethidium conjugates [19], modified aminoglycosides with arginine-like moieties [20], and carboxylate-containing tetrahydropyrimidines [21].

HIV, a retrovirus of critical significance to human health, presents two major RNA targets: the *trans*-activation responsive element (TAR), which interacts with the regulatory protein Tat (*trans*-activator of transcription), and the Rev response element (RRE), which recognizes another regulatory protein Rev. The Rev/RRE system is involved in the movement of unspliced and partially spliced HIV mRNA, while the Tat/TAR complex is involved in transcription [22].

The NMR-derived solution structure of Tat [23] indicates that is a fairly flexible and unstructured protein that can be loosely divided into five regions. Two of these are a flexible and somewhat α-helical basic fragment that is required for binding to RNA, and a glutamine-rich region that may also be involved in RNA binding. The former domain of basic amino acids (L-arginine and L-lysine) mediates RNA recognition by Tat, and small peptides that compromise this domain bind specifically to TAR or a shortened form of TAR (ΔTAR) [24, 25]. In fact, L-arginine itself and its derivative L-argininamide inhibit Tat/TAR interaction with a single-digit millimolar inhibition constant (K_I) value, with the latter being more potent [26, 27]. A highly basic 24 amino acid Tat fragment (Tfr24), along with a 27-nucleotide RNA fragment (ΔTAR) derived from the 59-nucleotide sequence found on the 5' end of all HIV transcripts, have been shown to replicate the activity of the full-length Tat/TAR system in vitro [24, 25]. The dissociation constant (K_D) for the Tat/TAR complex is approximately 3-5 nM [28-30], while the K_D for the Tfr24/ ΔTAR interaction has been estimated to be as low as 16-400 pM [24, 25].

The structure of TAR features two double-stranded stems, a hexanucleotide loop region, and a three-nucleotide pyrimidine bulge. The bulge is critical for Tat binding, as its removal drastically affects both affinity and specificity. The unpaired nucleotides of the bulge segment cause a widening of the major groove when compared to canonical A-form RNA, whose major groove is quite shallow and is therefore usually inaccessible for ligand recognition via hydrogen bonding [31]. The minor groove is more accessible but contains less functional information for base discrimination.

It is known that arginine residues in the basic region of Tat initiate contacts leading to complex formation, although several lysine residues in the same region do contribute to electrostatic interactions [27, 32]. One key arginine residue, R52, positions itself between one of the bulge nucleotides (U23) and one of the base pairs (A27-U38), which form a base-triple (Figure 1). Bidentate hydrogen bonds can be seen to form between the guanidinium group of the arginine and a guanosine base, similar to those occurring in the zinc finger domains that bind to certain stretches of DNA. This interaction is stabilized by electrostatic forces between the positively charged groups of the basic residues and the negatively charged phosphates of the RNA molecule. The Tat/TAR recognition also relies on sets of intermolecular hydrogen bonds and van der Waals' interactions [33].

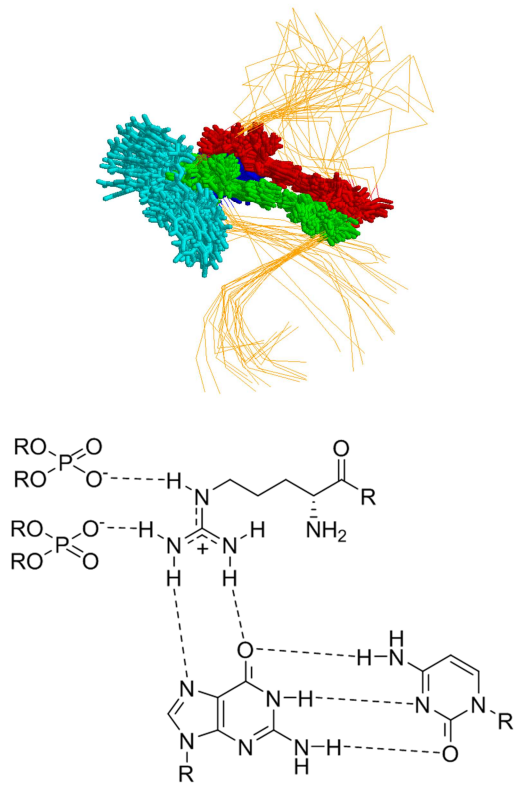

Figure 1. *Top*: Solution structure of HIV-1 TAR bound to arginine (pdb entry 1ARJ) [34]. The RNA backbone is shown in orange, U23 is shown in blue, C24 and U25 are in cyan, A27 and U38 are in red, and G26 and C39 are in green. *Bottom:* Putative interaction of the guanidinium group of arginine with a G:C base pair and the phosphate backbone.

2. Experimental Section

2.1. General

Unless otherwise stated, all reactions were carried out under

an atmosphere of nitrogen (house nitrogen dried with Drierite® and KOH). Commercially available reagents and solvents were used as received from Aldrich Chemical Company, Inc., Fisher Scientific, Acros Organics, Mallinckrodt Baker, Inc., EM Science, Alfa Aesar, and Pharmco Products, Inc. Vacuum filtration was accomplished using a porcelain Büchner funnel fitted with Whatman filter paper of corresponding size or a glass Büchner funnel with medium frit and Celite® 454 filter agent. All reactions were magnetically stirred and monitored by thin-layer chromatography. Routine thin-layer chromatography was done using Si250F pre-coated silica gel plates from JT Baker or Silica Gel 60 F_{254} pre-coated plates from EM Science. Unless otherwise noted, flash column chromatography was performed with 230-400 mesh Ultra Pure silica gel from SiliCycle using hexanes-ethyl acetate as the eluent. ^{1}H and ^{13}C NMR spectra were obtained on a GE QE-300 spectrometer. Chemical shifts were reported with $CDCl_3$ (δ 7.26 ppm for ^{1}H and δ 77.23 ppm for ^{13}C), DMSO-d6 (δ 2.50 ppm for ^{1}H and δ 39.51 ppm for ^{13}C) or MeOH-d4 (δ 3.31 ppm for ^{1}H and δ 49.00 ppm for ^{13}C) as internal references (Cambridge Isotope Labs, Inc.). Melting points were taken on an Electrothermal® melting apparatus and are uncorrected. Infrared spectra were taken on a Midac Corporation M series FT-IR spectrometer. Elemental analyses were performed by Atlantic Microlab (Norcross, GA) and mass spectrometry was conducted at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign, School of Chemical Science. X-ray crystallographic analysis was provided by the Yale University Chemical Instrumentation Center X-ray crystallography facility.

2.2. Determination of Inhibition Constant (IC_{50})

All analyses were performed using Microcal Origin 6.0. The data was fit using a logistic function of the form $y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2$, where A_1 and A_2 are the initial and final values of the % complex formation, x_0 is the midpoint (IC_{50}), p is an arbitrary power, x refers to an inhibitor concentration, and y is the % complex found at that inhibitor concentration.

3. Results and Discussion

We sought to develop a peptidomimetic approach that would allow the initial use of known RNA attractive functional groups, such as the guanidine of arginine and the carboxylic acid of aspartic acid, on a diversity scaffold (Figure 2). By initially employing these functional groups in an effort to obtain good affinity, the diversity scaffold could later be used in an effort to identify additional functional groups that could enhance affinity and/or selectivity. Even in the absence of being able to eventually remove the basic or charged groups from the scaffold, this approach would allow the development of a molecule with alterable features at three different positions in the molecules in a synthetically accessible fashion [35]. This versatility is not present in aminoglycoside-based inhibitors [36, 37].

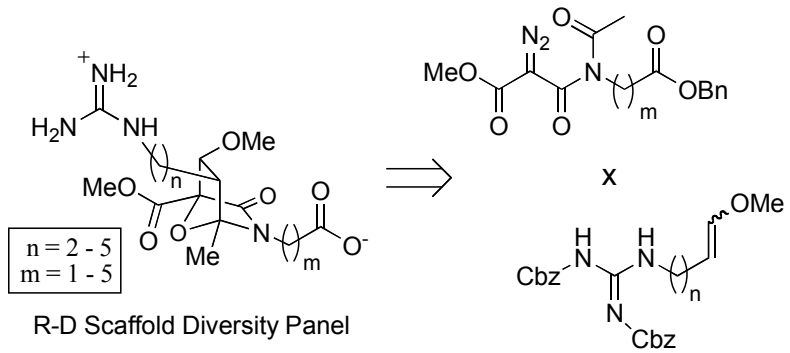

Figure 2. Synthetic strategy for the construction of the bicyclic scaffold.

Guanidines are common molecular recognition elements in natural systems [38-40]. For this reason, a guanidinium group was chosen as the first recognition element in our modular scaffold design. The second functional group chosen for the inhibitor scaffold was a carboxylate, whose role in RNA recognition is much less characterized. The charge repulsion with the phosphate backbone might suggest a negative effect on inhibitory power. Binding analysis of 2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine with TAR (K_I = 50-100 nM), however, hints at a potentially beneficial contribution from the carboxylate, which may be engaged in hydrogen bonding with amino group protons distal to the nucleic acid bases [21].

As seen in Figure 2, the scaffolds originate from reactions between diazoimides (dipole precursors) and guanidine-containing dipolarophiles. Homologous dipole precursors (m = 1-5) were synthesized from commercially available linear amino acids as shown in Figure 3. Sonication-assisted acylation of amino acids 1a-e, from glycine to 6-

aminocaproic acid, with subsequent esterification, generated a series of amides 2a-e. These were efficiently converted to diazoimides 4a-e via an imidation-diazotransfer sequence using methyl malonyl chloride 3 and mesyl azide.

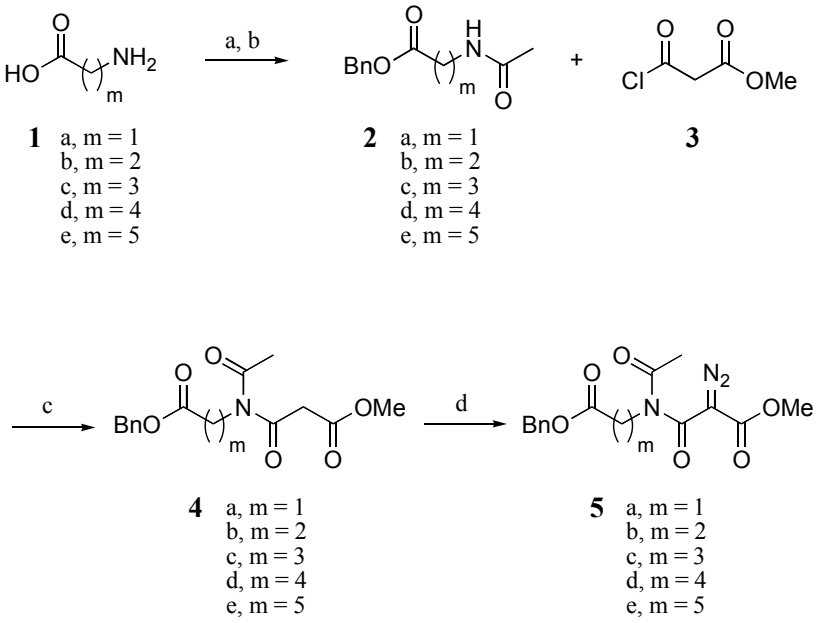

Figure 3. Synthesis of linear scaffold precursors 5a-e. Conditions: (a) Ac_2O, sonication, 23°C, 15 min.; (b) BnOH, CDI, Et_3N, DCM, 5 h, 23°C; (c) Toluene, N_2, 30 min, 70°C; (d) MsN_3, Et_3N, THF, 8 h, 23°C.

For the assembly of a mimetic library, a series of homologous vinyl ethers and primary amines (n = 2-5) was synthesized as shown in Figure 4. Phthalimide alcohols 6a-d, were treated with Swern oxidation conditions followed by acetal formation to give the phthalimide protected acetals 7a-d. Trimethylsilyl iodide-promoted elimination, followed by one-pot deprotection/guanidine installation [41], furnished bis(carbonylbenzyloxy)-enol ethers 10a-d. It is worth mentioning that the steric nature of the base in the elimination step can significantly affect the *E/Z* ratio of the resulting vinyl ethers. A maximal *E/Z* distribution (1:1) was obtained with *N*-methylmorpholine, while Hünig's base has strongly favored undesired *Z* isomer (1:9), and trimethylamine has led to an intermediate distribution [41]. The *E* and *Z* isomers were not separated at this point, since previous cycloaddition studies have indicated that a significant kinetic bias in the cycloaddition step was expected to enrich the formation of the desired *trans* product [41].

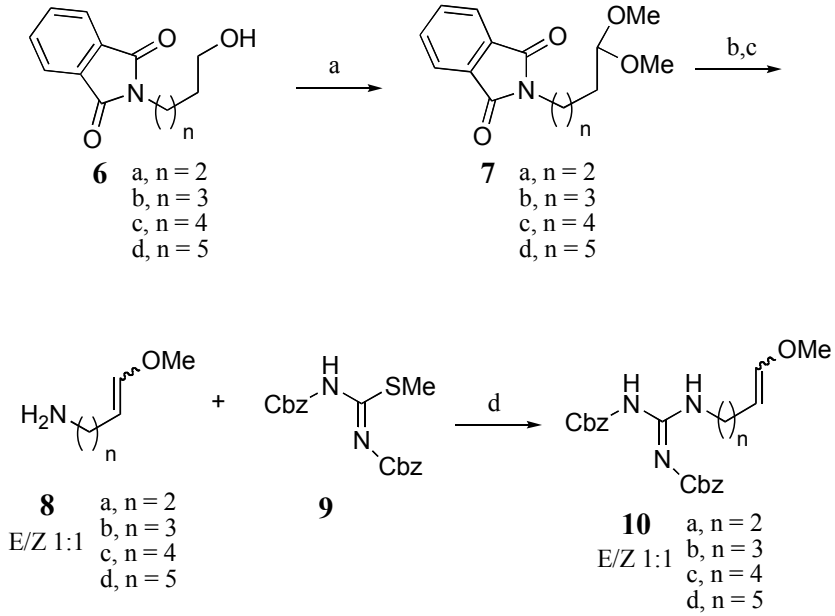

Figure 4. Synthesis of guanidine dipolarophiles 10a-d. Conditions: (a) Oxalyl chloride, DMSO, DCM, Et_3N, 30 min, -78°C to 23°C, then MeOH, Amberlyst-15, 4Å molecular sieves, 8h, 23°C.; (b) TMSCl, NaI, *N*-methylmorpholine, pyridine, 3 h, 23°C to 80°C; (c) NH_2NH_2, MeOH, 2 h reflux; (d) 5 h, 23°C.

A rhodium(II)perfluorobutyramidate $[Rh_2(C_3F_7CONH_2)_4]$ mediated 1,3-dipolar cycloaddition reaction was used to construct the R-D panel as shown in Figure 5 [34,41]. The array was assembled by twenty reactions involving each of the five dipole precursors (5a-e) with each of the four dipolarophiles (10a-d), yielding a single diastereomeric set of *cis/trans* cycloadducts. The major *trans* species were readily separated and isolated in pure form through flash chromatography. Simultaneous deprotection of benzyl ester and bis(carbonylbenzyloxy)-guanidine via hydrogenolysis yielded the potential mimetics 11-30 as water-soluble zwitterions. Since the 20-member library (n = 2-5, m = 1-5) was synthesized in a parallel manner, each molecule can be individually evaluated for potential inhibitory activity.

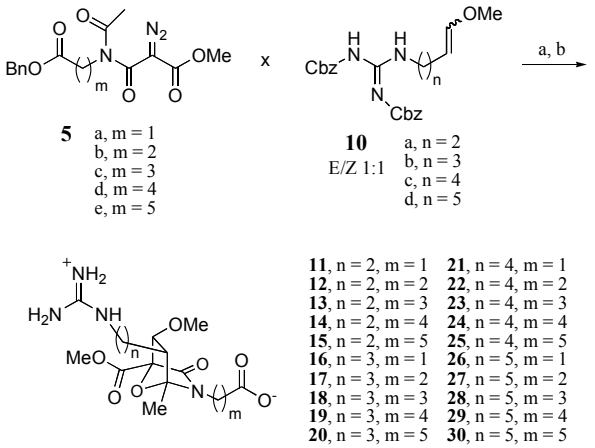

Figure 5. Synthesis of R-D panel using rhodium(II) catalyzed 1,3-dipolar cycloaddition. Conditions: (a) $Rh_2(C_3F_7CONH_2)4$, 4Å molecular sieves, BnH, N_2, 3h, 60°C.; (b) 10% Pd/C, 1,4-cyclohexadiene, CH_3CN/H_2O, 24 h, 23°C.

Initial binding studies were carried out using a mobility-shift gel electrophoresis assay with the Tfr24 peptide and ΔTAR.Tfr-24 peptide was chemically synthesized, and ΔTAR RNA was prepared enzymatically as previously described [42]. The RNA was snap-cooled (heated for a short period and then quickly frozen) so that it would adopt the necessary hairpin structure and not be a linear single-stranded chain. The peptide was titrated against radiolabeled 5' [γ-^{32}P]ΔTAR RNA at 60 pM in 10 µL [10 mM Tris (pH 7.5), 70 mM NaCl, 0.2 mM EDTA, 5% glycerol at 22°C] to establish a concentration at which all the RNA was bound when subjected to gel electrophoresis [10% w/v acrylamide and 75:1 w/w acrylamide/bis(acrylamide) in 45 mM Tris-borate, 1 mM EDTA, 20°C]. Inhibition of binding was performed under conditions that gave approximately 80% binding in the absence of inhibitor. After an initial equilibration period, the bicyclic compounds 11-30 and argininamide were added to the mixtures containing the peptide (0.65 nM) and RNA fragment, and the solutions were incubated for an additional 20 minutes at 22°C. After gel electrophoresis, the relative amounts of complexed and free RNA were quantified using a Phosphorimager. A preliminary screen was performed at 200 µM inhibitor concentration (Figure 6). Of the twenty member array, compounds 11, 16, 17, 22, 26, 27 and 29 were found to inhibit the Tfr24 gel-shift at this concentration and were selected for further analysis in a concentration-dependent gel-shift assay (Figures 7 and 8) [34,41].

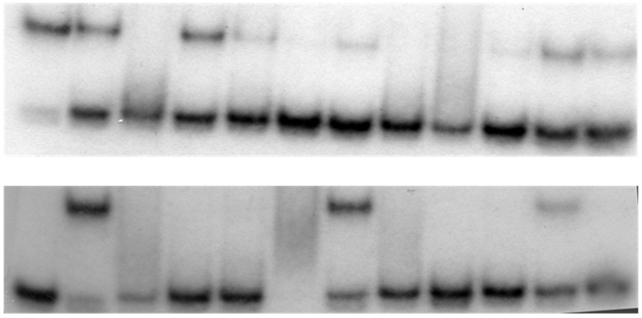

Figure 6. Gel-shift screen for binding activity (inhibitor concentrations were all at 200 µM). In each lane the upper band corresponds to the ΔTAR-Tfr24 complex, and the lower band corresponds to the free ΔTAR RNA. *Top* (l-r): ΔTAR-Tfr24 complex only, complex + arginine, complex + 11, complex + 12, complex + 13, complex + 14, complex + 15, complex + 21, complex + 22, complex + 23, complex + 24, complex + 25. *Bottom* (l-r): complex + 16, complex + 17, complex + 18, complex + 19, complex + 20, blank, complex + 26, complex + 27, complex + 28, complex + 29 (twice), complex + 30.

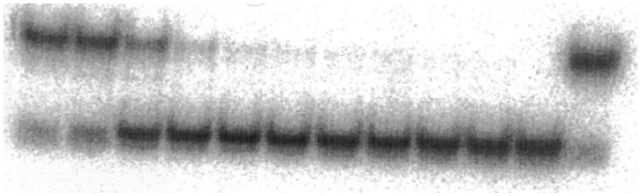

Figure 7. Dose-dependent gel-shift experiment for inhibitor 11. Inhibitor concentrations in the first ten lanes are as follows (l-r): 1 µM, 50 µM, 100 µM, 200 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, 800 µM, 900 µM. The last two lanes correspond to free ΔTAR and ΔTAR complexed withTfr24, respectively.

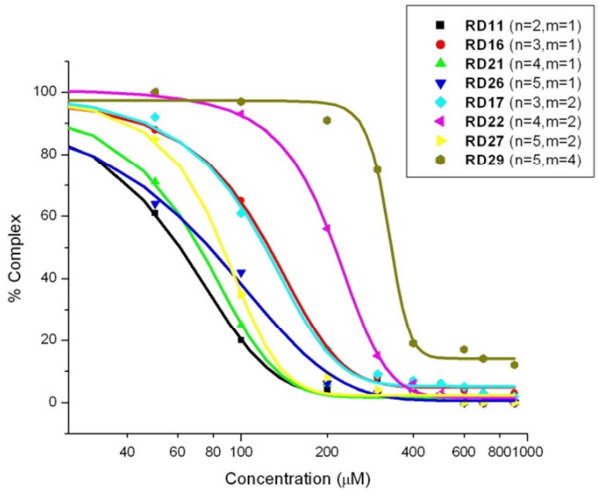

Figure 8. Dose-dependent gel-shift curves for the most active scaffold molecules.

Interestingly, several library members performed significantly better than argininamide, a known inhibitor that lacks a carboxylic acid functional group. Several of the more potent compounds revealed an IC_{50} of 58-82 µM for the most active molecules 11 (n=2, m=1), 21 (n=4, m=1), 26 (n=5, m=1), and 27 (n=5, m=2), as shown in Table 1 [34,42].

Table 1. Gel-shift IC_{50} values (in µM) for the R-D scaffold array.

	m=1	m=2	m=3	m=4	m=5
n=2	58	-	-	-	-
n=3	119	113	-	-	-
n=4	68	210	-	-	-
n=5	76	82	-	324	-

In the case of aminoglycoside-based inhibitors, much of their activity seems to be due to the preponderance of positively charged groups which can make favorable electrostatic contacts with RNA. This phenomenon tends to make these compounds fairly specific for RNA vs. DNA or proteins, but not very selective within the RNA world. This lack of selectivity is particularly disheartening when the plethora of RNA present in the cellular *milieu* is considered, given that approximately 80% of the total RNA is ribosomal RNA and another 10-15% of it is tRNA [43]. They have fallen out of favor clinically because of toxicity and widespread bacterial resistance [44]. They are also fairly promiscuous, conceivably due to the electrostatic nature of their recognition of RNA. Exacerbating matters, the charged nature of these compounds limits cellular uptake. Peptidomimetics can be synthesized with high (nM) affinity for RNA, but these molecules typically contain a large number (as many as nine) of guanidinium groups [17]. It is worth mentioning that several leads for RNA-based therapeutics are in the µM range [45-51].

With m ≤ 5 and n ≤ 5, it was assumed that adequate conformational space was covered for the necessary intermolecular forces in RNA-small molecule binding. In order to evaluate the conformational flexibility of the larger scaffold panel members, a molecular mechanics calculation was used to evaluate all conformations within 3 kcal/mol. The conformational flexibility of each member (n = 2-5 an m = 1-5) was calculated separately in order to obtain the complete conformational freedom of each position, without regard to interaction with each other. Two representative members (26 and 30) are shown in Figure 9. The dependence on n, the distance from the scaffold to the carboxylate, is clearly dominated by either n = 1 or n = 2. Interestingly, there seems to be negligible dependence on m, provided that n is small. A proposed model for the activity of these inhibitors is shown in Figure 10.

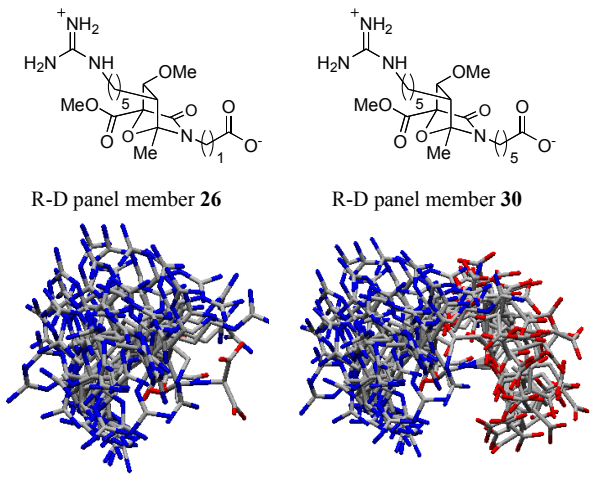

Figure 9. Graphical representation of the conformational flexibility of the higher n,m R-D panel members and the more restricted m = 1 position.

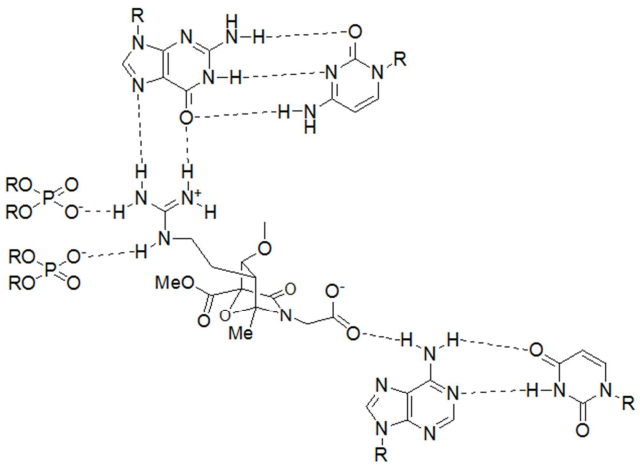

Figure 10. A possible model for key contacts made between inhibitor 11 and ΔTAR RNA. Shown here are putative contacts made with a G:C base pair, an A:U base pair, and the phosphate backbone.

4. Conclusions

In principle, drugs that distinguish RNA could work by any number of mechanisms: altering the conformation of the nucleic acid (and therefore its function), modifying its interaction with effector proteins, and forming covalent adducts are just some of the possibilities. Initial attempts at designing these drugs include replacing the amide backbone of the Tat protein with an oligocarbamate-based polymer [52], using D-amino acids [53], and peptoids [17]. While these approaches did result in several good (µM-nM) binders *in vitro*, their *in vivo* properties were disappointing due to their size and lack of "drug-like" properties. Aminoglycosides serve as the model compounds for a great deal of RNA-based work, and their prevalence has led to a number of very important advances. Unfortunately, their lack of specificity and highly-charged state renders them suboptimal starting points for rational drug design. Intercalators which stack on DNA can in some cases

acknowledge local deformations in the RNA which permit their entry, for example, into the major groove or near bulge regions. Regrettably, there is not (yet) an apparent correlation between structure, *in vitro* binding data, and *in vivo* function. Combinatorial, semi-synthetic, biosynthetic and bioinformatics approaches seem to be the most promising, as an incredible amount of structural diversity can be accessed in a relatively simple format, thus allowing one to elucidate structure-activity (SAR) profiles.

In conclusion, the ability of a compact, bicyclic framework to present chemical functionality in a manner that is amenable to RNA affinity has been demonstrated. From a panel of twenty initial compounds, three lead molecules with good affinity were observed. Six molecules showed better inhibition than the known TAR binder argininamide. It should be noted that each panel molecule with affinity can be further elaborated at three remaining diversity positions. Three combinatorial positions with twenty members at each site will allow for 20x20x20=8,000 library members. In addition, since the construction of these molecules is modular, each member only requires the same number of steps for synthesis as the original panel. The structural characterization and functional elaboration of these molecules, as well as the evaluation of the specificity of these molecules for ΔTAR RNA, are the focus of current research efforts.

Acknowledgments

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