Communications to the Editor

High Affinity and Specific Binding of HIV-1 TAR RNA by a Tat-Derived Oligourea

N. Tamilarasu, Ikramul Huq, and Tariq M. Rana*

Department of Pharmacology
Robert Wood Johnson Medical School
675 Hoes Lane, Piscataway, New Jersey 08854

Received November 4, 1998

Protein−nucleic acid interactions are involved in many cellular functions such as transcription, RNA splicing, and translation. Small peptides with unnatural backbones that can bind with high affinity to a specific sequence or structure of nucleic acids and interfere with protein−nucleic acid interactions would provide useful tools in molecular biology and medicine. Recently, minor groove binding polyamide ligands have been designed for sequence-specific recognition of DNA.[1] In contrast to DNA, RNA molecules can fold into extensive structures containing regions of double-stranded duplex, hairpins, internal loops, bulged bases, and pseudoknotted structures.[2] The complexity of the RNA structure makes it difficult to design ligands for sequence-specific RNA recognition. Three-dimensional structures of RNA create binding sites for specific interactions with proteins. One example of such interactions is the mechanism of trans-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the trans-activation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all nascent HIV-1 transcripts.[3] Inhibition of Tat−TAR interactions is a potential approach for anti-HIV therapeutics. Since structural information is now available for TAR RNA and TAR−Tat peptide complexes from NMR,[4] photo-cross-linking,[5] and affinity cleaving studies,[6] it is possible to design small molecules to interfere with Tat−TAR function.

We have recently begun to examine TAR RNA recognition by unnatural biopolymers.[7] In this report, we synthesized an oligourea containing the basic arginine-rich region of Tat by solid-phase synthesis methods, and tested for TAR RNA binding. Oligoureas have backbones with hydrogen-bonding groups, chiral centers, and a significant degree of conformational restriction. Introducing additional side chains at the backbone NH sites can further modify biological and physical properties of these oligomers. This Tat-derived unnatural biopolymer binds specifically to TAR RNA with affinities significantly higher than the wild-type Tat peptide. These results identify a new class of unnatural peptides for structure-specific recognition of RNA.

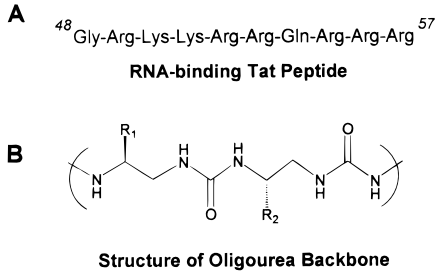

Figure 1. (A) Tat-derived peptide, amino acids 48−57, contains the RNA binding region of Tat protein. (B) Structure of the oligourea backbone. Sequence of the oligourea corresponds to the Tat peptide shown in (A), except the addition of an L-Tyr amino acid at the carboxyl terminus.[15] Tat-derived oligourea was synthesized on solid support by using activated *p*-nitrophenyl carbamates of protected amines.[9,10] The oligourea was purified by HPLC and characterized by mass spectrometry; 1850 (M + H).[15]

Recently, two methods have been reported for solid-phase synthesis of oligourea.[8,9] To synthesize Tat-derived oligourea on solid support, we used activated *p*-nitrophenyl carbamates of protected amines in the form of azides, which were reduced with $SnCl_2$-thiophenol-triethylamine on solid support (Figure 1).[9,10] After HPLC purification and characterization by mass spectrometry, the oligourea was tested for TAR RNA binding (Figure 2). The Tat-derived oligourea was able to bind TAR RNA and failed to bind a mutant TAR RNA without the bulge residues.

Equilibrium dissociation constants of the oligourea−TAR RNA complexes were measured using direct and competition electrophoretic mobility assays.[11] Dissociation constants were calculated from multiple sets of experiments which showed that the oligourea binds TAR RNA with a K_D of 0.11 ± 0.07 μM. To compare the RNA binding affinities of the oligourea to natural peptide, we synthesized a Tat-derived peptide (Tyr47 to Arg57) containing the RNA binding region of Tat protein (Figure 1). Dissociation constants of the Tat peptide−RNA complexes were determined from multiple sets of experiments under the same conditions used for oligourea−TAR RNA complexes. These experiments showed that the Tat peptide (47−57) binds TAR RNA with a K_D of 0.78 ± 0.05 μM. A relative dissociation constant (K_{REL}) can be determined by measuring the ratios of wild-type Tat peptide to the oligourea dissociation constants (K_D) for TAR RNA. Our results demonstrate that the calculated value for K_{REL} was 7.09, indicating that the urea backbone structure significantly enhanced the TAR binding affinities of the unnatural biopolymer.

Specificity of the oligourea−TAR RNA complex formation was addressed by competition experiments (Figure 2c). Oligourea−RNA complex formation was inhibited by the addition of unlabeled wild-type TAR RNA and not by mutant TAR RNAs. Mutant TAR RNA without a trinucleotide bulge (Figure 2c) or with a one base bulge (data not shown) was not able to compete for oligourea binding to wild-type TAR RNA.

* To whom correspondence should be addressed.

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(11) Supporting Information, Figure 4S.

10.1021/ja983822t CCC: $18.00
Published on Web 02/04/1999

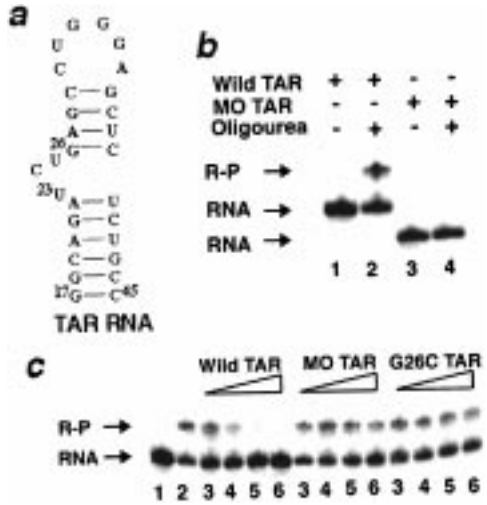

Figure 2. (a) Secondary structure of wild-type TAR RNA used in this study. Wild-type TAR RNA spans the minimal sequences that are required for Tat responsiveness in vivo[14] and for in vitro binding of Tat-derived peptides.[12] Wild-type TAR contains two nonwild-type base pairs to increase transcription by T7 RNA polymerase.[5] Mutant M0 TAR contained no bulge residue in its sequence. In mutant G26C, a base pair in the upper stem of TAR RNA, G26-C39 was substituted by C26−G39. (b) Electrophoretic mobility shift analysis for the Tat-derived oligourea binding to wild-type and trinucleotide bulge mutant (M0) TAR RNA. Experimental conditions for RNA−protein complex formation and analysis are described in Figure 4S (Supporting Information) and ref 5. (c) Specificity of the oligourea−TAR complex formation determined by competition experiments. Oligourea-RNA complexes were formed between ^{32}P-5′-end-labeled TAR RNA (40 nM) and the oligourea (150 nM) in the presence of increasing concentrations of unlabeled wild-type or mutant TAR RNAs. Concentrations of the competitor RNAs in lanes 3, 4, 5, and 6 were 50, 100, 150, and 200 nM, respectively. Lanes 1 and 2 were marker lanes showing RNA and oligourea−RNA complexes. Oligourea−RNA complexes are labeled as R−P.

Two base pairs immediately above the pyrimidine bulge are critical for Tat recognition.[12] To determine whether the oligourea recognizes specific base pairs in the stem region of TAR RNA or only a trinucleotide bulge containing RNA, we synthesized a TAR mutant where the G26−C39 base pair was substituted by a C26−G39 base pair (Figure 2a). Competition experiments showed that this mutant TAR (G26C) did not inhibit oligourea binding to TAR RNA (Figure 2c). These results indicate that the Tat-derived oligourea can specifically recognize TAR RNA.

To probe the oligourea−RNA interactions and determine the proteolysis stability of oligourea, we synthesized TAR RNA containing 4-thio-uracil at position 23 and performed photo-cross-linking experiments as described earlier (Figure 3).[5] Irradiation of the oligourea−RNA complex yields a new band with electrophoretic mobility less than that of the RNA (lane 4). Both the oligourea and UV (360 nm) irradiation are required for the formation of this cross-linked oligourea−RNA complex (see lanes 3 and 4). Since the cross-linked oligourea−RNA complex is stable to alkaline pH (9.5), high temperature (85 °C) and denaturing conditions (8 M urea, 2% SDS), we conclude that a covalent bond is formed between TAR RNA and the oligourea during the cross-linking reaction. To test the protease stability of the oligourea−RNA complexes, we subjected the oligourea−RNA cross-link products to vigorous proteinase K digestion which showed that the complexes were completely stable and there were no signs of oligourea degradation (lane 5). Under similar proteinase K treatment, a degradation of the RNA−protein cross-link products was observed (see lanes 7 and 8).

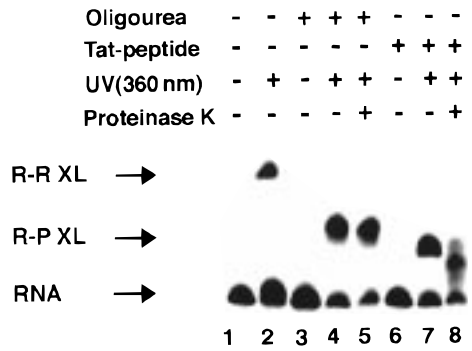

Figure 3. Site-specific photo-cross-linking reaction of TAR RNA labeled with 4-thio-uracil at position 23 with the oligourea. For photochemical reactions, RNA duplex was prepared by hybridizing two strands.[5] Strand 1 of the duplex was 5′-end-labeled with ^{32}P. Preformed RNA duplexes (40 nM) in the presence of the oligourea (100 nM) (lanes 4−5) or the Tat peptide (lanes 7−8) were irradiated (360 nm) and analyzed by denaturing gels as described earlier.[5] R-R XL indicates the RNA−RNA cross-links. RNA−oligourea and RNA−peptide cross-link products are shown as R-P XL.

Further competition experiments showed that the oligourea was able to compete with a Tat peptide for TAR RNA binding.[13] These findings show that a small Tat-derived oligourea binds TAR RNA specifically with high affinity and interacts in the widened major groove of TAR RNA similarly to Tat peptides. Due to the difference in backbone structure, oligoureas may differ from peptides in hydrogen-bonding properties, lipophilicity, stability, and conformational flexibility. Moreover, oligoureas are resistant to proteinase K degradation. These characteristics of oligoureas may be useful in improving pharmacokinetic properties relative to peptides. RNA recognition by an oligourea provides a new approach for the design of drugs which will modulate RNA−protein interactions.

Acknowledgment. We thank Seongwoo Hwang for helpful discussions. This work was supported in part by the National Institutes of Health Grants AI-41404, AI-43198, and TW-00702. T.M.R. is a recipient of a Research Career Development Award from NIH.

Supporting Information Available: Figures 4S−12S (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA983822T

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(15) For the structure, purification, and characterization of the oligourea, see Figures 6S−12S in Supporting Information.