Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 2839–2842

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of the U1A–RNA Complex by an Aminoacridine Derivative

Alicia Y. Gayle and Anne M. Baranger*

Department of Chemistry, Wesleyan University, Middletown, CT 06459, USA

Received 22 June 2002; accepted 19 July 2002

Abstract—The RNA recognition motif (RRM) is one of the most common RNA binding domains. There have been few investigations of small molecule inhibitors of RRM–RNA complexes, although these inhibitors could be valuable tools for probing biological processes involving RRM–RNA complexes and would have the potential to be effective drugs. In this paper, the inhibition by small molecules of the complex formed between the N-terminal RRM of the U1A protein and stem loop 2 of U1 snRNA has been investigated. An aminoacridine derivative has been found to promote dissociation of the U1A-stem loop 2 RNA complex with an IC_{50} value of 1 μM. Fluorescence experiments indicate that two aminoacridine ligands bind to each RNA target site. RNase A footprinting suggests that one binding site may be near the base pair that closes the loop and the other may be in a more flexible region of the loop. The addition of the aminoacridine derivative to stem loop 2 RNA increases the susceptibility of other portions of the loop to digestion by RNase A, which implies that binding of the ligand changes the conformation or dynamics of the stem loop target site. Either direct binding to the RNA or indirect alteration of the structure or dynamics of the loop would be likely to inhibit binding of the U1A protein to this RNA.

RNA–protein complexes play essential roles in fundamental reactions of gene expression and are important in the life cycles of many pathogens, including retroviruses.[1] Therefore, the development of small molecules able to perturb these interactions is of considerable importance. Due to the ability of RNA to form diverse, stable structures, the RNA targets in RNA–protein complexes vary in both sequence and structure and form distinct, potential target sites for small molecules.[2] Many previous investigations have focused on targeting RNA in the ribosome and in two RNA–protein complexes from HIV, the Rev–RRE and the Tat–TAR complexes.[3,4] However, there have been fewer investigations of the interruption of other RNA–protein complexes by small molecules. In this paper, we describe experiments that probe the inhibition of the complex formed between RNA and the N-terminal RNA recognition motif (RRM) of the U1A protein by small molecules. We find that an aminoacridine derivative (AD1) is able to inhibit the U1A–RNA complex with an IC_{50} of 1 μM.

Complexes formed between RNA recognition motifs (RRMs) and RNA are attractive targets for small molecule inhibition because the RRM is one of the most common RNA-binding domains and proteins containing this domain participate in many steps of gene expression.[5] The RRM is comprised of approximately 100 amino acids that form an antiparallel β-sheet flanked by two α-helices. RRMs bind to single-stranded RNAs in a variety of structural contexts. In general, the RNA target sites are not well structured in the absence of protein and thus form challenging targets for small molecule recognition.[6]

The U1A protein is a component of the spliceosome that splices most eukaryotic pre-mRNA.[7,8] The U1A protein binds with high affinity and specificity to stem loop 2 of U1 snRNA and to two related internal loops in the 3′-untranslated region (UTR) of its own pre-mRNA.[9–11] The U1A protein has been structurally characterized bound to stem loop and internal loop target sites by X-ray crystallography and NMR spectroscopy and these structures are nearly identical.[12–14] The structure of the U1A protein–stem loop 2 RNA complex is shown in Figure 1 along with the sequence of the stem loop 2 RNA used in experiments described in this paper. The U1A protein recognizes the AUUGCAC sequence in the loop and the CG base pair that closes the loop. Although the RNA has not been structurally

*Corresponding author. Tel.: +1-860-685-2739; fax: +1-860-685-2211; e-mail: abaranger@wesleyan.edu

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PII: S0960-894X(02)00636-4

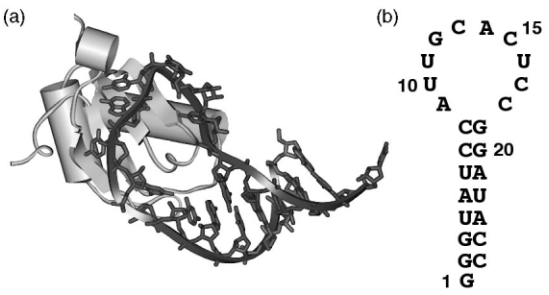

Figure 1. (a) Diagram of the U1A–RNA complex from the X-ray cocrystal structure.[12] (b) Stem loop 2 of U1 snRNA used in these experiments. The sequence of the stem has been changed from the wild type sequence so that the stem is fully complementary.

characterized in the absence of the U1A protein, NMR experiments and molecular dynamics simulations have suggested that the loop is flexible in the absence of the protein and undergoes substantial conformational rearrangements and conformational restriction upon binding the protein.[10,12,15,16]

We investigated the ability of the aminoacridine derivative AD1 and 2,4,5,6-tetraaminoquinozaline, shown in Figure 2, to destabilize the U1A–stem loop 2 RNA complex. We selected these molecules because both AD1 and 2,4,5,6-tetraaminoquinozaline have been found to inhibit formation of the Tat–TAR complex by binding to the closing GC or CG base pair and adjacent nucleotides of loops in the TAR RNA.[17,18] A similar association with stem loop 2 RNA would be likely to inhibit complex formation with the U1A protein, since binding with high affinity requires contacts between U1A and the closing CG base pair of the loop. AD1 was found to inhibit the Tat–TAR complex with an IC_{50} value of 22 nM, while 2,4,5,6-tetraaminoquinozaline inhibited the Tat–TAR complex with an IC_{50} value of 10 μM.[17,19] This difference in inhibitory potency may result from the greater positive charge of AD1 than 2,4,5,6-tetraaminoquinozaline, which should promote strong ionic interactions with the TAR RNA. In addition, the flexible linkers in AD1 may allow for favorable interaction with a greater diversity of RNA structures.

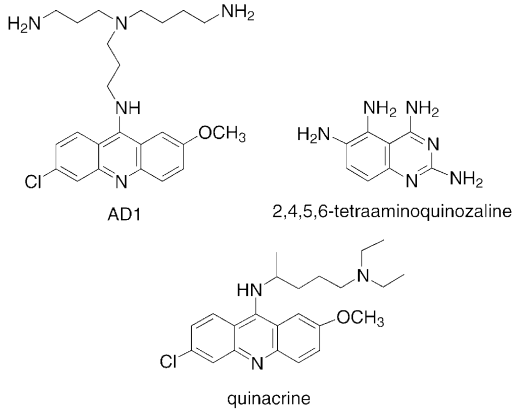

Figure 2. Small molecules used in these investigations. AD1 and 2,4,5,6-tetraminoquinozaline were synthesized following published methods.[19,23,24]

We measured the ability of AD1 and 2,4,5,6-tetraaminoquinozaline to inhibit the formation of the U1A–stem loop 2 RNA complex using gel mobility shift assays. The wild type sequence of stem loop 2 RNA contains UG and UU pairs in the stem. We changed the stem sequence of to be fully complementary, as shown in Figure 1. Previous experiments have shown that the sequence of the stem is not important for binding of the U1A protein.[10,20] We confirmed that the incorporation of a complementary stem sequence did not change the ability of U1A to bind to stem loop 2 RNA. To measure the ability of the small molecules to promote dissociation of the U1A–stem loop 2 RNA complex, the U1A–stem loop 2 RNA complex was formed and the concentrations of U1A and stem loop 2 RNA were adjusted so that between 50 and 80% binding was achieved. After addition of AD1 the reaction mixture was equilibrated at room temperature for 1 h and then loaded onto a running non-denaturing 12% polyacrylamide gel maintained at 25 °C. The results obtained from a typical experiment with AD1 are shown in Figure 3. The concentration of AD1 required to reduce binding by 50% was 1.0 ± 0.3 μM. No dissociation of the U1A–RNA complex was observed upon addition of 2,4,5,6-tetraaminoquinazoline up to 10 mM. Acridine, spermidine, and the related acridine derivative quinacrine did not promote complex dissociation, suggesting that both the acridine group and the flexible, positively charged polyamino group are important for inhibition of the U1A–stem loop 2 RNA complex by AD1.

The binding of AD1 to stem loop 2 RNA was characterized by fluorescence spectroscopy. AD1 has an excitation maximum at 416 nm and an emission maximum at 488 nm. Upon binding RNA, the emission signal is quenched and the emission maximum shifts from 488 to 476 nm (Fig. 4). The stoichiometry of the binding reaction was determined by adding increasing concentrations of stem loop 2 RNA to a constant concentration of AD1. The results from one of these experiments are shown in Figure 5. From the average of three such experiments performed with 0.5 and 0.3 μM AD1, the stoichiometry of the binding reaction was determined to be two AD1 molecules to one RNA. In a subsequent set of experiments, the concentration of

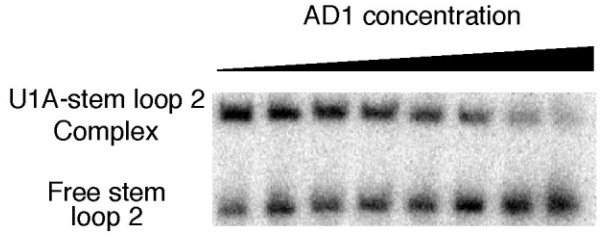

Figure 3. The inhibition of the U1A–stem loop 2 RNA complex by AD1 was monitored by gel electrophoresis on 15 cm×40 cm×1.5 mm 8% acrylamide, 80:1 mono/bisacrylamide gels. Binding reactions were carried out in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 125 mM NaCl, 0.5% Triton X-100, and 5% glycerol. The concentrations of U1A protein and stem loop 2 RNA were 40 and 4 pM, respectively. The highest concentration of AD1 was 3.6 μM and a 2-fold serial dilution was performed to create a series of binding reactions containing progressively less AD1.

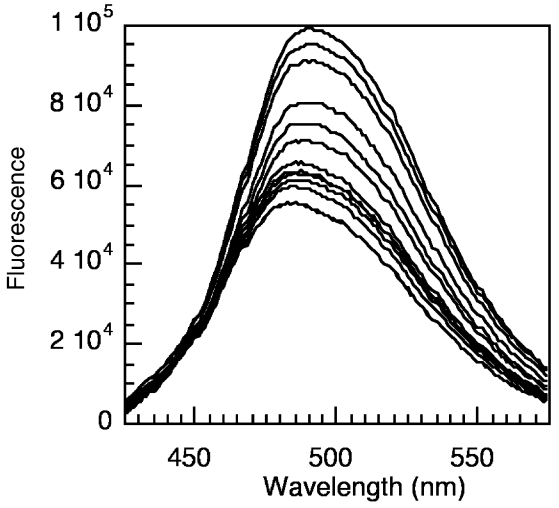

Figure 4. Fluorescence spectra of AD1 with progressively more RNA added. The initial concentration of RNA is 0 μM and the final concentration is 0.8 μM. The measurements were performed in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 125 mM NaCl, 0.5% Triton X-100, and 5% glycerol. The volume of RNA added did not change the volume of the AD1 solution by more than 5%. The excitation wavelength was 390 nm. The scans were performed at 20 °C and the solutions were continuously stirred during the emission scan.

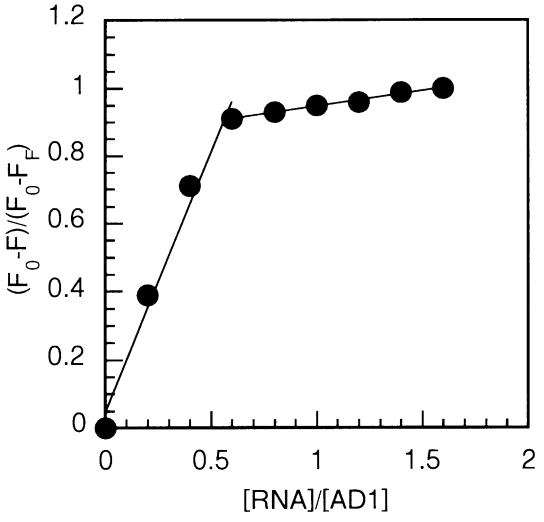

Figure 5. A plot of the fraction AD1 fluorescence signal quenched versus the ratio of RNA to AD1. F_0 is the initial fluorescence intensity of AD1, F_F is the final fluorescence intensity of the AD1–RNA complex, and F is the observed fluorescence intensity. This plot was used to determine the stoichiometry of the reaction. The conditions were identical to those described in the legend of Figure 4.

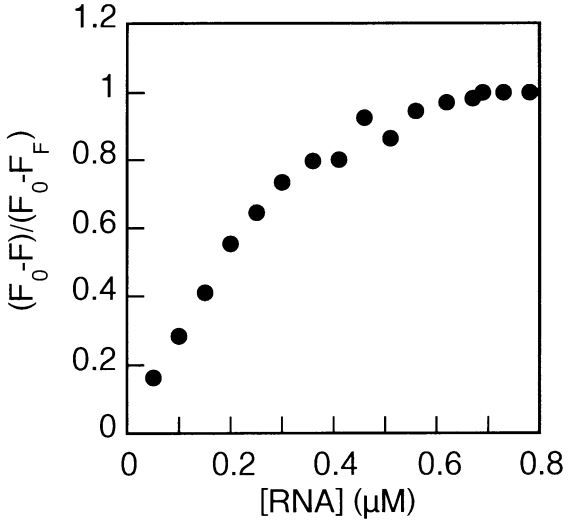

Figure 6. A plot of the fraction AD1 fluorescence signal quenched versus RNA concentration. The plot is the average of three experiments. The measurements were performed under the conditions described in the legend of Figure 4 except the concentration of NaCl was 250 mM and the concentration of AD1 was 0.3 μM.

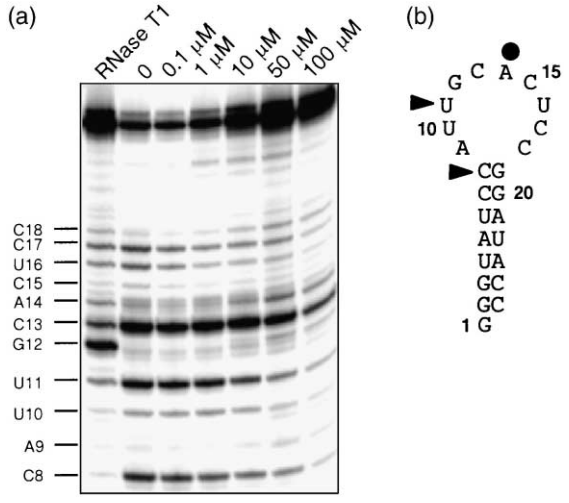

Figure 7. (a) RNase A footprinting of the AD1–stem loop 2 RNA complex. Stem loop 2 RNA was 5′-end labeled with ^{32}P, incubated with the indicated concentrations of AD1 for one h at room temperature in 10 mM Tris–HCl (pH 8.0), 125 mM NaCl, 0.5% Triton X-100, and 5% glycerol, and digested with RNase A. The reactions were separated on 20% denaturing polyacrylamide gels. The concentration of AD1 added to each reaction is indicated above each lane. The RNase T1 lane is stem loop 2 RNA treated with RNase T1, an enzyme that preferentially digests single-stranded RNA 3′ to G′s. (b) Secondary structure of stem loop 2 RNA indicating the sites of protection (▶) and enhanced digestion (●) by RNase A upon AD1 binding.

sodium chloride was raised to 250 mM. As expected, this increase in salt concentration decreased binding affinity and a binding curve was obtained under these conditions (Fig. 6). Although the individual equilibrium binding constants cannot be determined from this titration, maximum binding is achieved at 0.6 μM RNA, which is comparable to the concentration of AD1 required to inhibit the U1A–RNA complex. These results suggest that binding of AD1 to stem loop 2 RNA prevents binding of the U1A protein.

RNase A footprinting experiments were performed to suggest possible binding sites of AD1 on stem loop 2 RNA (Fig. 7). From five such experiments we determined that nucleotides C8 and U11 are protected from RNase A activity upon binding of AD1. These positions are therefore, likely to be the binding sites of AD1 on stem loop 2 RNA, although we can not distinguish between direct binding and an indirect effect that decreases the reactivity of C8 or U11 with RNase A. One of these positions, C8, is similar to the binding site

of AD1 on the TAR RNA, which has been proposed to be a GC base pair closing an internal loop.[18] However, the other protected site, U11, is in a flexible part of the loop. A14 became more susceptible to RNase A cleavage upon binding AD1, which suggests that association with AD1 changes the conformation of the RNA target site and increases the susceptibility of some portions of stem loop 2 RNA to RNase A cleavage. C8, U11, and A14 have been shown to contact the U1A protein and to be important for binding affinity.[10,12,13,21,22]

These results demonstrate that AD1 is an effective inhibitor of the U1A–RNA complex. The correlation between the results of the fluorescence and inhibition studies provides evidence that inhibition of the U1A–RNA complex arises from the binding of AD1 to the RNA target site. Footprinting experiments suggest that one of the two AD1 molecules that binds stem loop 2 RNA may bind at the base of the loop, which is similar to the proposed binding site of AD1 on the TAR RNA.[18] However, the protection of U11 from RNase cleavage implies that either AD1 is able to bind directly to the unstructured portion of the loop or that binding of AD1 to the base of the loop alters RNA conformation so that RNase activity is decreased at this position. Either direct binding of AD1 or indirect structural changes as a result of AD1 association would be likely to prevent the association of the U1A protein with stem loop 2 RNA. Since many RRMs bind to RNAs that are less structured than stem loop 2 RNA, the development of small molecules that directly associate with these target sites will be challenging. The indirect alteration of RNA conformation or flexibility may be a more straightforward approach for the inhibition of the formation of these ubiquitous RNA–protein complexes.

Acknowledgements

This research was supported, in part, by the NIH (grant GM-56857). A.M.B. is an Alfred P. Sloan Research Fellow. A.Y.G. was supported by awards for undergraduate research from Bristol-Myers Squibb and the Howard Hughes Medical Institute.

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