Specific binding of Hoechst 33258 to site 1 thymidylate synthase mRNA

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Received December 30, 1999; Revised March 8, 2000; Accepted March 14, 2000

ABSTRACT

The translational initiator codon in thymidylate synthetase (TS) mRNA is located in a stem–loop structure with a CC bubble. TS is an important target for anticancer drugs. Aminoglycoside antibiotics have been shown to specifically bind to TS mRNA site 1 constructs and, furthermore, specific binding requires the non-duplex CC bubble region. It is shown here that DNA intercalating agents and DNA minor groove-binding drugs also bind to a TS mRNA site 1 construct. This binding is competitive with aminoglycosides, suggesting that the binding sites overlap. Hoechst 33258 binds with a dissociation constant of 60 nM, a value significantly lower than the ~1 µM values found for aminoglycosides. Footprinting and direct binding studies show that the CC bubble is important for binding of the Hoechst compound. However, the exact structure of the bubble is unimportant. Interestingly, mutations in regions adjacent to the bulge also affect binding. These studies point to the important role of non-duplex RNA structures in binding of the DNA minor groove binder Hoechst 33258.

INTRODUCTION

Aminoglycoside antibiotics operate by binding to target RNA structures and inhibiting their ability to function biochemically. The prokaryotic 16S rRNA decoding region is the pharmacologically relevant target for the antibiotic aminoglycosides (1,2). The binding of aminoglycosides to this region results in protein mistranslation, leading to the resulting bacteriocidal effects of the drugs (3). The local domain of RNA to which the aminoglycosides bind contains an accessible non-Watson–Crick base paired bubble structure (4). As a general rule, aminoglycosides bind specifically to non-duplex regions of RNA, such as bulge and bubble regions (4,5–7). The dissociation constants for binding can vary between nanomolar (8) and micromolar values (5,11,14). Gradually converting the bubble region into an A-form duplex diminishes aminoglycoside binding in 3- to 5-fold steps in affinity over a 75-fold range (15). Thus, aminoglycoside recognition is not unique to a particular RNA structure, but rather aminoglycoside binding is a general feature of non-duplex RNA. The minimal RNA bubble structure reported which supports aminoglycoside binding is the internal CC bubble found in the 5′-untranslated region (UTR) of the mRNA for thymidylate synthetase (TS) (16). It was of interest to further explore drug binding to the TS and to determine whether drug molecules, other than aminoglycosides, can also recognize structural features of an RNA construct based on the mRNA of TS.

TS catalyzes the reductive methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) to form thymidine 5′-monophosphate (dTMP) (17). TS represents the sole de novo source of dTMP formation in cells and is critical for DNA synthesis and is an important target enzyme in cancer chemotherapy (17–21). Interestingly, TS has been shown to bind to numerous RNA constructs (22,23). Of particular interest is the observation that TS specifically binds to its own mRNA with an affinity in the 1–2 nM range (24,25). This binding has functional consequences, because translation of the human TS mRNA is controlled by TS via an autoregulatory feedback mechanism (24,25). Thus, the binding of apo-TS to its own message prevents its translation. TS is one of the first eukaryotic enzymes demonstrated to be regulated in this fashion (26).

There are two distinct domains in the TS mRNA which are involved in protein recognition (Scheme 1A) (27). The first site (site 1) is confined to a predicted hairpin loop located within the first 188 nt of the message and includes the initiation codon AUG (27). The second site (site 2) appears to be confined to an ~200 nt region corresponding to nt 434–634 in the protein coding region. The sequence of the site 1 hairpin loop is known (27) and the predicted secondary structure of the
hairpin loop is shown in Scheme 1B. Aminoglycosides bind in a specific and saturable fashion with dissociation constants of ~1 µM to a TS mRNA site 1 construct and the binding site for aminoglycosides is located in the CC bubble region (16). It is of further interest to explore the binding of other possible antagonists to the TS mRNA site 1 construct to determine if they might bind more tightly to the construct than aminoglycosides and also to determine which features of the construct are recognized. It is reported here that the DNA minor groove binder Hoechst 33258 (Scheme 2) binds to the TS mRNA site 1 construct with a dissociation constant of ~60 nM. While binding requires the CC bubble, protection experiments demonstrate that the site of binding is adjacent to the bubble. However, binding is competitive with aminoglycosides, suggesting that there is overlap in their respective binding sites.

MATERIALS AND METHODS

Materials

Distamycin A, DAPI, Hoechst 33258, quinacrine, proflavine, neomycin sulfate and paromomycin sulfate were purchased from Sigma and were used without further purification. 5-Carboxytetramethylrhodamine succinimidyl ester was purchased from Molecular Probes. CRP was prepared as previously reported (11). Oligonucleotides were obtained from Oligo’s Etc. PCR reactions were carried out using the Gene Amp PCR kit with AmpliTag DNA polymerase from Perkin Elmer. RNase CL3 was purchased from Boehringer Mannheim. RNA transcripts were generated using the RiboMAX large scale RNA production kit with T7 RNA polymerase from Promega.

RNA synthesis and purification

TS RNA constructs were transcribed in vitro by T7 RNA polymerase using synthetic oligonucleotide templates (28). All RNA contained GGG at the 5′-end to increase the efficiency of transcription. Purified RNA was resuspended in sterile deionized water. The RNA was renatured by incubating in binding buffer (150 mM NaCl, 1 mM MgCl2 and 20 mM HEPES, pH 7.5) for 1 min at 80°C followed by slow cooling to 25°C.

Fluorescence measurements

Fluorescence intensity and anisotropy measurements were performed on a Perkin Elmer LS-50B luminescence spectrometer equipped with a thermostat accurate to ±0.1°C. The CRP solution (10 nM) was excited at 550 nm and monitored at 580 nm. A Hoechst 33258 solution (0.2 mM) was excited at 350 nm and monitored at 460 nm. The integration time was 5 s. For every single point six measurements were made and their average values were used for calculation. Measurements were performed in a buffer solution containing 150 mM NaCl, 1 mM MgCl2 and 20 mM HEPES, pH 7.5. CRP concentration was determined spectroscopically at 550 nm using a molar extinction coefficient of 6.00 × 10^4 M⁻¹ cm⁻¹.
**Determinations of dissociation constants**

Equation 1 was used for the determination of the dissociation constant for the interactions between RNA and CRP ($K_D$).

$$A = A_0 + \Delta A(([{\text{RNA}}_0] + [{\text{CRP}}_0] + K_0) - ([{\text{RNA}}_0] + [{\text{CRP}}_0] + K_0)^2 - 4[{\text{RNA}}_0][{\text{CRP}}_0])^{1/2}/2$$

where $A$ and $A_0$ are the fluorescence anisotropy of CRP in the presence and absence of RNA, respectively, $\Delta A$ is the difference between the fluorescence anisotropy of 10 nM CRP in the presence of an infinite concentration of RNA minus the fluorescence anisotropy in the absence of RNA. $[{\text{RNA}}_0]$ and $[{\text{CRP}}_0]$ are the initial concentrations of RNA and CRP, respectively.

In the competitive binding assay, equation 2 was used to calculate the $K_D$ values.

$$[{\text{aminoglycoside}}_0] = \frac{(1)}{(1)}$$

where $K_D$ is the dissociation constant between the RNA and the aminoglycosides and $[{\text{aminoglycoside}}_0]$ is the initial concentration of the aminoglycosides. Both $K_d$ and $K_D$ were determined by non-linear curve fitting using the equations described above and presented as a mean value from three independent measurements.

In the direct fluorescence binding assay equation 3 was used for the determination of the dissociation constant for the interactions between RNA and Hoechst 33258 ($K_D$).

$$I = I_0 + \Delta I(([{\text{RNA}}_0] + [{\text{L}}_0] + K_0) - ([{\text{RNA}}_0] + [{\text{L}}_0] + K_0)^2 - 4[{\text{RNA}}_0][{\text{L}}_0])^{1/2}/2$$

where $I$ and $I_0$ are the fluorescence intensity of Hoechst 33258 in the presence and absence of RNA, respectively, $\Delta I$ is the difference between the fluorescence intensity of 200 nM Hoechst 33258 in the presence of an infinite concentration of RNA minus the fluorescence intensity in the absence of RNA. $[{\text{RNA}}_0]$ and $[{\text{L}}_0]$ are the initial concentrations of RNA and Hoechst 33258, respectively.

**RNase CL3 footprinting assay of TS RNA**

5'-32P-labeled TS RNA (20 000 c.p.m.) in 40 µl of incubation buffer (150 mM NaCl, 1 mM MgCl2 and 20 mM HEPES, pH 7.5) was treated with RNase CL3 in the presence or absence of Hoechst 33258 for 4 min at 25°C. The reaction was stopped by addition of a 3 M sodium acetate solution containing 5 mM EDTA followed by precipitation of the RNA with ethanol. Electrophoresis was carried out for 4 h on 15% polyacrylamide–8 M urea gels.

**RESULTS**

**Binding of intercalators and minor groove-binding drugs to the TS construct**

It had previously been shown that aminoglycosides are capable of binding to the TS construct (Scheme 1, TS 1) with $K_D$ values in the micromolar range (16). Fluorescence anisotropy measurements with CRP (Scheme 2) were used to determine affinities of binding of competitive aminoglycoside antagonists (16). Figure 1A shows a typical fluorescence titration curve using CRP to measure binding. The $K_D$ for CRP in these measurements is 0.87 ± 0.12 µM. Molecules that compete with CRP for binding to the RNA molecule are readily determined by competition assays (16). A group of putative antagonists were chosen for study which fell into two distinct drug classes: the intercalators and the minor groove binders (Scheme 2). Quinicine and proflavine are representative of the intercalator class of drugs, while Hoechst 33258, DAPI and distamycin A are representative of the DNA minor groove binders. When assayed as competitive antagonists of CRP in the quantitative RNA binding assay (16), all of the molecules exhibited competitive binding behavior. A typical competition curve for the minor groove binder Hoechst 33258 is shown in Figure 1B. The curve fits a simple competitive model (11) and curve fitting reveals a $K_D$ of 60 nM. This value is substantially lower than the dissociation constants measured for aminoglycosides, which typically give $K_D$ values in the low micromolar range (16). A summary of the $K_D$ values for the different drugs is shown in Table 1. All of the analogs were competitive with CRP, with the Hoechst compound exhibiting the highest affinity. The other analogs exhibited affinities similar to that of neomycin (Table 1). The specificity of binding of the high affinity binding Hoechst compound was explored further.
The specificity of Hoechst 33258 binding

The series of TS constructs shown in Scheme 3 were prepared and studied with respect to binding of the Hoechst compound. TS 1 is the wild-type construct already described. All of the constructs, save for TS 5, in which the CC bubble is replaced by a GC base pair, bind CRP with comparable dissociation constants. This is expected, because aminoglycosides, while not able to specifically bind to duplex RNA, readily bind to RNA containing internal bubbles and bulges (15). When the various constructs were studied with respect to the binding of Hoechst 33258, the results provided in Table 2 were obtained. As can be seen here, the structural nature of the bubble (TS 1–TS 4) was largely irrelevant for competitive binding. A significant diminution in affinity (~2-fold) was observed in constructs TS 6–TS 8, in which the base pair adjacent to the bubble was changed. The next base pair also appeared important in binding, because TS 9 and TS 10 showed substantial decrements in binding affinities. The remaining mutants (TS 11–TS 14), in which base pair alterations were made in the remaining part of the stem, proved to bind the drug with affinities similar to TS 1 and thus the mutated regions appeared to be unimportant for Hoechst 33258 binding.

It was not possible to directly determine whether Hoechst 33258 could bind to the fully duplex stem–loop construct TS 5 by competition with CRP because the latter is unable to bind to the fully duplex RNA. Direct fluorescence binding measurements on the Hoechst compound were made. When the Hoechst compound binds to TS RNA, fluorescence enhancement is observed, as shown in Figure 3. Binding of this compound to DNA can be estimated by following the observed fluorescence enhancement (29,30). In the case of TS 1, a clear saturable binding isotherm was observed which provided a measured $K_D$ value of 186 ± 25 nM. In contrast, the Hoechst compound did not show saturable binding to the duplex construct TS 5. Here the binding appeared to be non-specific, suggesting that the Hoechst compound, like aminoglycosides, can only specifically bind to RNA molecules containing non-duplex structural elements.

In order to reveal the approximate binding site of the Hoechst compound, RNase CL3 footprint studies were performed on TS 1. RNase CL3 is known to cleave 3′ to cytidine in the single-strand region of RNA (31). As shown in Figure 4, the GC base pair adjacent to the CC bubble is the most significant site of protection, suggesting that this base pair constitutes part of the Hoechst 33258 binding site. These results are consistent with the mutational studies described in Table 2, where the base pair in question proved to be of importance in binding.

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DISCUSSION

The design of potent antagonists of specific RNA molecules is of great interest and importance. Thus far, aminoglycosides appear to be the only molecules generally able to bind to a variety of RNA structures (32–34). Aminoglycosides are polycationic in nature, and bind to RNA through electrostatic and hydrogen bonding interactions (33). The naturally occurring aminoglycosides appear not to be highly selective with respect to RNA structure. They appear to bind to many non-duplex RNA structures with modest affinities (~K_D values in the micromolar range) (15). This is not to say that aminoglycosides are not capable of specificity, because they potentially are. For example, aptamers have been selected against the aminoglycoside tobramycin with sub-nanomolar affinities and with great specificity (35). However, in general it is still true that standard aminoglycosides are quite promiscuous with respect to RNA structure, as long as there are non-duplex regions present (15). The major groove of duplex RNA presumably presents a steric barrier to the binding of aminoglycosides. An issue that arises is whether other nucleic acid-directed molecules will show similar specificities in their binding to RNA. In this article, the binding of a group of DNA-intercalating and minor groove-directed agents to a TS mRNA construct is reported. It is shown that Hoechst 33258 binds to this construct with a substantial affinity and that specific binding requires the CC bubble of the TS RNA to be present.

The binding of drug molecules to the TS construct is monitored by competition with CRP, a fluorescent aminoglycoside analog (16). This molecule, like the other aminoglycosides which bind to TS mRNA constructs, requires the CC bubble for specific binding. In a competition assay, neomycin B is found to bind to the construct with a K_D of 1.8 µM. Interestingly, the two intercalating agents quinacrine and proflavine are also competitive with CRP and bind with ~1 µM affinities. Of the three DNA minor groove binders DAPI, distamycin A and Hoechst 33258, only the latter showed high affinity binding. The K_D value was measured to be 60 nM in the competition assay. Because of this significant affinity, the binding behavior of this drug was explored further.

The first issue of concern is whether the binding of Hoechst 33258 requires the CC bubble as a structural feature in the TS RNA construct. Since aminoglycosides require this bubble for binding, it was not possible to carry out competition experiments.
of Hoechst 33258 with CRP. Since Hoechst 33258 undergoes a fluorescence intensity change when it binds to RNA and DNA, it was possible to directly follow its binding by fluorescence spectroscopy (29,30). It could be shown that Hoechst 33258 binds saturaingly to the construct. However, the measured $K_D$ is some 3-fold larger than the value measured by direct competition with CRP. However, direct binding measurements of Hoechst 33258 by fluorescence methods are known to be problematical and may provide only approximate values (29,30). It is interesting to note that the mutant TS 5, which lacks the CC bubble, does not saturaingly bind Hoechst 33258. These direct binding experiments suggest the importance of the non-duplex element in TS for binding of the drug. It is interesting to note that a similar observation was made on the binding of Hoechst 33258 to a TAR RNA construct derived from HIV-1 (36). Here it was found that the uridine bulge of the TAR construct was important for specific binding of the drug (36). Deletion of the bulge markedly altered the binding of the drug, although binding still occurred, and changed its orientation upon binding (36). Thus, the binding of Hoechst 33258 to RNA molecules may generally require non-duplex structural elements.

To further explore the binding of Hoechst 33258 to the TS constructs which contain non-duplex elements, binding studies were performed on a series of TS mutants. These studies showed that the exact structure of the bulge was not important inasmuch as TS constructs 1–4 all showed comparable binding affinities. Interestingly, mutations in regions adjacent to the bulge affected binding. A decrease in affinity of ~2-fold was determined for constructs TS 6–TS 8, in which the base pair adjacent to the bubble was substituted. The next base pair also appeared to be important for binding, because TS 9 and TS 10 showed substantial decrements in binding affinities compared to TS 1. Other mutations in the TS stem proved to be of insignificant importance with respect to Hoechst 33258 binding. These substitution experiments suggest that drug binding involves the GC base pairs adjacent to the CC bubble, in addition to the bubble itself.

This conclusion is supported by RNase CL3 footprinting. These experiments showed that the GC base pair adjacent to the CC bubble in TS 1 is clearly involved as part of the binding site of Hoechst 33258. It is already known that the Hoechst compound can bind to GC-rich regions of double-stranded DNA (29,37,38). In the case of TS RNA it is likely that the CC bubble allows the drug to gain access to the GC base pairs which otherwise would be inaccessible for steric reasons. Thus, the binding of aminoglycosides and Hoechst 33258 to RNA molecules may share some features in common insofar as the specific binding of both compounds appears to require non-duplex structural elements in the target RNA molecules. The fact that aminoglycosides and the Hoechst compound appear to have similar, but non-overlapping binding sites suggests the possibility that hybrid molecules could be constructed with exceedingly high affinities for TS RNA.

ACKNOWLEDGEMENT

These studies were funded by US Public Health Service National Institutes of Health Grant 1 RO1 EY-12375

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