

RNA Sequence Determinants for Aminoglycoside Binding to an A-site rRNA Model Oligonucleotide

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The codon-anticodon interaction on the ribosome occurs in the A site of the 30 S subunit. Aminoglycoside antibiotics, which bind to ribosomal RNA in the A site, cause misreading of the genetic code and inhibit translocation. Biochemical studies and nuclear magnetic resonance spectroscopy were used to characterize the interaction between the aminoglycoside antibiotic paromomycin and a small model oligonucleotide that mimics the A site of *Escherichia coli* 16 S ribosomal RNA. Upon chemical modification, the RNA oligonucleotide exhibits an accessibility pattern similar to that of 16 S rRNA in the 30 S subunit. In addition, the oligonucleotide binds specifically aminoglycoside antibiotics. The antibiotic binding site forms an asymmetric internal loop, caused by non-canonical base-pairs. Nucleotides that are important for binding of paromomycin were identified by performing quantitative footprinting on oligonucleotide sequence variants and include the C1407-G1494 base-pair, and A-U base-pair at positions 1410/1490, and nucleotides A1408, A1493 and U1495. The asymmetry of the internal loop, which requires the presence of a nucleotide in position 1492, is also crucial for antibiotic binding. Introduction into the oligonucleotide of base changes that are known to confer aminoglycoside resistance in 16 S rRNA result in weaker binding of paromomycin to the oligonucleotide. Oligonucleotides homologous to eukaryotic rRNA sequences show reduced binding of paromomycin, suggesting a physical origin for the species-specific action of aminoglycosides.

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Introduction

Antibiotics interact with many biological macromolecules to affect cellular processes and ultimately kill bacteria. A wide variety of antibiotics with various targets in the cell have been developed, yet their widespread use has led to the appearance of resistant bacteria. Organisms have developed resistance to antibiotics by three major mechanisms: enzymatic modification and inactivation of antibiotics, altering the target of the antibiotics, or active efflux of antibiotics from the cell (Neu, 1992). Structural and biochemical studies of antibiotic interactions with their binding site should aid in the design of better antimicrobial agents.

The ribosome is the target of a class of structurally related antibiotics, the aminoglycosides (Figure 1; Gale *et al.*, 1981). These antibiotics affect protein synthesis by inducing codon misreading and inhibiting translocation (Davies *et al.*, 1965; Davies & Davis, 1968) by binding directly to 16 S ribosomal RNA (rRNA) in the 30 S subunit. The aminoglycosides are composed of amino sugars linked to a deoxystreptamine ring. The neomycin-class aminoglycosides, which contain conserved functional groups on rings I and II (Figure 1(a) and (b)), cause a characteristic miscoding pattern, whereas streptomycin (Figure 1(c)), which does not contain these conserved functional groups, induces a pattern of miscoding that is distinct from that of the neomycins (Davies *et al.*, 1966).

Ribosomal RNA plays a pivotal role in the selection of transfer RNAs (tRNA) and in catalysis of peptide bond formation by ribosomes (Noller, 1991). Translational accuracy requires specific

Abbreviations used: DMS, dimethyl sulfate; DEPC, diethyl pyrocarbonate; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; ppm, parts per million.

interaction between the tRNA anticodons and mRNA codons. The codon-anticodon interaction occurs in the decoding region of the small (30 S) subunit, which is formed by two short phylogenetically

conserved sequences near the 3' end of 16 S rRNA (the 1400-1500 region: Figure 2(a)). These sequences define a portion of the A and P sites that respectively contain the aminoacyl-tRNA and

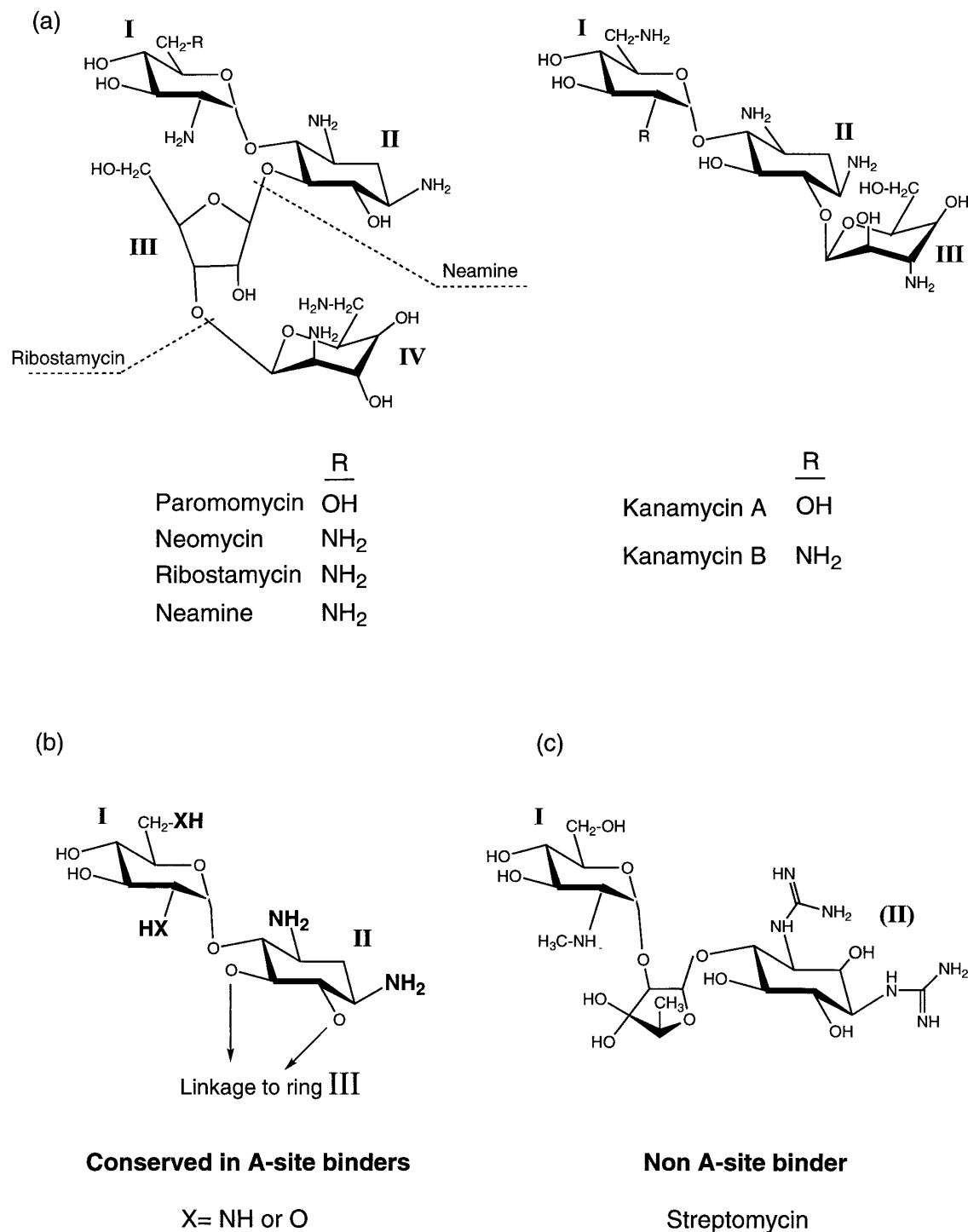


Figure 1. (a) Structures of the aminoglycoside antibiotics that bind in the A site of the decoding region. The A-site binding aminoglycosides all contain rings I and II. The neomycin group of aminoglycosides include paromomycin, neomycin, ribostamycin and neamine. Ribostamycin contains all rings except ring IV, while neamine lacks both rings III and IV. (b) Rings I and II of the aminoglycosides indicating positions of amino groups or hydrogen bond donors (XH) common to all A-site binding antibiotics. (c) Structure of the aminoglycoside antibiotic streptomycin. The rings that correspond to rings I and II of the A-site binding aminoglycosides are indicated.

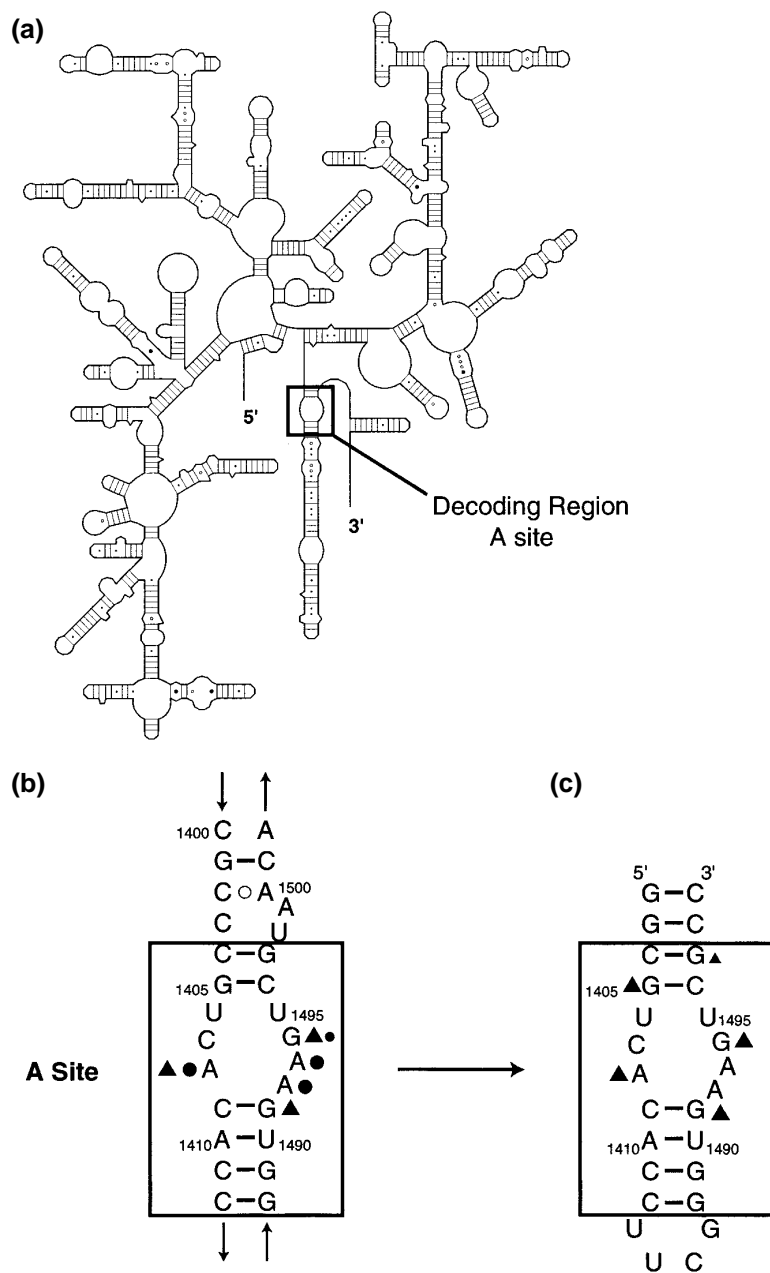


Figure 2. (a) Secondary structure of 16S rRNA from *E. coli* with the decoding region boxed. (b) Sequence of the decoding region; nucleotides protected from DMS modification by A-site tRNA (●) and neomycin-class aminoglycoside antibiotics (▲) are indicated. (c) Sequence of A-site oligonucleotide with portions of 16S rRNA sequence boxed. Nucleotides in the oligonucleotide protected from DMS modification by neomycin-class aminoglycoside antibiotics are indicated (▲).

peptidyl-tRNA bound to adjacent codons (Moazed & Noller, 1986, 1990). Although the P site contains RNA sequences that are dispersed in the secondary structure of ribosomal RNA, the A site is formed by nucleotides in just the decoding region and 530 loop (Moazed & Noller, 1986, 1990). Interaction of tRNA in the A site affects the reactivity of nucleotides in the 530 loop, yet these changes are likely an indirect result of conformational changes in the ribosome (Powers & Noller, 1994a,b). Therefore, we define the small subunit A site in this work as only the nucleotides in the decoding region.

The A site is where aminoglycosides bind to the ribosome, as determined by biochemical and genetic methods. Chemical modification experiments on the ribosome indicate that neomycin-

class aminoglycosides footprint the A site (Moazed & Noller, 1987; Woodcock *et al.*, 1991; and see Figure 2(b)). Aminoglycoside resistance mutations in a variety of organisms occur in the small subunit A-site RNA. Yeast resistance to the neomycin-class antibiotic paromomycin is conferred by a C to G mutation of the mitochondrial rRNA at the position corresponding to C1409 of *Escherichia coli* 16S rRNA (Li *et al.*, 1982). Paromomycin resistance in *Tetrahymena thermophila* results from a G to A mutation at the position corresponding to G1491 of *Escherichia coli* 16S rRNA (Spangler & Blackburn, 1985). Disruption of the 1409-1491 base-pair in *E. coli* 16S rRNA confers resistance to a variety of aminoglycoside antibiotics (DeStasio *et al.*, 1989; DeStasio & Dahlberg, 1990).

The A site has been implicated as the aminogly-

coside binding site by the action of specific RNA methylases that modify bases in this region and confer resistance to certain aminoglycosides (Beauleck & Cundliffe, 1987). Methylation at N-7 of G1405 confers resistance to gentamicin, whereas methylation at N-1 of A1408 has been shown to confer resistance to kanamycin, neamine and ribostamycin (Cundliffe, 1990).

The *E. coli* small ribosomal subunit is composed of a 1542 nucleotide 16 S rRNA and 21 proteins. Several studies have demonstrated that the ribosome can be reduced to individual, functional domains. The 3' domain of 16 S rRNA assembles *in vitro* into an independent ribonucleoprotein particle that contains the proteins normally associated with this domain in the full 30 S subunit (Samaha *et al.*, 1994). Based on footprinting experiments, no ribosomal protein binds in the decoding region (Powers & Noller, 1995). The decoding region has been reduced to a small functional domain (64 nucleotides) able to bind both antibiotic and RNA ligands of the 30 S subunit in a manner similar to the 30 S subunit (Purohit & Stern, 1994). Reductionist approaches can delineate both structure and interactions of RNA molecules. A well-designed system can produce an oligonucleotide that maintains the local structure that exists in the context of the whole RNA (Puglisi *et al.*, 1992; Battiste *et al.*, 1994; Szewczak & Moore, 1995).

This piece-wise approach to understanding a biological system has many advantages. RNA-ligand interactions can be investigated by affinity chromatography and gel mobility shift assays on an oligonucleotide that would be difficult to perform on the intact RNA. Structural analyses by NMR or X-ray crystallography can often be applied more readily to the simplified model than the intact system. Mutations made in the decoding region of rRNA are lethal or impair ribosome function (Krzyszosiak *et al.*, 1987; Thomas *et al.*, 1988; Denman *et al.*, 1989; DeStasio & Dahlberg, 1990; Cunningham *et al.*, 1992a,b; Cunningham *et al.*, 1993). In an oligonucleotide system, there are no lethal effects of mutagenesis, so mutations that would otherwise impair ribosome function can be studied for their antibiotic binding properties.

The present study demonstrates that a 27 nt RNA corresponding only to the A site of 16 S rRNA adopts the same conformation as in the 30 S subunit and binds aminoglycosides specifically. This oligonucleotide system has been used to identify the nucleotides of the A site that are important for antibiotic binding. The reduced size of this molecule enables the structure determination of the RNA-antibiotic complex by NMR.

Results

Design of a small A-site RNA fragment

The interaction between aminoglycosides and the A site of the decoding region was characterized

using an RNA oligonucleotide (Figure 2(c)) corresponding to the highly conserved A site of the 16 S rRNA sequence (bases 1404-1412 and 1488-1497 of the *E. coli* 16 S rRNA sequence). Phylogenetic variation of mitochondrial rRNA sequences and mutational studies have demonstrated that the A site is separated from the P site by two Watson-Crick base-pairs (Figure 2(b); Cunningham *et al.*, 1993). These two base-pairs were critical to the design of the A-site model oligonucleotide and, along with two additional G-C base-pairs, form the upper stem (Figure 2(c)). The lower stem was closed by a tetraloop UUCG, whose structure has been determined (Allain & Varani, 1995; Varani *et al.*, 1991).

The structure of the 27 nt RNA was assayed using chemical modification with dimethyl sulfate (DMS). Although DMS modifies RNA at the guanine N-7, the adenine N-1, and at the cytidine N-3 positions, only the N-7 and N-3 modifications can be detected by direct strand scission (Peattie, 1979; Peattie & Gilbert, 1980). To allow a direct comparison with previous data obtained on the ribosome, primer extension was used to detect all sites of DMS modification. A longer oligonucleotide (44 nt) was designed, which is identical with the original (27 nt) molecule but has an additional 17 nucleotides at the 3' end to allow for annealing of a DNA primer during primer extension (see Materials and Methods). The folding of this oligonucleotide was initially compared with the 27 nt RNA molecule using direct strand scission. The RNAs were modified using diethyl pyrocarbonate (DEPC), which modifies N-7 of adenine (and more weakly at guanine). Both oligonucleotides showed the same modification pattern and this pattern remains unchanged upon increasing the Mg^{2+} concentration from 0 to 10 mM (data not shown). The oligonucleotide with the primer annealing site was used only in biochemical studies, whereas the 27 nt RNA was used for NMR.

A-site oligonucleotide, a correctly folded piece of the ribosome

To show whether the model oligonucleotide mimics the conformation of the A site within the ribosome, chemical probing with DMS was performed on the oligonucleotide and compared with the known pattern obtained for the ribosome, the 30 S subunit (Moazed *et al.*, 1986), and an oligonucleotide analog of the A and P sites of the decoding region (Purohit & Stern, 1994). In the absence of antibiotic, N-1 of A1408, A1492 and A1493 as well as N-7 of G1491 and G1494 are reactive to DMS. The reactivity of A1492 is higher than that of A1493, as observed in the 30 S subunit. The N-3 positions of C1407, C1409 and C1496 are not reactive to DMS, which suggests that these nucleotides could be base-paired in the oligonucleotide. These reactivities correlate well with what is observed for 16 S rRNA in the 30 S subunit (Moazed *et al.*, 1986). In addition, the N-7 positions

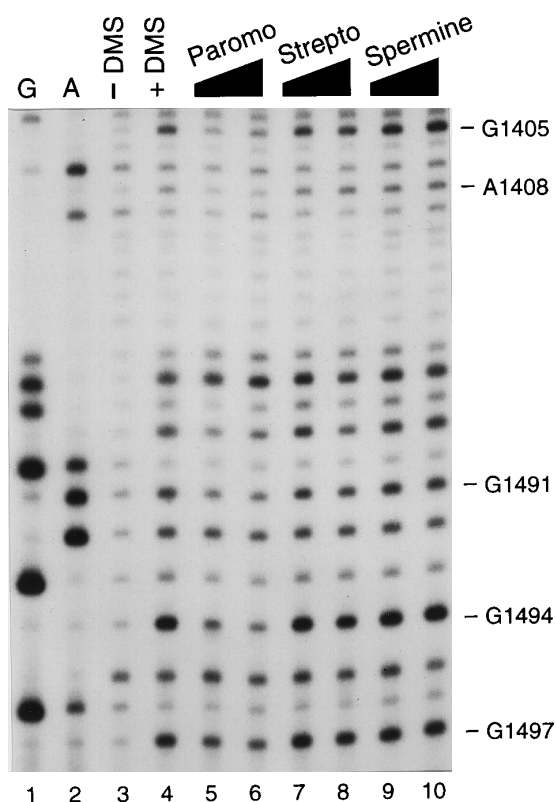


Figure 3. Autoradiograph of DMS probing reactions on the wild-type A-site oligonucleotide. Lanes 1 and 2 are G and A dideoxy sequencing reactions. Lane 3 is a primer extension reaction using unmodified oligonucleotide RNA. Oligonucleotide alone (lane 4) and in the presence of 10 or 100 μM paromomycin (lanes 5 and 6), 10 or 100 μM streptomycin (lanes 7 and 8), or 10 or 100 μM spermine (lanes 9 and 10).

of G1405 and G1497 are reactive to DMS (see Figure 3, lane 4), but these modifications are not observed in the ribosome.

Binding of the neomycin-class aminoglycosides to the A-site oligonucleotide was assayed by chemical probing with DMS at pH 7.0. In the presence of 10 μM paromomycin, residues G1405, A1408 and G1494 are strongly protected from chemical modification by DMS, whereas G1491 and G1497 are weakly protected (Figure 3, lane 5). The paromomycin footprint is observed at a similar concentration on the ribosome (Moazed & Noller, 1987). Similar protection patterns were observed with 10 μM neomycin or gentamicin, and 100 μM ribostamycin, neamine or kanamycin (data not shown). The protection pattern observed with 10 μM paromomycin is unchanged in the presence of 10 mM Mg^{2+} , suggesting that there is no requirement for, or competition by, Mg^{2+} for antibiotic binding (data not shown). An approximate K_d (1 μM) for paromomycin binding to the oligonucleotide was estimated from the modification level as a function of antibiotic concentration.

To obtain a more precise value for the K_d of paromomycin binding to the oligonucleotide, quantitative footprinting experiments were per-

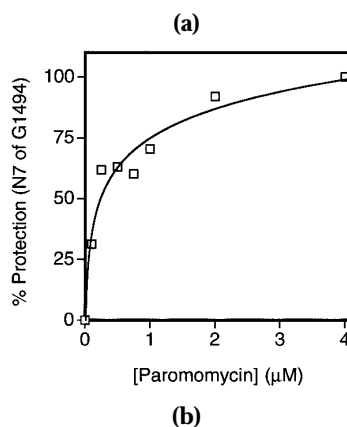
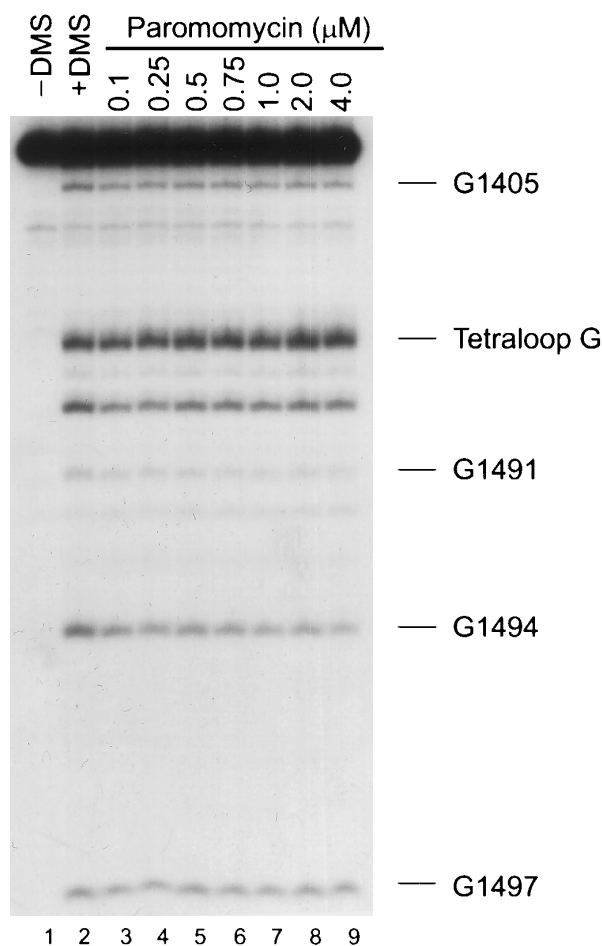


Figure 4. (a) Autoradiograph of DMS probing reactions on the 3' end labeled 27 nt RNA. Lane 1 is a control reaction with no DMS added. Lane 2 is a DMS probing reaction in the absence of paromomycin. Lanes 3 through 9 are reactions in the presence of 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 and 4.0 μM paromomycin. In all reactions, oligonucleotide is present at a concentration of 5 nM. (b) Graph showing the reactivity at N-7 of G1494 as a function of increasing paromomycin concentration. Reactivity between lanes was normalized using the reactivity of the tetraloop G as the standard.

formed on 5 nM 3' end-labeled 27 nt RNA by direct strand scission to detect the modifications at N-7 of guanine (Figure 4). The observed K_d is 0.2 μM , similar to that obtained using primer extension

detection of modifications on the 44 nt oligonucleotide. Since a greater number of modification signals can be detected using primer extension, footprinting experiments using the 44 nt oligonucleotide and primer extension were performed to compare qualitative binding affinities for paromomycin to the wild-type and variant oligonucleotides.

Specificity of antibiotic binding to the oligonucleotide was tested by performing chemical modification in the presence of the aminoglycoside streptomycin, which does not bind in the decoding region (Moazed & Noller, 1987), or the polycation spermine. Addition of 10 or 100 μM streptomycin or spermine did not alter the reactivities of any bases to chemical modification (Figure 3, lanes 7 to 10), indicating that the neomycin class of aminoglycosides binds specifically to the A-site oligonucleotide. Neither streptomycin nor spermine effectively compete for the aminoglycoside binding site on the oligonucleotide. The footprinting pattern in the presence of 10 μM paromomycin was unchanged on addition of up to 1 mM spermine or streptomycin (data not shown).

The assignment of the exchangeable imino protons of the A-site RNA oligonucleotide by NMR spectroscopy allowed the determination of its secondary structure. The region of a two-dimensional NOESY experiment that highlights important NOEs between imino protons is shown in Figure 5(a). The upper and lower helices, and -UUCG- tetraloop all form as observed by NOE connectivities between imino protons on adjacent base-pairs in a helix. Two uridine imino protons, with sharp resonances between 10 and 11 ppm, give a strong NOE between each other, indicating formation of a U1406·U1495 base-pair. These uridine imino protons also give NOEs to the imino protons of G1494 and G1405. The NMR data thus indicate that the asymmetric loop is closed by a canonical C1407·G1494 base-pair as well as the non-canonical U1406·U1495 base-pair (Figure 5(b)). No imino-imino proton NOE between G1491 and G1494 was observed. Stacking NOEs observed between the H-8 protons of A1408, A1492 and A1493, and the H-1' protons of adjacent residues indicate that these three adenine residues are stacked within the helical stem between the two C·G pairs. More detailed NMR experiments suggest formation of a dynamic A1408·A1493 base-pair, consistent with the lower reactivities of these nucleotides to DMS relative to A1492. Full discussion of heteronuclear NMR data will be presented in detail elsewhere. Addition of 10 mM Mg^{2+} gave no major change in the imino proton spectrum. In summary, the secondary structure as determined by NMR correlates with the chemical modification results.

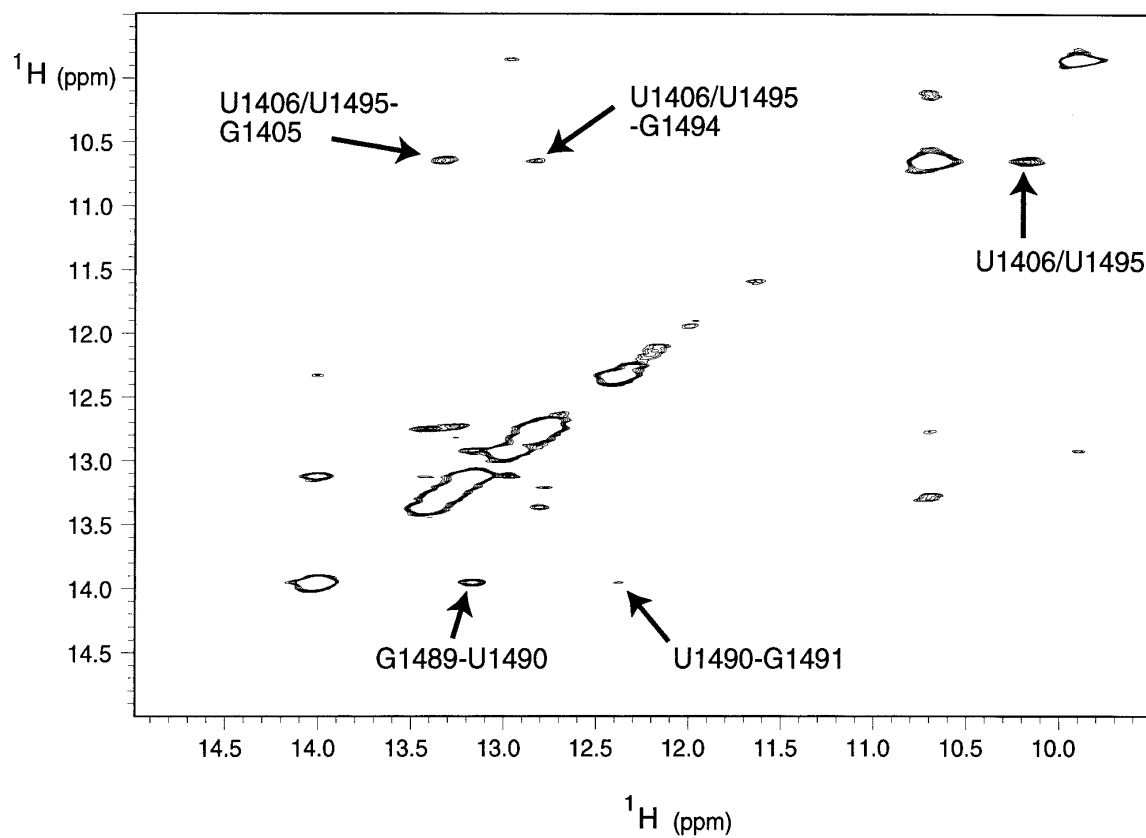
To characterize further the specific binding of aminoglycosides to the A-site RNA, chemical shifts of imino proton RNA resonances were monitored as a function of antibiotic concentration (Figure 6(a)). A change in chemical shift indicates

a perturbation of the local environment of the imino protons that reflects either conformational change of the RNA upon drug binding or proximity of the antibiotic to RNA protons. Imino protons of the A-site RNA alone and bound to paromomycin were assigned by two-dimensional proton NMR. A 1:1 complex is formed between the A-site RNA and paromomycin (Figure 6(a)), consistent with the $>\text{mM}$ RNA concentration and the approximate K_d for paromomycin binding to the oligonucleotide. At a paromomycin to RNA ratio of 0.5:1, two resonances were observed for the U1490 imino proton that indicate slow exchange on the NMR time-scale ($k_{\text{ex}} < 10^3 \text{ s}^{-1}$) between the free and bound form of the RNA. The imino protons of U1490 and G1491 are shifted downfield by 0.4 and 1 ppm, respectively, upon paromomycin binding. The imino protons whose chemical shifts change significantly upon binding of paromomycin border the nucleotides that are protected from chemical modification upon addition of paromomycin. Titration of the RNA by paromomycin in the presence of 5 mM Mg^{2+} (data not shown) led to the same spectrum, suggesting that the conformation of the antibiotic-RNA complex is not very sensitive to divalent ions.

Upon binding of other neomycin-class aminoglycosides (neomycin and ribostamycin) the RNA imino protons showed chemical shift changes similar to those observed upon addition of paromomycin (U1490 and G1491 resonances were shifted downfield by more than 0.2 ppm and 0.5 ppm, respectively). Two-dimensional NOESY experiments data indicate that neomycin and ribostamycin bind in the same way as paromomycin (data not shown). For neamine and gentamicin, the U1490 resonance was shifted downfield by 0.1 and 0.15 ppm, respectively. The G1491 resonance was either shifted downfield by more than 0.5 ppm or broadened upon addition of either antibiotic. Further NOESY experiments are still in progress to determine if these antibiotics bind the RNA like paromomycin. Streptomycin and the polycation spermine gave no large change in the NMR spectrum of the RNA upon addition to 1:1 stoichiometry (chemical shift changes for the U1490 and G1491 resonances were less than 0.05 and 0.1 ppm, respectively; data not shown). As a control for non-specific RNA-antibiotic interaction, paromomycin and the microhelix acceptor stem of tRNA^{Met} (Puglisi *et al.*, 1994) were mixed in 1:1 stoichiometry and no major change in the NMR spectrum was observed (data not shown).

More detailed NMR experiments gave insight into the A-site RNA-paromomycin interaction. NOESY experiments were performed in $^2\text{H}_2\text{O}$ to assign the non-exchangeable protons of the RNA and paromomycin. As shown in Figure 10(a), intermolecular NOEs are observed between H-5 of U1495 and ring II of paromomycin, and between H-5 of U1490 and the paromomycin ring IV. Additional intermolecular NOEs were observed between H-5, H-6, H-8, H-2' and H-3' in nucleotides

(a)



(b)

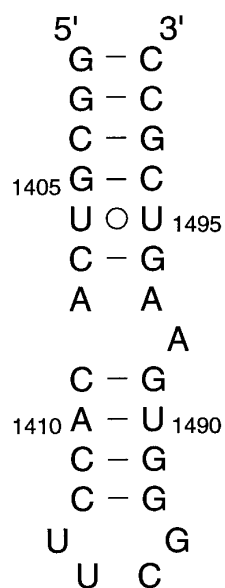


Figure 5. (a) Region of a SSNOESY spectrum performed in H_2O at 5°C with a mixing time of 300ms on the wild-type A-site oligonucleotide. NOEs between imino protons are indicated with arrows. (b) Secondary structure of the A-site RNA as determined from NMR data.

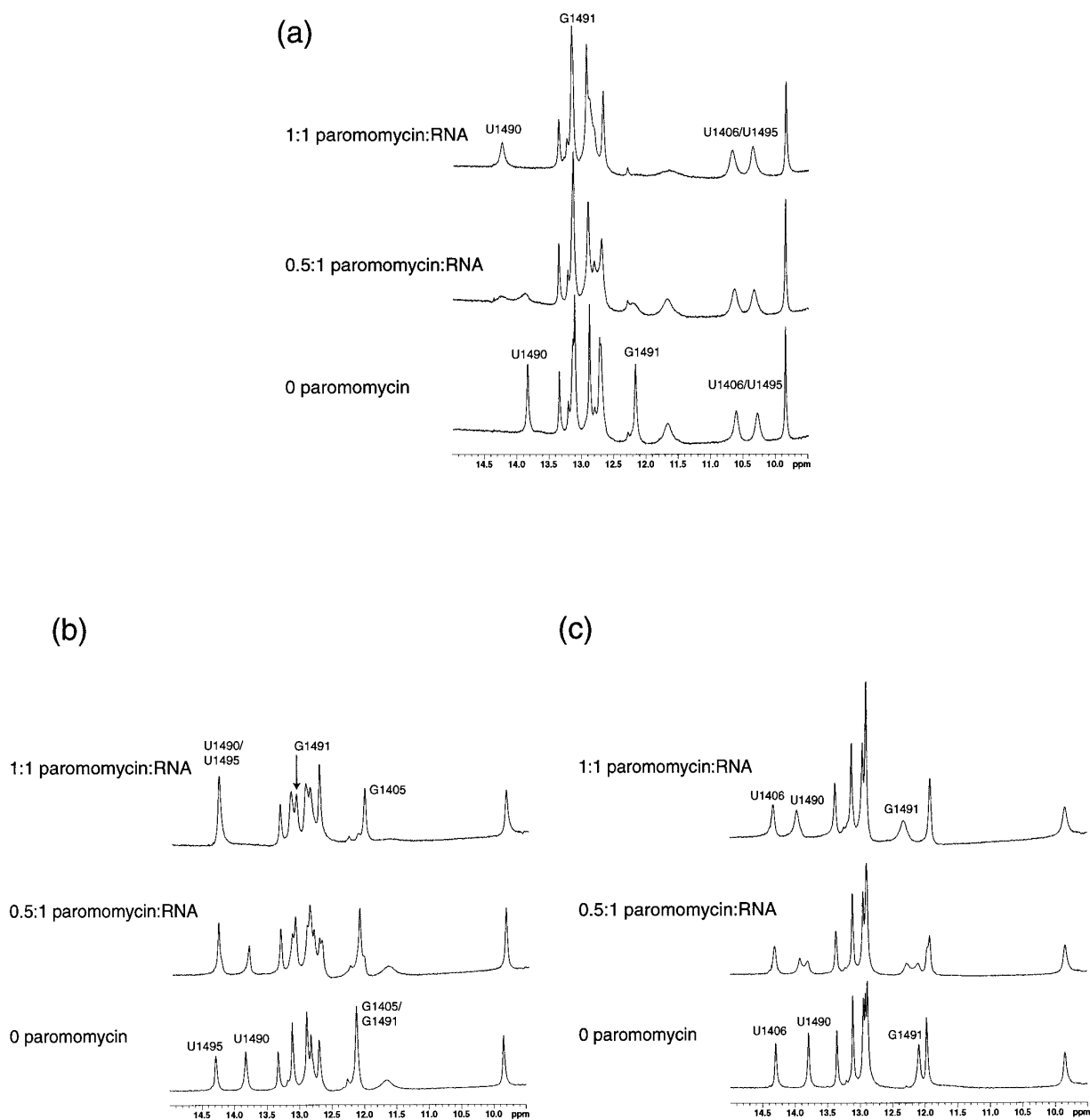


Figure 6. (a) Titration of wild-type A-site oligonucleotide with paromomycin yields a specific 1:1 complex. Imino proton spectra for the free RNA, 0.5:1 paromomycin:RNA and for the 1:1 complex. Imino proton resonances that change significantly upon binding of paromomycin are indicated. (b) Titration of the U1406A mutant RNA with paromomycin yields a specific 1:1 complex. Imino proton spectra of the free RNA, 0.5:1, and 1:1 stoichiometry are shown. (c) Titration of the U1495A mutant RNA with paromomycin to a 1:1 ratio. The small shifts in the imino proton resonances of U1490 and G1491 are indicated.

from the U1406·U1495 to the A1410·U1490 base-pair and the four rings of paromomycin; these results will be discussed in detail elsewhere. The NMR results demonstrate specific binding of paromomycin in the major groove of the A-site RNA, in the region of the asymmetric internal loop. In combination with the chemical footprinting experiments, these results demonstrate that the A-site RNA oligonucleotide binds aminoglycosides specifically and in a similar way as the ribosome.

Nucleotides of the A-site RNA important for antibiotic binding

To define the critical sequence components of rRNA for aminoglycoside antibiotic binding, chemical modification as a function of paromomycin concentration was performed on variant oligonucleotides containing the base substitutions indicated in Table 1.

Nucleotides in the upper helix and the internal loop that are universally conserved in all ribosomes

Table 1. Effect of nucleotide substitutions on binding of paromomycin to the A-site oligonucleotide

Position	Sequence	Antibiotic binding
1406	U (wt)	++
	A	++
1407/1494	C·G (wt)	++
	U·A	-
	G·C	-
1408	A (wt)	++
	G	+
	U	-
1409/1491	C·G (wt)	++
	G·C	+
	UC	-
1410/1490	A·U (wt)	++
	U·A	++
	G·C	-
1492	A (wt)	++
	G	++
	C	++
	Δ	-
1493	A (wt)	++
	G	-
1495	U (wt)	++
	G	++
	A	-
	C	-

wt, Wild-type; ++, antibiotic footprint observed at 10 μ M paromomycin; +, antibiotic footprint observed at 100 μ M paromomycin; -, no antibiotic footprint observed at 1 mM paromomycin.

are required for specific aminoglycoside binding. Sequence variations at all positions except A1492 and U1406 disrupt high-affinity binding of paromomycin (Figure 7). Nucleotide U1406 can be changed to adenine, forming an A1406·U1495 base-pair, with no effect on the paromomycin footprint (Figure 8(a), lanes 5 to 8). The change in

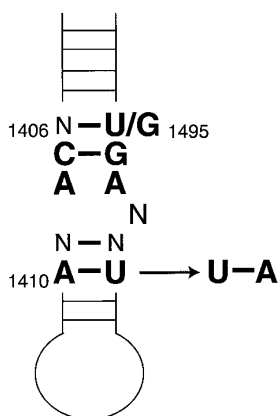


Figure 7. Critical nucleotides for paromomycin binding to the A-site oligonucleotide. Antibiotic binding was assayed by quantitative footprinting of variant oligonucleotides. High-affinity binding was defined by protection of G1405, A1408, G1491, G1494 and G1497 (or a subset of these positions if the given nucleotide was not present in the variant oligonucleotide) at 10 μ M paromomycin. Nucleotides whose identity is critical for high-affinity binding of paromomycin are indicated in bold. N, any nucleotide; N-N, any Watson-Crick base-pair.

sequence has little effect on the pattern of chemical modification in the absence of paromomycin (Figure 8(a), lane 4), as the N-7 of G1405, G1491, G1494 and G1497 are all reactive. The reactivity of N-1 of A1492 is greater than that of A1493, just as in the wild-type sequence. The low reactivity at N-1 of A1406 suggests that it is involved in a base-pair.

Position 1495 can be changed to G, creating a potential U1406·G1495 base-pair, with no detectable effect on antibiotic binding. Substitution of this uridine by adenine (Figure 8(b)), forming a U1406·A1495 base-pair, or by cytidine reduces paromomycin binding affinity by more than tenfold. The DMS modification pattern for the U1495 \rightarrow A variant in the absence of aminoglycoside is quite similar to that of the wild-type sequence (Figure 8(b); lane 4), and the reactivity at N-1 of A1495 is no greater than background, suggesting it is involved in a base-pair. Upon addition of paromomycin, a weak footprint is observed at N-7 of G1491, G1494 and G1497 at 1 mM antibiotic (Figure 8(b), lane 8).

The universally conserved C1407·G1494 base-pair must be maintained for the binding of paromomycin to the oligonucleotide. If the base-pair is switched to a G1407·C1494 base-pair or substituted with a U1407·A1494 base-pair, no paromomycin footprint of the RNA is observed at up to 1 mM antibiotic.

The asymmetry of the A-site internal loop is required for high-affinity aminoglycoside binding. A pair of universally conserved adenine residues (A1492, A1493) maintains this asymmetry in the ribosome. The oligonucleotide system reveals that A1493 is important for aminoglycoside binding, as an A to G substitution at position 1493 results in no observable antibiotic footprint at 1 mM paromomycin. Position 1492 can be changed to G or C with no effect on binding of paromomycin; strong footprints appear at 10 μ M antibiotic. Deletion of the nucleotide at position 1492, which creates a symmetric internal loop, also disrupts binding of paromomycin, as evidenced by a weak footprint observed at 1 mM paromomycin.

Position 1408 is A in all prokaryotic ribosomes but G in all eukaryotic ribosomes (Van de Peer *et al.*, 1994). An A1408 \rightarrow G substitution reduced the binding affinity of paromomycin, with an antibiotic footprint appearing at 100 μ M paromomycin. Mutation of 1408 to a U, which leads to a possible U1408·A1493 base-pair, results in no observable paromomycin footprint at 1 mM antibiotic.

The lower helical stem of the A site is composed of phylogenetically non-conserved nucleotides. Disruption of the C1409·G1491 base-pair in the ribosome confers paromomycin resistance in yeast and resistance to a variety of aminoglycosides in *E. coli* (Li *et al.*, 1982; DeStasio & Dahlberg, 1990). Change of this base-pair in the A-site oligonucleotide from C1409·G1491 to U1409·C1491 severely diminished specific binding of paromomycin (Figure 9); a weak footprint at positions G1405 and

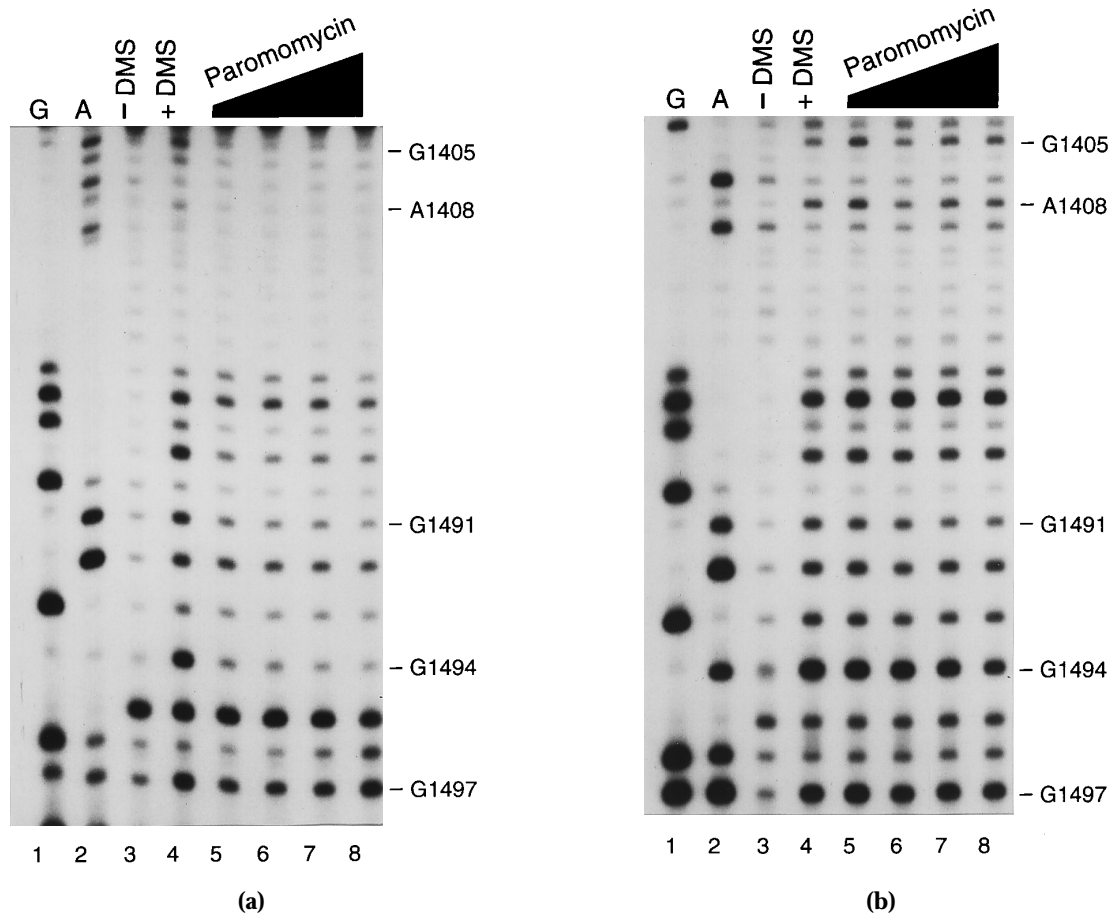


Figure 8. Autoradiographs of DMS probing reactions with (a) U1406A and (b) U1495A oligonucleotides. Lanes 1 and 2 are G and A dideoxy sequencing reactions. Lane 3 is a primer extension reaction using unmodified oligonucleotide RNA. Oligonucleotide alone (lane 4) and in the presence of 1, 10, 100 μ M and 1 mM paromomycin (lanes 5 to 8).

G1494 was observed in the presence of 100 μ M paromomycin. The N-3 of C1491 and N-1 of A1410 were reactive to DMS, which suggests that the lower stem of the U1409-C1491 variant is less stable than that of the wild-type sequence. No footprint was observed at N-3 of C1491 or N-1 of A1410 with increasing paromomycin concentration (Figure 9, lanes 5 to 8). Change of the C1409-G1491 base-pair to G1409-C1491 reduced binding of paromomycin slightly, with a wild-type footprint apparent at 100 μ M paromomycin (data not shown).

Alteration of the next base-pair in the lower stem from A1410-U1490 to G1410-C1490 appeared to abolish binding, with no footprint apparent at 1 mM paromomycin. However, the G1410-C1490 variant had an altered modification pattern from the wild-type sequence in the absence of paromomycin; the reactivity at N-1 of A1408 and A1493 was reduced in the absence of antibiotic. The altered chemical modification pattern suggests that there may be an overall structural change of this RNA variant, which may affect paromomycin binding. In contrast, change of the A1410-U1490 base-pair with a U1410-A1490 base-pair had no

effect on binding of paromomycin, as a strong footprint was observed at 10 μ M paromomycin.

NMR characterization of aminoglycoside binding to variant oligonucleotides

To test independently the footprinting results of antibiotic binding to variant RNA oligonucleotides, we again used NMR as a tool to study this interaction. As with the wild-type sequence, NMR can probe the secondary structure of the variant RNAs, and demonstrate specific binding of paromomycin. The footprinting experiments revealed a striking effect on paromomycin binding for variants of the U1406-U1495 base-pair. Two of these variants, U1406 \rightarrow A and U1495 \rightarrow A, were studied further by NMR.

The secondary structure of these mutants was determined using two-dimensional proton NMR as described above for the wild-type sequence. In the U1406A variant (Figure 6(b)), the U1406-U1495 base-pair is replaced by the A1406-U1495 pair with the chemical shift of the U1495 imino proton resonance (14.3 ppm) in the normal range for an

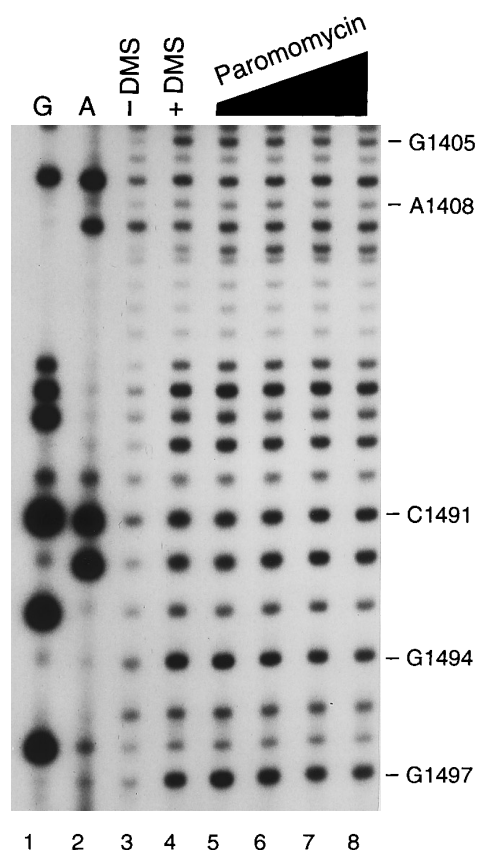


Figure 9. Autoradiograph of DMS probing reactions with C1409U + U1491C oligonucleotide. Lanes 1 and 2 are G and A dideoxy sequencing reactions. Lane 3 is a primer extension reaction using unmodified oligonucleotide RNA. Oligonucleotide alone (lane 4) and in the presence of 1, 10, 100 μ M and 1 mM paromomycin (lanes 5 to 8).

A·U pair. A similar chemical shift was observed for U1406 in the U1495A variant (Figure 6(c)), in which a U1406·A1495 base-pair is formed. These were the only modifications of the secondary structure observed for the free form of the U1406A and U1495A RNA variants.

The NMR data show that the U1406A variant forms a specific, 1:1 complex with paromomycin, as suggested from the biochemical experiments (Figure 6(b)). Upon addition of paromomycin, similar downfield shifts of U1490 and G1491 imino proton resonances are observed for the U1406A variant as for the wild-type sequence. The free and paromomycin-bound forms of the U1406A variant are in slow exchange on the NMR time-scale. Moreover, similar intermolecular paromomycin-RNA NOEs were observed for the U1406A variant as for the wild-type complex. The NOEs between H-5 of U1490 and ring IV of paromomycin, and between H-5 of U1495 and ring II are observed in the variant complex (Figure 10(b)); the NOEs between U1495 and ring II of paromomycin are more intense than in the wild-type complex. Additional NOEs between the RNA and paromomycin were observed and indicate a specific

binding of the drug in the major groove of the RNA, similar to that observed in the wild-type complex.

The U1495A variant interacts with paromomycin in a different manner from the wild-type sequence. At the high concentrations of RNA and antibiotic used for NMR, the U1495A variant forms a 1:1 complex with paromomycin, but the imino proton spectrum is almost unchanged from the free form of the RNA. The imino proton of G1491 in the U1495A variant shifts weakly downfield (0.2 ppm) on addition of paromomycin (Figure 6(c)), compared with the large (1 ppm) shift for the wild-type sequence. Moreover, the intermolecular NOEs between H-5 of U1490 and ring IV of paromomycin are very weak (Figure 10(c)). If this variant binds paromomycin in the same way as the wild-type, one would expect an intermolecular NOE between H-8 of A1495 and ring II of paromomycin. This NOE was not observed (data not shown), contrasting with the strong NOEs originating from H-5 of U1495 in the wild-type and U1406A variant sequences (Figure 10(a) and (b)). The paromomycin-U1495A variant complex does not correspond to the specific complex observed for the wild-type sequence or the U1406A variant, consistent with the results of chemical probing experiments. Furthermore, titration of the C1409U·G1491C variant (a previously described paromomycin resistance mutation) with paromomycin indicates a non-specific interaction at the concentration used for the NMR experiment (data not shown). Therefore, there is a direct correlation between the biochemical and NMR results.

Discussion

Relevance of model system

The A-site oligonucleotide mimics the chemical modification pattern and aminoglycoside binding properties of the decoding region A site in the ribosome. The oligonucleotide is reactive at two additional N-7 positions that are not reactive in the ribosome, G1405 and G1497. However, these nucleotides are located within a region of many reverse transcriptase stops in the 16 S rRNA. The RNA specifically binds aminoglycosides that interact with the A site of the ribosome.

NMR and biochemical data indicate that the structure of the A-site internal loop is not dependent on the presence of divalent cations; this is a practical advantage for long-term study by multidimensional NMR methods. The ribosome is functional and correctly folded only in the presence of high (>5 mM) Mg^{2+} , which is required for formation of long-range tertiary structure. The formation of local, linear structures in RNA such as the A-site internal loop, is often independent of divalent cation (Wyatt *et al.*, 1990; Laing *et al.*, 1994). The binding of paromomycin is also unaffected by low concentrations of divalent cations.

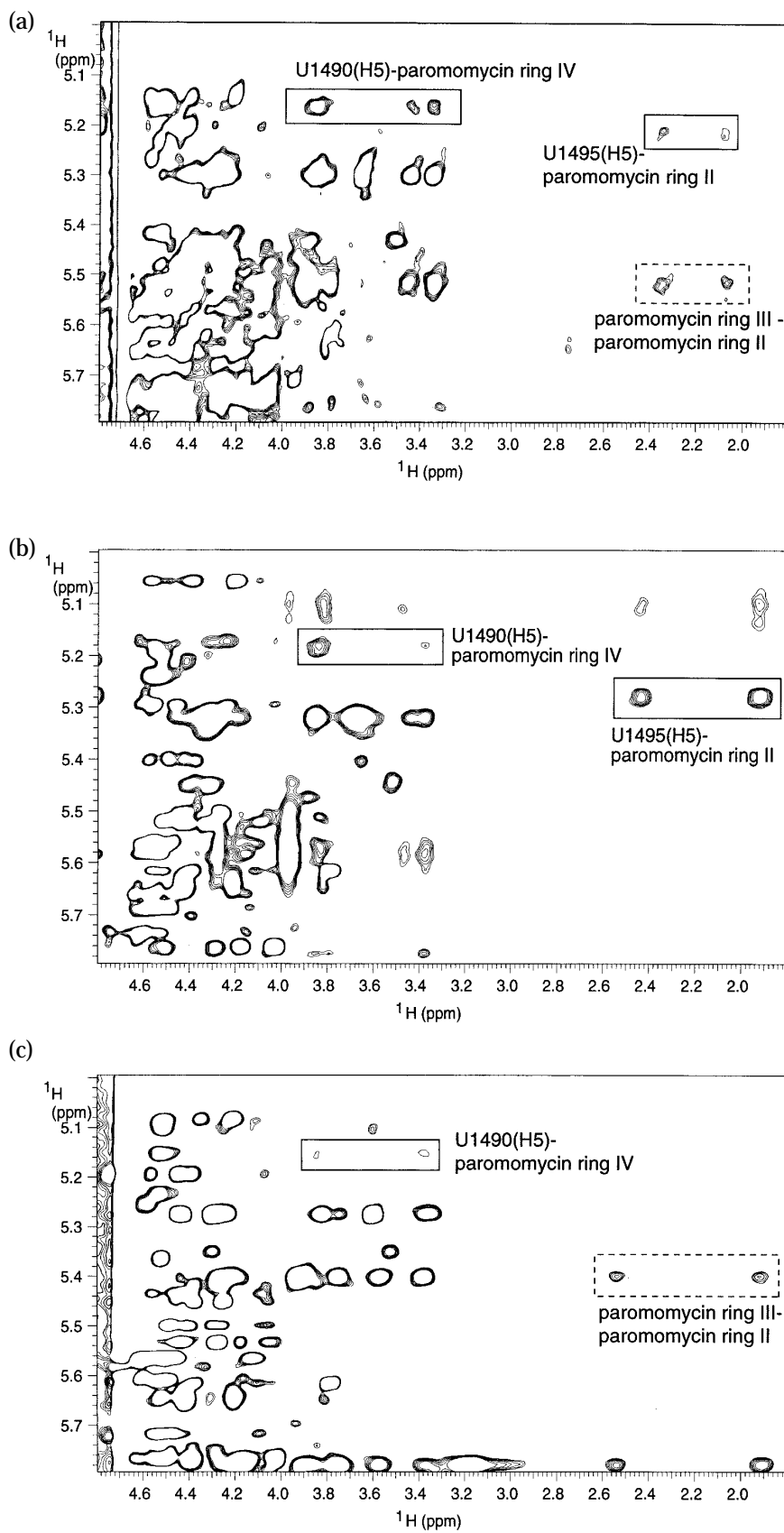


Figure 10. Portion of 2D NOESY spectra in $^2\text{H}_2\text{O}$ showing RNA-paromomycin NOEs for the (a) wild-type oligonucleotide and (b) U1406A and (c) U1495A variants; the RNA to paromomycin stoichiometry is 1:1 for all three variants. The intermolecular NOEs, when present, between H-5 of U1490 and the ring IV of paromomycin, and H-5 of U1495 and the ring II of paromomycin are boxed with a continuous line. Paromomycin-paromomycin intramolecular NOEs are indicated with a broken line. NOESY experiments were performed at 35°C with a mixing time of 250 ms.

Interactions with ligands tend to occur in regions of unusual RNA structure. The chemical modification pattern suggests that the A-site RNA is

composed of two helical stems with an asymmetric internal loop of three adenine residues, and this result is confirmed by NMR studies. The internal

loop is closed by non-canonical U·U and A·A base-pairs, leaving a single bulged adenine residue (A1492). Paromomycin binds in the major groove in the region of the internal loop. Asymmetric internal loops have been observed in other ligand-binding RNAs, notably the Rev response element (RRE; Battiste *et al.*, 1994). The RRE RNA binds both the Rev protein as well as the aminoglycoside antibiotic neomycin B (Zapp *et al.*, 1993) in the major groove of its asymmetric loop. Formation of the Rev-RRE complex induces the formation of purine-purine base-pairs in the internal loop (Battiste *et al.*, 1994). The NMR data on the A-site RNA suggests that binding of paromomycin to the oligonucleotide induces local conformational changes in the RNA.

Specific interaction of aminoglycoside antibiotics with the oligonucleotide and ribosome show a similar dependence on RNA sequence. Footprinting studies performed on mutant ribosomes (DeStasio *et al.*, 1989) revealed that paromomycin does not bind tightly to the A site of aminoglycoside-resistant ribosomes. When a corresponding sequence was introduced into the oligonucleotide, no footprint was observed and a similar enhanced reactivity is observed at position 1491, suggesting disrupted base-pairing in the lower stem. Due to the conserved function and sequence of the decoding region, there is limited information concerning the RNA determinants for antibiotic binding on the ribosome.

Determinants for recognition of RNA by aminoglycosides

A group of conserved nucleotides within the A site is critical for paromomycin binding, as shown by chemical probing on variant oligonucleotides. The universally conserved C1407·G1494 base-pair, A1408, and A1493 are required for wild-type binding of paromomycin to the oligonucleotide. In addition, there must be an asymmetric internal loop produced by a nucleotide at position 1492, which is a universally conserved adenine residue in the ribosome. Base-pairing is required in the lower stem, although the specific sequence is not critical for specific binding. The results obtained by NMR are consistent with the footprinting experiments. In each instance, when a wild-type footprint pattern is observed in the chemical modification experiment, formation of a specific complex is observed by NMR.

The sequence determinants for aminoglycoside binding may differ from conserved nucleotides. Positions 1406 and 1495 are universally conserved uridines in the ribosome, and form the U·U pair in the oligonucleotide. Paromomycin binds both the A1406·U1495 and U1406·G1495 base-pairs in addition to the normal U1406·U1495 base-pair, but does not bind tightly to the U1406·A1495 base-pair variant. No major structural change in the RNA was detected by chemical modification for either adenine substitution (A1406 or A1495), indicating that the differences in paromomycin binding

affinity between the two is the result of a more subtle change. The reduced binding seen with the U1495C mutation is not unexpected, since this change causes hygromycin resistance in *T. thermophila* (Spangler & Blackburn, 1985).

Antibiotic resistance and species specificity

In addition to their activity against prokaryotes, some aminoglycosides are also effective against eukaryotes. Paromomycin causes codon misreading in some eukaryotes but at concentrations higher than those required for prokaryotes (70 μM versus 6 μM ; Davies *et al.*, 1965; Palmer & Wilhelm, 1978; Wilhelm *et al.*, 1978; Palmer *et al.*, 1979). Paromomycin binds with reduced affinity to an oligonucleotide resembling eukaryotic A site rRNA sequences (A1408G), with a footprint appearing at about 100 μM paromomycin. Position 1408 represents the major difference between prokaryotic and eukaryotic ribosomes in the A site: all eukaryotes possess guanine at position 1408, whereas in prokaryotes this nucleotide is always adenine (Van de Peer *et al.*, 1994).

Although the nucleotides at positions 1409 and 1491 are not conserved in all ribosomes, these positions are important for ribosome function and antibiotic resistance. Disruption of the Watson-Crick base-pair at these positions is correlated with resistance to paromomycin (DeStasio & Dahlberg, 1990). Footprinting experiments with the oligonucleotide indicate that changing this C1409·G1491 base-pair to a U C mismatch or a G1409·C1491 base-pair reduces binding of paromomycin to the A site. In the U C mismatch oligonucleotide, there is a weak antibiotic footprint at 1 mM paromomycin, whereas the G1409·C1491 oligonucleotide variant shows a strong footprint at 100 μM paromomycin. The U1409·C1491 sequence variant, based on the chemical modification data, showed changes in the structure of the lower stem that may reduce the affinity of paromomycin for the RNA. The biochemical data on the G1409·C1491 variant oligonucleotide suggests that this sequence should show low-level paromomycin resistance. The results on the oligonucleotide system do not completely agree with previously reported *in vivo* results (DeStasio & Dahlberg, 1990). *In vivo*, the U1409·C1491 mismatch conferred high-level resistance to a variety of aminoglycosides, but the G1409·C1491 base-pair conferred low-level resistance to neamine, kanamycin and gentamicin but not to paromomycin or neomycin.

In addition to conferring resistance, mutations that disrupt the base-pair at the 1409-1491 positions have a detrimental effect on growth in *E. coli*. There appear to be slightly different rRNA sequence requirements for ribosome function in prokaryotes versus eukaryotes; sequences that conferred paromomycin resistance in *T. thermophila* and yeast mitochondrial rRNA (purine-purine mismatches at 1409-1491) were lethal when introduced into *E. coli*. (DeStasio & Dahlberg, 1990). In addition to G1408,

higher eukaryotes tend to have mismatches at position 1409-1491 of the small subunit rRNA (Van de Peer *et al.*, 1994). The RNA sequence differences between prokaryotes and eukaryotes correlate with the different potency of aminoglycoside action in various organisms.

The results obtained using the oligonucleotide system can be used to predict the effects of ribosomal RNA sequence mutations *in vivo*. Paromomycin resistance is predicted for sequences with reduced affinity in the oligonucleotide.

Materials and Methods

Preparation of A-site oligonucleotide RNAs for biochemical studies

RNA for biochemical experiments (44 nucleotides) was prepared by *in vitro* transcription with phage T7 RNA polymerase and purified on 20% (w/v) polyacrylamide (19:1 (w/w) acrylamide:bis)/7 M urea gels using standard methods (Puglisi & Wyatt, 1995). DNA templates (61 nucleotides) were synthesized at 0.2 μ mol scale and gel-purified on 10% polyacrylamide (19:1 acrylamide:bis)/7 M urea gels.

An extended version of the A-site oligonucleotide with a 17-base primer annealing site (5'-GGTTGGCGTG-GCTCGCG-3') added to the 3' end was used for primer extension detection of the chemical modifications. A 17 nucleotide DNA primer (5'-CGCGAGCCACGCCAACCC-3') complementary to the priming site was used.

Chemical modification

Chemical modification reactions (20 μ l) were performed in 80 mM potassium cacodylate (pH 7.0), with 75 nM RNA oligonucleotide. Antibiotics (Sigma) or spermine were added and modification was performed by addition of DMS (1 μ l, 1:10 dilution in EtOH) followed by incubation at room temperature for five minutes. Reactions were stopped by precipitation in ethanol. Sodium borohydride reduction and aniline-induced strand scission was performed as described (Peattie, 1979), except that lyophilization steps were replaced by extraction with phenol and precipitation in ethanol. In brief, modified RNA was resuspended in 10 μ l of 1 M Tris-HCl (pH 8.2). Upon addition of 10 μ l of freshly prepared 0.2 M NaBH₄, the samples were incubated on ice in the dark for 30 minutes. The reaction was quenched by addition of 300 μ l of 0.3 M sodium acetate followed by precipitation in ethanol. Pellets were dissolved in 20 μ l of 1.0 M aniline/acetate (pH 4.5) followed by incubation in the dark for 20 minutes at 60°C. The reaction was terminated by addition of 110 μ l of 50 mM Tris-HCl (pH 8.2) and 110 μ l of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) followed by vigorous mixing and centrifugation. The RNA was concentrated by addition of ethanol to the aqueous phase and pellets were washed with 100 μ l of 70% (v/v) EtOH.

Modification reactions using 3' end labeled 27 nt A site RNA were identical with those described above for the 44 nt RNA, except that the RNA concentration was 5 nM.

Primer extension

RNA was suspended in 2.5 μ l of deionized, filtered water and primer extension performed as described

(Moazed *et al.*, 1986; Stern *et al.*, 1988) with the following modifications: (1) 4 μ l of 1 mM dNTPs was added to each tube prior to addition of reverse transcriptase; and (2) five units (0.2 μ l) of AMV reverse transcriptase (Seikigaku) per tube were used in the extension. DNA primer was 5' end labeled using [γ -³²P]ATP. Extension was performed for one hour, the reaction stopped by addition of 20 μ l of 0.1 M NaOH followed by incubation for 30 minutes at 90°C to digest remaining RNA. The DNA was precipitated and pellets washed with 70% ethanol. DNA was resuspended in 10 μ l of 7 M urea loading dyes, heated to 65°C, and loaded on a 20% polyacrylamide (19:1 acrylamide:bis)/7 M urea gel.

Modification data analysis

Gels were typically exposed on film for 12 to 24 hours. Modification data were quantified by exposing the gel on a Molecular Dynamics PhosphorImager screen and analyzed using Molecular Dynamics ImageQuant software.

For quantification of the data, the total number of cpm loaded per lane was normalized. Additionally, to normalize for the efficiency of primer extension/strand scission, the data were normalized using either the C (N-3) or G (N-7) of the tetraloop as the standard.

Preparation of the A-site oligonucleotide RNA for NMR

Milligram quantities of the A site RNA (27 nucleotides) were prepared by *in vitro* transcription from an oligonucleotide template and purified as described (Puglisi & Wyatt, 1995). After electroelution and precipitation with ethanol, the resuspended RNA was dialyzed against the buffer used for the NMR experiment in a microdialysis apparatus with a 3500 Da cut-off membrane.

Proton NMR

NMR experiments were recorded on a Varian Unity + 500 MHz spectrometer with a Varian inverse detection probe. NMR data were processed using Varian software. For all NMR experiments performed on the free A-site RNA (no antibiotic bound), the sample concentration was 4.5 mM in 10 mM sodium phosphate (pH 6.4) at 25°C.

RNA titration by paromomycin

The free form of the A-site RNA was titrated by adding 5 μ l of a concentrated solution of paromomycin. The antibiotic was dissolved in 10 mM sodium phosphate (pH 6.4), the same buffer as that used for the RNA sample.

Exchangeable proton NMR spectra

One-dimensional NMR spectra of exchangeable protons from the RNA were obtained in 90% H₂O/10% ²H₂O at 5, 10 and 25°C, and water suppression achieved using binomial (1331) pulse sequence. Two-dimensional NMR experiments (SSNOESY; Smallcombe, 1993) were recorded at 5°C with 75 and 300 ms mixing time. The same experiments were recorded on the 1:1 RNA to paromomycin complex.

Non-exchangeable proton NMR spectra

Standard NOESY, ^{31}P decoupled DQF-COSY and TOCSY experiments on the free form RNA or complexed with the paromomycin in 99.996% $^2\text{H}_2\text{O}$ were acquired. NOESY experiments were acquired with mixing times of 50, 100, 150, 200 and 400 ms. NMR experiments were performed in 10 mM sodium phosphate (pH 6.4) at an RNA concentration of 4.5 mM at 35°C.

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