# *In vitro* selection of RNA lectins: using combinatorial chemistry to interpret ribozyme evolution

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**Background:** It has been hypothesized that the fact that both ribosomal RNA and the group I intron can bind to aminoglycoside antibiotics implies that these RNAs are evolutionarily related. This hypothesis requires the assumption that there are relatively few ways for RNA molecules to form aminoglycoside-binding sites.

**Results:** We have used *in vitro* selection to determine the diversity of aminoglycoside-binding sites that can be formed by RNA molecules. We have generated RNA 'lectins' that can bind aminoglycosides tightly and specifically. Sequence analysis indicates that there are many different ways to form tight and specific aminoglycoside binding sites. These artificially selected binding sites are functionally similar to those that have arisen from natural selection.

**Conclusions:** Our results suggest that the presence of aminoglycoside-binding sites on RNA molecules may not be a useful trait for determining evolutionary relatedness. Instead, the fact that RNA molecules can bind these 'low molecular-weight effectors' may indicate that natural products such as aminoglycosides have evolved to exploit sequence- and structure-specific recognition of nucleic acids, in much the same way that lexitropsins have been designed by chemists to recognise specific nucleic acid sequences.

**Chemistry & Biology** May 1995, **2**:291–303

Key words: aminoglycoside, aptamer, lectin, molecular evolution, ribozyme

## Introduction

Aminoglycoside antibiotics such as streptomycin or kanamycin have long been known to bind to and inhibit the function of ribosomal RNA [1,2]. More recently, members of this class of compounds have also been shown to inhibit the activities of other functional RNAs, such as the group I self-splicing intron and the hammerhead ribozyme [3–5]. These observations have prompted speculations that aminoglycoside-binding sites on RNAs may be evolutionarily related to one another [6]. One popular hypothesis suggests that 'low molecular-weight effectors' (LMEs) present in the prebiotic milieu were involved in the evolution of the earliest RNA molecules by augmenting their structures and modulating their functions [7]. These primordial LME-binding sites may have persisted into modern times, and it is possible that their descendants can still be seen in functional RNAs. Although they no longer serve their original function, ancient LME-binding sites can be identified by virtue of the fact that they continue to be exploited by modern LMEs, such as aminoglycoside antibiotics. If true, this model would have profound implications for how we envision the evolution of the RNA world and the origin of the translation apparatus. For example, the fact that both rRNA and group I introns can bind similar aminoglycoside antibiotics has been taken to mean that these molecules may have had a common catalytic ancestor [8,9].

The alternative possibility is that the aminoglycoside-binding sites found in natural RNAs are unrelated to one another. This possibility is bolstered by recent results from the field of combinatorial chemistry. The variety of sequences and structures found in synthetic libraries of peptides, nucleic acids, or other compounds is equal to or greater than the variety found in nature. When such combinatorial libraries have been sieved for individual molecules that can bind to target compounds, the binding species that are recovered frequently differ from natural binding sites and from one another. In particular, nucleic-acid aptamers (binding species or sites) that can specifically interact with target ligands such as nucleotides, amino acids, vitamins and organic dyes have been selected from random-sequence RNA pools [10]. Analysis of the selected molecules has revealed that there are often several sequence 'solutions' to a given ligand-binding 'problem.' For example, ≥100 000 different RNAs were found to form specific complexes with Cibacron Blue ($K_d$ ≈100 μM) [11]. Similarly, 100–1000 different RNAs were found to bind specifically to tryptophan agarose ($K_d$ ≈10 μM) [12]. The number of different aptamers recovered is generally a function of the stringency of the selection. However, even in cases where fewer binding species with higher affinities were found, multiple sequence classes could still be observed. For example, a population of aptamers that bound to ATP ($K_d$ <50 μM) [13] contained at least 13 different sequence classes, and aptamers that bound tightly to cyanocobalamin ($K_d$ <1 μM) [14] populated at least four divergent sequence classes. In contrast, RNAs derived from selections using theophylline ($K_d$ <1 μM) [15] or nicotinamide mononucleotide (NMN) ($K_d$ ≈5 μM) [16] tended to fall into single sequence classes.

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Given these results, the presence of aminoglycoside antibiotic binding sites on rRNA, group I introns and other RNAs might not indicate that all of these RNAs are evolutionarily related, but rather that there are multiple, distinct ways that RNAs can form ligand-binding sites. To distinguish between historical and stochastic explanations for the origins of aminoglycoside-binding sites, the diversity of possible binding sites must be determined. We have used *in vitro* selection to identify RNA sequences that can bind tightly and selectively to two different aminoglycoside antibiotics, kanamycin A and lividomycin. We find that thousands to millions of different RNA species can bind aminoglycoside antibiotics with much the same affinity and selectivity as ribosomal RNA or the group I self-splicing intron.

The selection of antibiotic-binding RNAs from random sequence pools also has important implications for drug discovery. To our knowledge, this is the first selection of RNA 'lectins' that can specifically bind to oligosaccharides. If RNA aptamers can be selected that specifically bind to aminoglycosides, then it may also be possible to find aptamers that bind to other, similar targets, such as the oligosaccharide moieties of glycoproteins or bacterial cell wall polysaccharides.

## Results and discussion

### Choice and immobilization of aminoglycoside antibiotics

The aminoglycosides lividomycin and kanamycin A were initially chosen as targets for *in vitro* selection experiments (Fig. 1). Both of these drugs are LMEs known to bind to ribosomal RNA. These two compounds were particularly interesting because they differentially interact with the translation apparatus and group I ribozymes; both efficiently inhibit translation, but only lividomycin affects self-splicing [6]. These compounds are structurally as well as functionally dissimilar; lividomycin has two additional sugars, and has five amino groups as opposed to four for kanamycin A. Thus, this pair of compounds can help establish the range of aminoglycosides that can elicit binding sites with high affinities and specificities. For example, if RNAs can be selected to bind kanamycin A, then it is likely that they can be selected to bind other members of the 'kanamycin family' and members of structurally similar families, such as the gentamycins. Finally, just as the positively charged amino acid arginine proved to be an excellent ligand for polyanionic RNAs, the fact that the aminoglycosides contain a number of positively charged moieties made them good targets for our initial attempts to derive RNA lectins that could bind oligosaccharides.

These aminoglycosides were immobilized via their primary or secondary amino groups. Several different affinity matrices were constructed and tested to determine which would give optimal separation characteristics for different RNA molecules. Aminoglycosides immobilized on Affi-gel 10 (Biorad, Hercules, CA) and Toyopearl (Tosohaas, Montgomeryville, PA) bound a large fraction of an unselected random-sequence pool non-specifically, even at high salt concentrations (≤5 M NaCl) or in the presence of free ligand (25 mM). However, epoxy-activated agarose (Pierce, Rockford, IL) or Sepharose (Pharmacia, Piscataway, NJ) matrices proved to have much lower non-specific retention of pool RNAs (suggested by Michael Famulok, U. Munich). At a NaCl concentration of 500 mM, less than 1% of an unselected RNA population bound to these affinity matrices. Such resin-specific effects have previously been noted for other ligands, such as ATP [13].

### *In vitro* selection of RNA aptamers that bind specifically to aminoglycoside antibiotics

Lividomycin and kanamycin A have molecular weights of 762 and 583 Daltons, respectively. Aptamers selected to bind other ligands of roughly the same size and functional complexity have been found to contain core binding sequences that are generally 20–30 bases long [10]. Therefore, we chose to employ an RNA pool that had a core of 30 random-sequence positions as a starting point for our selections (Fig. 2).

In the first selection cycle (Fig. 2), 15 μg of N30 RNA (sequence complexity of $2 \times 10^{13}$; ~15 library equivalents) was applied to a glycine pre-column to absorb species that bound non-specifically to the matrix or

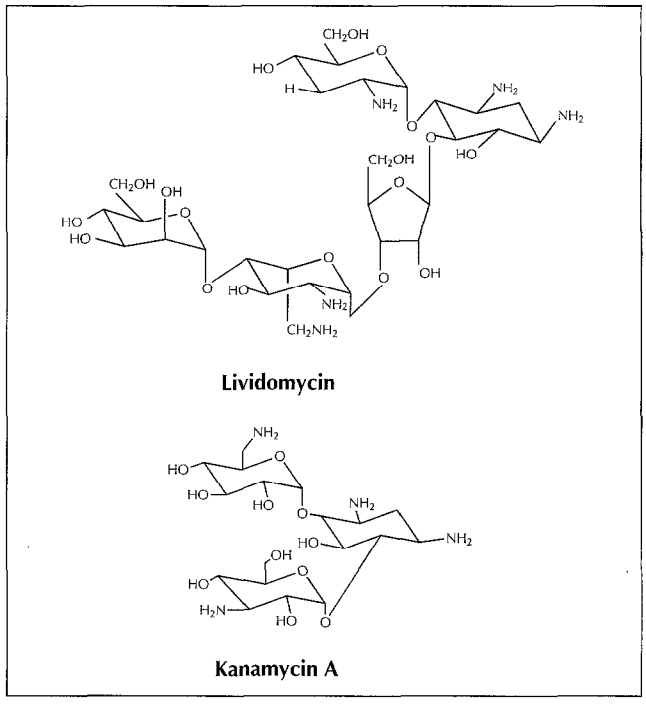


**Fig. 1.** Structures of aminoglycoside targets. Lividomycin and kanamycin A were immobilized on epoxy-agarose via their primary and secondary amines. The target ligand for selection was a composite of the agarose backbone, a 12 atom hydrophilic spacer arm, a secondary or tertiary amine linkage to the aminoglycoside antibiotic, and the aminoglycoside antibiotic itself. Interactions between RNA and the column matrix, spacer arm, and ligand linkage were minimized by pre-absorbing unselected and selected pools to a glycine affinity column made from the same activated resin, and by affinity elution, which would have favored the isolation of species that could bind the solution structure of the ligand.

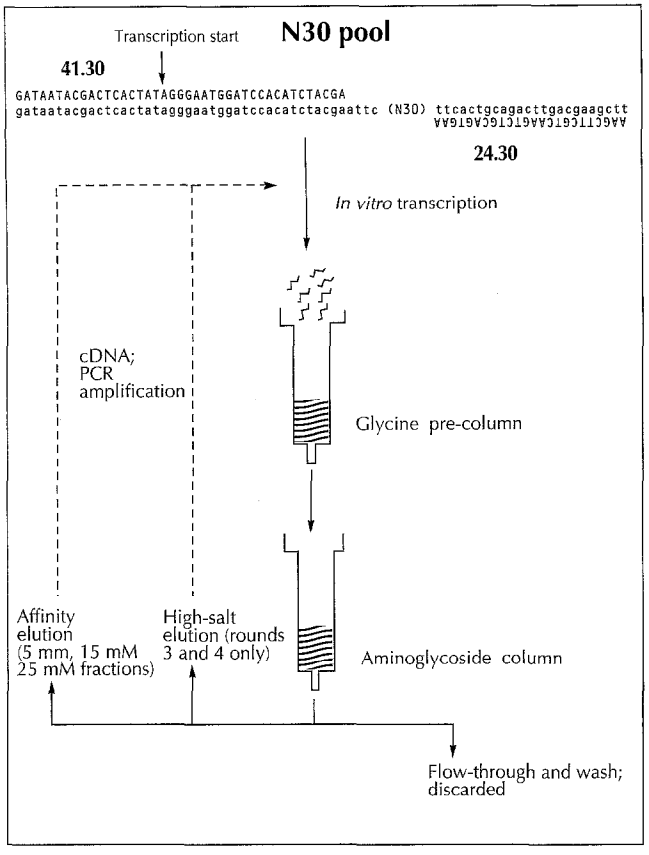


**Fig. 2.** Schema for selection. Overall view of the steps involved in the selection of aminoglycoside-binding sites. The sequences of the pool and primers (41.30 and 24.30) used are shown at the top. The RNA pool iteratively progressed through pre-column separations, affinity column separations and amplification procedures. In general, only those RNA molecules in the fraction eluted with 25 mM antibiotic were amplified and used for further selections. However, in round 3 a separate high-salt elution followed the affinity elution. The RNAs from this high-salt elution were amplified, sieved by affinity chromatography, and the high salt fraction was again collected separately. Details of this schema are discussed in the text and in Materials and methods.

spacer arm. The eluate from the pre-column was directly loaded onto affinity columns containing either lividomycin or kanamycin A. The affinity columns were washed with buffer to remove weakly bound RNAs and then progressively eluted with 5 mM, 15 mM and 25 mM solutions of the cognate antibiotic to isolate specifically bound RNAs. The tightest-binding species from each cycle were amplified by a combination of reverse transcription, PCR and *in vitro* transcription. Subsequent cycles were similar to the first, except that only 10 μg ($2 \times 10^{14}$ sequences; ~10 library equivalents) of nucleic acid was used. Since <1% of the applied RNA population binds in the first round, and since the amplification procedure results in the production of 40–100 copies of RNA for each cDNA formed, there should have been no false selection of RNA species due to population 'bottlenecks'.

As the RNAs were radiolabeled in each round, the selections could be followed by counting the fraction of each population that was eluted both specifically and non-specifically (Fig. 3). The ability of the populations to bind to the antibiotic columns improved in each cycle; by the fourth cycle of selection and amplification a majority of the RNAs were retained on the columns and could only be eluted with free ligand.

Starting in round 3, we noted that a fraction (20% for lividomycin, 5% for kanamycin A) of the RNA population remained on the columns following affinity

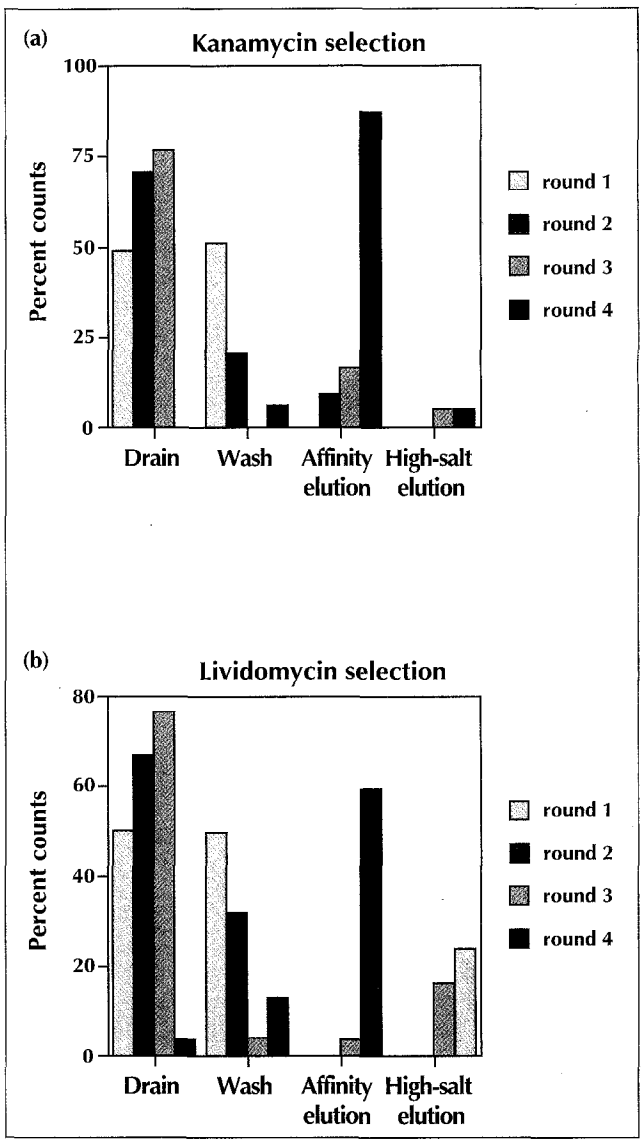


**Fig. 3.** Iterative selection of aminoglycoside binding sites. **(a)** Kanamycin selection. **(b)** Lividomycin selection. The column fractionation of RNAs as a function of selection is shown. Radiolabeled RNAs in the initial flow-through, wash, affinity elution and high-salt elution steps (cycles 3 and 4 only) were quantitated. Radioactive counts from each of the affinity-elution steps (5 mM, 15 mM and 25 mM antibiotic) were pooled for the purposes of this graph, as were the 5 M NaCl and 4 M GuSCN high-salt elution steps. The data for each fraction in each cycle were expressed as a percent of the total number of counts eluted. Fractions from different rounds are color-coded. For both kanamycin A and lividomycin, there is a significant improvement in the ability of the RNA population to bind to immobilized and soluble aminoglycosides as selection proceeds.

**Table 1.** Relative binding abilities of aminoglycoside-binding aptamers.

| Sample | Column fractions (% total)[a] | | | |
|---|---|---|---|---|
| | Drain/wash | Affinity elution[b] | High salt[c] | Column resin |
| Kanamycin pool | 12 | 78 | 7.2 | 2.1 |
| sla 110 | 4.4 | 69 | 18 | 8.4 |
| sla 150 | 7.6 | 73 | 11 | 8.0 |
| sla 151 | 6.6 | 77 | 6.6 | 10 |
| sls 21 | 3.5 | 73 | 15 | 8.8 |
| sls 26 | 3.1 | 79 | 12 | 5.9 |
| sls 254 | 3.2 | 79 | 4.3 | 14 |
| Lividomycin pool | 25 | 66 | 5.2 | 3.6 |
| sla 352 | 74 | 20 | 1.5 | 4.1 |
| sla 355 | 4.9 | 85 | 3.2 | 6.9 |
| sls 453 | 47 | 46 | 3.3 | 3.3 |
| sls 457 | 12 | 65 | 15 | 8.2 |
| sls 458 | 8.1 | 69 | 15 | 7.3 |
| sls 460 | 13 | 69 | 13 | 5.4 |

[a]RNA was chromatographically partitioned as described in Materials and methods. Values are expressed as a percent of the total counts recovered.
[b]Sum of 5 mM, 15 mM, 20 mM and 25 mM aminoglycoside elutions.
[c]Sum of 4 M GuSCN, 10 mM Tris (pH 8.0) and 5 M NaCl elutions.

elution. To determine whether these RNAs are from a particularly high-affinity class, residual counts were eluted with 5 M NaCl and 4 M GuSCN. These 'high-salt' fractions were amplified and selected separately from the affinity-eluted fraction (see Fig. 2). After an additional cycle, the high-salt fraction was assayed in parallel with the RNA population selected by affinity elution. No difference was observed in the amount of material that could be affinity eluted (data not shown), indicating that this population was similar in composition to the population of affinity-eluted RNAs. Moreover, individual aptamers from the affinity-eluted population have binding characteristics similar to those from the population eluted with high salt (Table 1). These results were expected, in that the populations eluted by high salt could also be removed by further affinity elutions (data not shown); thus, the high-salt elutions merely facilitated the recovery of tightly bound RNAs.

### Selected RNAs comprise a large family of diverse sequences and structures

The total number of aminoglycoside-binding sites in the selected population can be roughly estimated by determining the fraction of the total sequence diversity that was lost at each cycle, a value that has been termed the 'selection coefficient' [11]. This estimate assumes that any given sequence is either completely retained or completely eliminated from the population during the course of the selection, which is generally not the case. It thus provides a lower limit for the total number of sequences in a population. In round 1, <0.1% of the population was eluted and amplified, while in subsequent rounds <5% of the population was carried on. Using these values, we estimate that there may be up to a million different lividomycin- and kanamycin-binding sites remaining in the selected population.

To determine whether the populations were as diverse as expected, a spot check of individual sequences was carried out. Following round 4, RNAs from both affinity-eluted and high-salt eluted pools were cloned and sequenced. The sequences of lividomycin-binding and kanamycin-binding aptamers are shown in Figure 4. Inspection of these sets of sequences revealed no duplications and no obvious sequence motifs. In addition, a multiple sequence alignment was carried out using the program CLUSTAL to determine whether there were subtle similarities within either of these aptamer sets; again, no significant similarities were observed. These results were not expected: both the LME hypothesis and artificial evolutionary experiments with RNAs that interact with the translation apparatus [17] suggest that the sequences should have fallen into a relatively small number of sequence classes.

To determine whether aminoglycoside-binding sites contained common secondary structural motifs rather than common sequence motifs, secondary structural predictions were generated using the program MULFOLD. Single and multiple stem–loops, internal loops, multi-arm junctions and stems with and without bulges are found; examples of predicted aptamer structures are shown in Figure 5. No predominant secondary structural feature appears in the selected RNAs. Further, in different aptamers the flanking constant sequence tracts are predicted to participate in different secondary structures. Since a particular secondary structure could have been most easily formed by using the 'fixed' information present in the constant sequence tracts, this observation confirms that selected sequences are truly dispersed in sequence space. As a counter-example, selections for arginine-binding RNAs generated identifiable secondary structural motifs, but these motifs were found to be formed in part from constant-region sequences and thus

represented an extremely skewed subset of the original random-sequence population [18,19].

### Selected sequences bind tightly to their cognate ligands

The functional as well as structural diversity of aminoglycoside binding sites was explored. Binding constants for RNA populations and individuals were determined using analytical affinity chromatography [11–14, 18]. It should be noted that aptamer affinities determined by chromatography correspond well with aptamer affinities determined by other methods, such as fluorescence quenching [16]. RNAs from round 4 were applied to their cognate antibiotic affinity columns and the columns were washed with 60 column volumes of buffer (as opposed to 6 for the selections themselves). Despite this extensive wash step, a large fraction of the selected RNAs remained bound to the column: >75% of RNAs selected to bind to lividomycin and >85% of RNAs selected to bind to kanamycin. However, these RNAs could be largely eluted by free antibiotic. For example, most of the RNAs that bound to the kanamycin column could only be eluted by ≥15 mM concentrations of free antibiotic (Fig. 6). The fact that affinity elution is required to remove the aminoglycoside-binding aptamers indicates that many of the selected sequences are interacting specifically with a particular ligand rather than non-specifically with a matrix of positive charge. Based on these results, the aggregate dissociation constant of all complexes between affinity-eluted RNAs and kanamycin is estimated to be no more than 220 nM. Similar values were obtained for RNAs selected to bind to lividomycin.

The binding characteristics of the selected populations were mirrored by individual sequences. Six lividomycin-binding and six kanamycin-binding aptamers were prepared and assayed by analytical affinity chromatography (Table 1). Again, aptamers remained bound to immobilized aminoglycoside antibiotics even after washing with 60 column volumes but could be readily eluted with free ligand; only a fraction (5–20%) of the applied RNA samples were eluted with a further high-salt wash or remained bound to the column resin. Individual sequences generally bound either slightly more tightly or slightly less tightly than the population as a whole. Although two of the six lividomycin-binding aptamers (sla 352 and sls 453) bound less well to the affinity column than the others, affinity elution still proved to be effective in removing the bound RNA fraction.

The aminoglycoside-binding aptamers interact with their cognate ligands almost as well as the best aptamers observed in other selection experiments. For example, a complex between a cyanocobalamin-binding aptamer and its ligand has a $K_d$ of 88 nM [14], while a complex between an aptamer and theophylline has a $K_d$ of 320 nM [15]. In addition, it is instructive to compare RNAs selected to bind organic dyes [11] with RNAs selected to bind aminoglycosides. The organic dyes and aminoglycosides are roughly the same size, and contain

(a)

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*sla_352    A.TCCCCCGTTCCTAGTCTTGTAAGGTAGTA...
*sla_355    TT.TGGGCATATG..ATGCAGGGAGCAAACGGG.
 sla_361    AGACAGTG.GAT..CGCAGGCTACGGGAAATGC.
 sla_362    AAGTTACTAACCGATGTCAGACCTTGGA.CG...
 sla_367    G.GTTGAGGTTGGAGTGCAGGTCTTGGAACC...
 sla_372    GTAGGGC...AGGACCAGTGGCTTGCTACCAGT.
 sla_377    GAAAGAGCAGGCCCAAGGTGCGACGTGAAC....
 sla_379    CTAAGGTTCGGA..GGTGCGTTTCGATGG..GC.
 sls_380    A...GCACGCTTGGTGTGCACGATG.GCCCATGG
 sla_3204   .CGAGAGCATGGCGCG.ATATACTGCCGGTA..C
 sla_3205   C....GGTAGATGG.ACATGGCGGCAAGGCGGTC
 sla_3207   TCACTC.TACGGGTCAGAAGGTGTTCTG.TG..C
 sla_3211   CAAAGGG...AGGGGAAGGGAAAGGCT..TGGTA
 sla_3214   AGTCCCCTAAAGCTGCAATTGGCAGTGCGT....
 sla_3215   C...CTACAGGCTGTGTGATAGGTA.TGTGCCGC
 sla_3220   TGAGGTCCG...CCAAGTCGGCACCTGTATGCT.
 sla_3227   ...CTGCTGGGTCAATAGTCTCAGGGGATCGAC.
 sla_3228   CTATGGA....GGCCGATTCAAGGGCAGGTATGT
 sla_3230   GC.CCAGTGCATGAGGGCTTTAGGTG.ACTG..A
 sla_3233   GGGAAAG...GACGCTTGTAGCCGACCAC.GCC.
 sla_3234   ACATCTA...CGTCCCAGGCAGAGGCA...GGGG
 sla_3236   CG.CCGGAAGGATATAAGTGGAGCAC.GAAG..C
 sla_3237   .C.TAGCTGCTAGAGAATCAGAGGTG.GGCG..A
 sla_3238   CAAGATAGTAAA..AGTAAGGCA.GCTGTTTGC.
 sla_3243   TAGTACGCACGTCGCACATGGTTGGCTGTC....
 sla_3244   C.GCGATGACGATCT.ATTCACATTCGGA.GGG.
 sla_3248   ATTCTCGT.CTGCTATGGGGGGTTCGGAGGG...
*sls_453    GGACCATGATGAAATGTGCCG.G...AGAATTGA
 sls_455    G.CCTGTAACCTCTTAAAACTCACAAAGCCGA..
*sls_457    ACCTTAGCCCATGCCA.ATAAGTTGGAGGC...C
*sls_458    .AAGGCGGTTTT..AGGAGGGAT.AAGGTCGTT.
*sls_460     TCGACTGAGG  ATGTACCGTTTTA AGAAGCA
 sls_471    CC..CACGA..GGATCCCCAT.GTTAAGTGGAGA
 sls_481    TGAGCACGACGAGGCCACTAA.GTTCTGTGG...
 sls_485    ...CTCGCACGATCCACATGGATTTCCGACG..A
 sls_486    T.ACCGTCAAA..GGGAACATTACTAAGACAGG.
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(b)

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 sla_16     .A..CCGCGGGGT.TGCGGACCGGGAGCTCCAGC
*sla_110    G...AATTGCCGTAATTTCCCGTGGAGCGAT.GC
*sla_150    AGATGGACCTATATGGTCATCCCGTGTC...GT.
*sla_151    AC.TACTCCAGTAGCGTCTAAGTCTGTC....C.
 sla_153    GGGCCAAAG..TAAAACGGAAGTTC.CGTTATGA
 sla_166    AGACTA.GCTGATAGATAGGTGCAACG.GTTG..
 sla_167    ...GTAACAGGTGGGATCTAAGACGCGGTAGA.G
 sla_1201   ...CAAGTGCG.GTGGCTGAACTACCCGTCGTGA
 sla_1202   TCAGGCTTGT.GT.TGTAAGT..GCACCTTCTCT
 sla_1203   AAAG.TGGG..AAATCGCGCAA.AAGTGAATATA
 sla_1204   GAGC.CGCATCACCTATAATTGCGTAGAG..G.C
 sla_1210   A....ACTTGCTGACACCACAAATTGGGGCCCTT
 sla_1213   GAAG.GGCA...GCAACAACAG.GGCTATCGCCA
 sla_1219   GTGACATGTGCAGACAACACAGTATGTGGA....
 sla_1220   TTGC.CTCACCAC.TACGAGCGAGCCTTC..GTC
 sla_1221   G.GTCGGATATGGGAAGTGCATTCGTAGGA...A
 sla_1223   TTGT..TGATGG.ACATTGGTGTACCCTGTCGAC
 sla_1224   AAGCATTTATCGC.TGAGAGC..ACCTCGGGAGC
 sla_1225   G..CGCCGAGTCGGTCTACCACTGGTGC...AGG
 sla_1226   CCCGGGATCT.ACCTGCC.CTTAAGCAGTT..GG
 sla_1227   ACGATCGGAA.ACATGCC.GT.AAGCATGT..GT
 sla_1229   .GAT.GGCA..TGGGAGGGAAC.AAGGGAACTAC
 sla_1230   G.GGGAAG.GTAAGGATCG.TAGACGGGCC...A
 sla_1231   .....AACTTACGGTGGTCGGGATATGGTAGCTG
 sla_1235   G.AACAAGAGTAAGGACGGGCCATCTCATG...G
 sla_1236   ...GTAGCGGCATATGGTAAATAGGGGGGATGAA
 sla_1239   G.GTGGTTTGCTCCCGTAGTTTTCTACC...ACC
 sla_1241   G.ATTGGCGACTAGGGCTAACTCCCGAGCAC...
*sls_21     CCCGATGACTTACGCGTA.GAACT.TTGGT..AA
 sls_22     GGGGGC.ACCGACGAGAA.GTTTGGCTCAA..GG
*sls_26     T...TTATTTGGTATACTCTCTATGAGTGATTGG
 sls_252    ...CTAACTTA.GCAGCTGGGGGTCCTGGATATG
*sls_254    GTGTGCGTCCAGTTTAAGAACAAGCAGCA..G..
 sls_258    ACGCGGCGATGA.GGATTTGT.CACC..ACTGGC
 sls_259    CGGT.AATA..TACAAGAGGGG.ATGGGAGGTCG
 sls_261    ...TTAG.GGCCGGTGATAGCCTCGGTAGATGAC
 sls_262    G...TATCGGGTAGG.CTAAGTTGCTAGCACGTG
 sls_266    .CATCCGCATGCT.TTCGGG..AGCACGCCTGTG
 sls_268    T..TCAACT..CAAGCGGGGTGTGCGCGGTTGCA
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**Fig. 4.** Sequences of aptamers that bind aminoglycoside antibiotics. **(a)** Aptamers that bind lividomycin. **(b)** Aptamers that bind kanamycin A. Sequence identifiers are shown on the left. * indicates sequences whose individual binding profiles were determined (Tables 1 and 2). 'Sla' indicates a species isolated by affinity elution; 'sls' indicates a species isolated by high salt elution. The aptamers have been aligned to maximize similarity using CLUSTAL; primers were removed to avoid biasing the alignment.

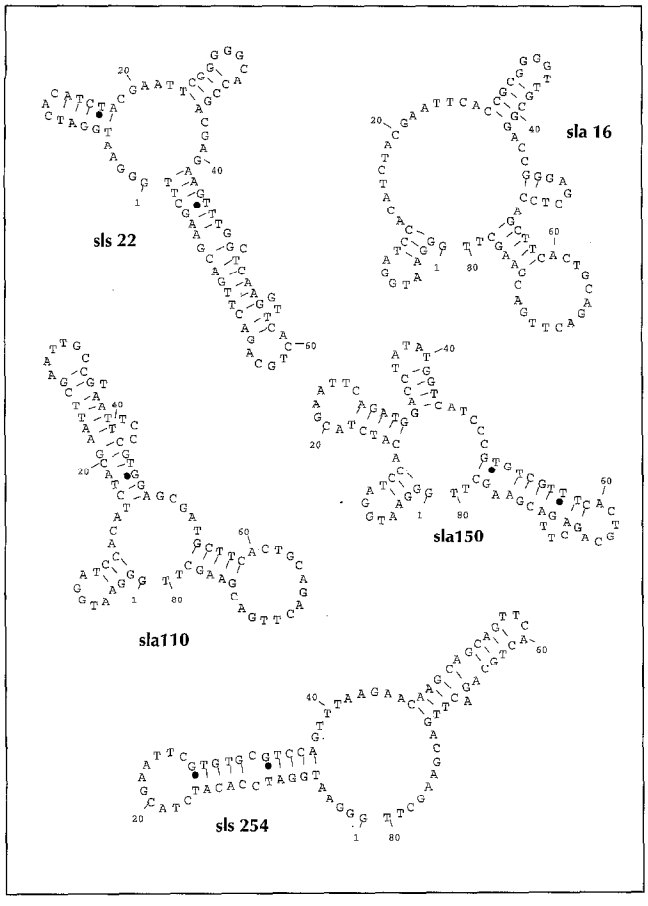


**Fig. 5.** Structures of aptamers that bind kanamycin. Representative structures generated using the program MULFOLD are shown for sequences derived from round 4 of the kanamycin A selection. Sequences derived from the N30 pool are in black, constant region sequences are in red. The proximity of the 5' and 3' ends of the sequences is a consequence of the preferences of the folding program.

similar numbers of functional groups. The selected RNA populations were sieved for similar numbers of cycles. However, the aggregate $K_d$ for the RNA–dye complexes is >100 μM, nearly 1000 times higher than that for the aminoglycoside–RNA complexes. This affinity difference can probably be directly attributed to the chemical differences between the two classes of ligands; the reactive dyes are all negatively charged, and contain multiple sulfonates, while the aminoglycosides are positively charged, and contain multiple amines. This result emphasizes a trend found in other selections, that positively charged ligands generally elicit tighter-binding aptamers.

### Selected sequences specifically recognize their cognate ligands

Since the target ligands were positively charged, it was especially important to determine whether the selected RNAs were merely forming non-specific electrostatic contacts with any small oligocation or were in fact specifically recognizing the shapes and structures of the aminoglycoside antibiotics. To determine whether selected RNAs could recognize gross structural features of their cognate ligands, the RNA populations from round 4 were assayed for their ability to bind to cognate and non-cognate aminoglycoside affinity columns. Aptamers selected to bind to lividomycin bound poorly to immobilized kanamycin A, while aptamers selected to bind to kanamycin A bound poorly to immobilized lividomycin (Fig. 7).

The binding specificity of selected RNAs was also assessed by affinity elution with a series of aminoglycoside antibiotics that were both structurally similar and dissimilar to lividomycin (Fig. 8a) and kanamycin A (Fig. 8b). The values for affinity elution of pool RNAs are the result of several independent determinations; clones were individually tested and compared with the values obtained for the pools (Table 2). In general, antibiotics that were structurally similar to the cognate antibiotic were more successful affinity eluants than structurally dissimilar antibiotics. For example, neomycin and paromomycin are from the same family as lividomycin and, overall, were found to be more successful at eluting lividomycin-binding RNAs than the less similar compounds ribostamycin and kanamycin (confirming the results of cross-binding studies), and the unrelated drug streptomycin (Table 2). Similarly, the kanamycin family members dibekacin and amikacin were generally better at eluting kanamycin-binding RNAs than the less structurally similar ribostamycin and the unrelated streptomycin (Table 2). The abilities of individual aptamers to

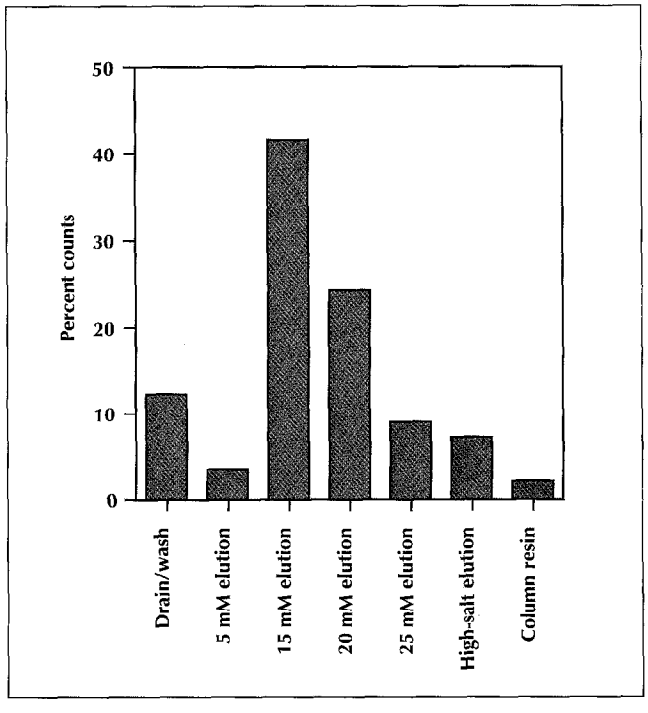


**Fig. 6.** Elution profile of RNAs bound to the kanamycin affinity column. The percent of RNAs that were eluted in each fraction is shown. The drain/wash fraction contained 60 column volumes of buffer. Six column volumes of 5 mM kanamycin were used for the first affinity elution, while three column volumes of antibiotic solution were used for each of the remaining affinity elution steps. The high-salt elution represents the sum of counts eluted with three column volumes of 5 M NaCl and three column volumes of 4 M GuSCN.

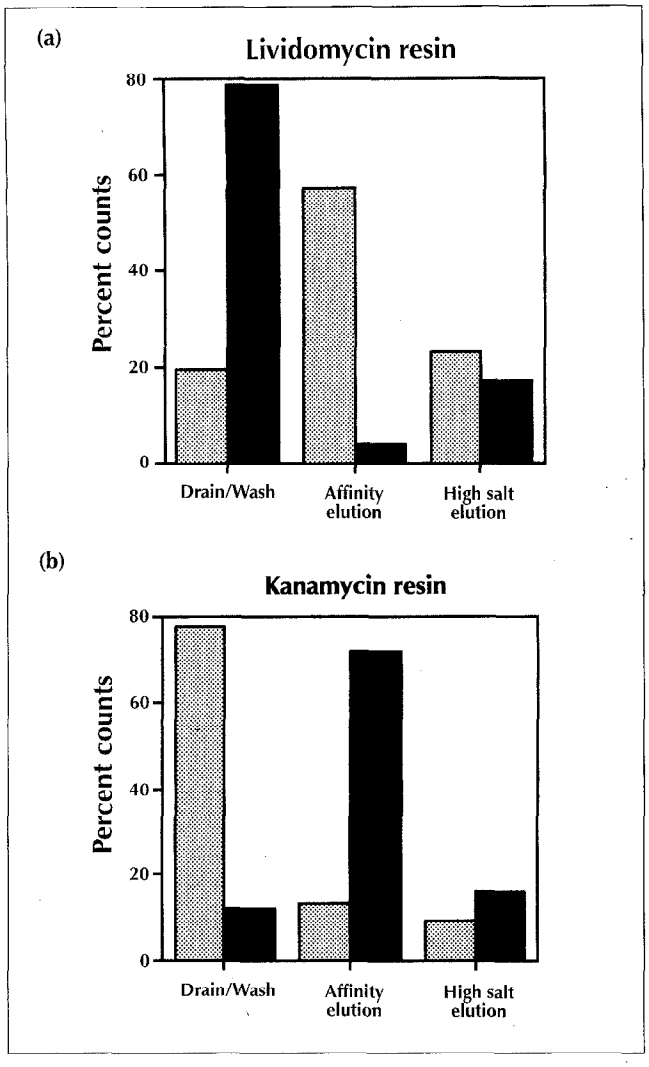


**Fig. 7.** Recognition of immobilized aminoglycosides by selected pools. **(a)** Selection on lividomycin resin. **(b)** Selection on kanamycin resin. RNAs from the fourth round of selection were applied to both cognate and non-cognate columns; for example, both lividomycin- (shaded columns) and kanamycin-binding (black columns) populations were applied to a kanamycin column (top panel). Following a wash step, the columns were eluted with free aminoglycoside antibiotic (kanamycin for kanamycin affinity columns, lividomycin for lividomycin affinity columns) and high salt. The number of counts in each fraction was normalized to the total number of counts eluted. While a fraction of the RNAs from both selections remain associated with either cognate and non-cognate columns (high-salt elutions), the majority of selected RNAs only stick to the column they were selected on and are only eluted by the antibiotic they were selected with.

distinguish between closely related ligand structures may provide clues to which epitopes on the aminoglycosides are recognized by these aptamers. For example, both the kanamycin pool and individual aptamers were surprisingly good at distinguishing between kanamycin A and the closely related kanamycin B (bekanamycin) even though these compounds differ by only one amino group (at the 2' position). Individual aptamers may interact with different epitopes; for example, the kanamycin-binding aptamer sla110 recognizes ribostamycin better than amikacin, but sla150 has the opposite preference.

Overall, these results indicate that RNAs selected to bind to aminoglycosides do not merely adhere non-specifically to agglomerations of positive charges, but can make structural distinctions between different positively charged ligands. The affinity-elution results cannot be rationalized by assuming that the most positively charged ligands elute more RNAs; for example, kanamycin B has more amino moieties than kanamycin A yet interacts poorly with RNA selected to bind to kanamycin A. Streptomycin contains two guanidino cations and has been shown to bind tightly to the group I intron and ribosomal RNA, but it cannot efficiently elute RNAs selected to bind to lividomycin or kanamycin A.

The large family of aminoglycoside-binding aptamers appear to interact with their cognate ligands much less specifically than aptamers selected to bind other small molecules, such as theophylline or ATP [10]. This may be due to the fact that previous *in vitro* selections have focused on repeating the enrichment procedure until only those few binding species that have the highest affinities and specificities remained. The affinities with which the selected populations and aptamers bind to their cognate ligands are already comparable to those observed in other selection experiments. Since the aggregate binding ability of a population has been shown to be the average of the binding ability of individual RNAs, highly specific RNAs may still be present as a subfraction within our large aminoglycoside-binding populations, and further selection may reveal them. We next set out to investigate whether the large number of LME sites that we had found by artificial selection were functionally similar to natural LME sites.

### Comparisons with natural RNAs

The calculated bounds on the dissociation constants for the selected RNA–aminoglycoside complexes compare favorably with the interactions observed for natural RNA–aminoglycoside complexes. Kanamycin A and lividomycin form complexes with individual aptamers that on average have $K_d$s of 300 nM or less. In comparison, kanamycin and lividomycin bind to and inhibit ribosomal RNA with $K_i$s of 10 μM or less, while lividomycin inhibits the group I intron with a $K_i$ of around 1 mM [6,20]. Other aminoglycosides (such as neomycin, ribostamycin and tobramycin) also inhibit these functional RNAs at concentrations of 1–1000 μM. It should be noted that the published inhibition and dissociation constants for complexes between aminoglycosides and natural RNAs were obtained using buffer conditions that differ from one another and from those used in our experiments. However, the salt concentration used in our selections and assays was uniformly higher than those used to derive the previously published values. Since the interaction energy between RNAs and aminoglycoside antibiotics probably has a large electrostatic component, aptamers from the selected populations should have LME binding sites that are on average as good as or better than those found on natural RNAs.

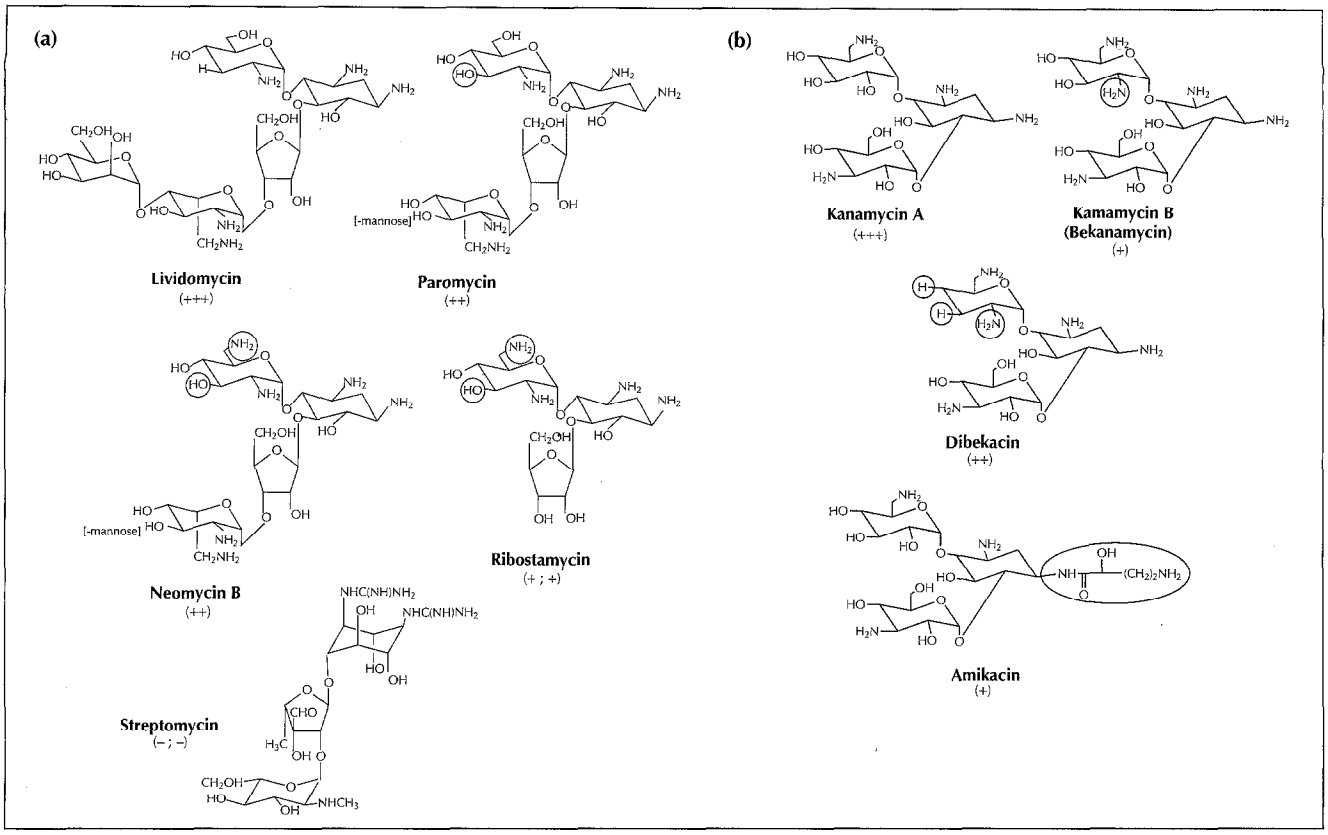


**Fig. 8.** Structures of aminoglycosides. **(a)** Lividomycin and related aminoglycosides. The panel of aminoglycosides used to elute lividomycin-binding RNAs included lividomycin, paromomycin, neomycin B, ribostamycin and streptomycin. Structural differences between lividomycin and similar compounds are circled; the mannose residue found in lividomycin but not in paromomycin or neomycin B is indicated by [–mannose]. Paromomycin is the aminoglycoside most structurally similar to lividomycin; neomycin B is next most similar, then ribostamycin (which lacks two sugar moieties). Streptomycin is from an unrelated family of aminoglycosides Symbols below each structure indicate the relative affinity of the lividomycin-binding RNA pool for this structure (see Table 2). For ribostamycin and streptomycin two values are shown; the first refers to the lividomycin-binding pool, the second to the kanamycin-binding pool. **(b)** Kanamycin A and related aminoglycosides. The panel of aminoglycosides used to affinity elute kanamycin A-binding RNAs included kanamycin B ('bekanamycin'), dibekacin and amikacin. Ribostamycin and streptomycin were also used (see (a)). Structural differences between kanamycin A and similar compounds are circled. Kanamycin B is the aminoglycoside most structurally similar to kanamycin A; dibekacin is next most similar, then amikacin. Ribostamycin has a furanose rather than pyranose sugar, which is linked 4,5 rather than 4,6. Streptomycin is from an unrelated family of aminoglycosides. Symbols below each structure indicate the relative affinity of the kanamycin-binding RNA pool for this structure (see Table 2).

The specificity of the selected populations for different aminoglycosides also rivals that of natural RNAs. For example, footprinting of the group I self-splicing intron indicates that neomycin B, episisomicin and streptomycin all protect the same subset of bases within the ribozyme's guanosine-binding site [21]. In comparison, the selected lividomycin-binding RNAs can recognize neomycin B, but have little or no affinity for streptomycin. Similarly, the selected kanamycin-binding RNAs can recognize structural distinctions between antibiotics that are much more closely related (Fig. 8b) than those that have been shown bind to the group I ribozyme. As another example, hygromycin and gentamycin protect the same subset of bases in *Escherichia coli* 16S rRNA from chemical modification [2,22], whereas artificially selected RNAs bind differently to antibiotics such as kanamycin and dibekacin, which are more structurally similar than hygromycin and gentamycin. Finally, it has been shown that the group I intron can also bind another type of LME, arginine, and that esterification of arginine has a similar effect on RNA binding [23]. We have shown here that RNAs selected to bind to aminoglycosides can give similar discrimination between sugars with different exocyclic moieties.

The finding that aminoglycoside-binding sites on aptamers were functionally similar to LME-binding sites on natural RNAs implied that they might be structurally similar as well. If true, this would suggest that our artificially selected populations might eventually converge on a set of sequences or structures similar to those generated through natural selection. To determine the overlap between artificially and naturally selected LME sites, we carried out direct sequence comparisons between aptamers and natural LME sites. For example, the kanamycin-binding site on rRNA has been mapped. Kanamycin binds adjacent to the 3' terminal domain of *E. coli* small subunit rRNA and strongly protects residues $A_{1394}$, $A_{1413}$ and $G_{1497}$ from

**Table 2.** Affinity elution of lividomycin-binding aptamers and kanamycin-binding aptamers by aminoglycosides[a]

| Sample | Neomycin | Paromomycin | Ribostamycin | Kanamycin | Streptomycin |
|---|---|---|---|---|---|
| **Lividomycin pool** | ++ | ++ | + | + | – |
| sla 352 | +++ | ++ | +++ | + | + |
| sla 355 | +++ | +++ | + | + | – |
| sls 453 | ++ | ++ | + | – | – |
| sls 457 | ++ | ++ | ++ | – | – |
| sls 458 | ++ | ++ | ++ | + | – |
| sls 460 | ++ | ++ | ++ | – | + |

| Sample | Bekanamycin | Dibekacin | Amikacin | Ribostamycin | Streptomycin |
|---|---|---|---|---|---|
| **Kanamycin pool** | + | ++ | + | + | – |
| sla 110 | – | ++ | + | ++ | – |
| sla 150 | – | ++ | + | – | – |
| sla 151 | + | ++ | + | + | – |
| sls 21 | – | ++ | ++ | ++ | – |
| sls 26 | – | ++ | + | + | – |
| sls 254 | + | +++ | + | ++ | – |

[a]Radiolabeled RNAs were affinity eluted from their cognate aminoglycoside affinity column by different aminoglycosides. Values are based on the sum of 5 mM and 25 mM aminoglycoside elutions and are normalized relative to a control affinity elution with the cognate aminoglycoside. Binding values are given as – (<10% of bound counts eluted), + (11–33% eluted), ++ (34–75% eluted) or +++ (>76% eluted).

chemical modification [22]. A segment of small subunit rRNA sequence involving residues 1390–1500 was compared with kanamycin-binding sequences by both computer alignment and visual inspection. No stretch of residues longer than seven bases was found to be similar between this segment of rRNA and any aptamer; the longest stretch of similar residues was found to lie within secondary structural contexts that are predicted to be different in the artificial and natural LME sites. Since other natural binding sites for kanamycin and lividomycin have not been as carefully mapped, we also compared the entire *E. coli* 16S rRNA sequence and the entire *Tetrahymena* group I intron sequence with the aptamer sequences. No significant similarities were found.

Although no similar sites were found on rRNA or the group I intron, other natural RNAs might contain aminoglycoside-binding sites similar to those in the selected population. The probability of finding a given aminoglycoside-binding, 30 nucleotide RNA, roughly 1 in $10^7$, implies that there will generally be a few, but not necessarily many (less than 100), such sites in a eukaryotic genome. Therefore, we probed the entire nucleic acid database (Genbank) with individual aptamer sequences. Although natural sequences with significant similarity to several of the aptamers were identified, none of them were self-splicing introns or ribosomal RNAs.

#### Assessing the LME hypothesis

These studies were originally undertaken to determine the diversity of LME-binding sites that can be formed by RNAs. If only a small number of high-affinity sites are possible, then the aminoglycoside-binding sites that are found on natural RNAs result from a relatively rare event, and so might all arise from one precursor (Fig. 9a). But if a wide variety of RNA sequences and structures bind to aminoglycosides, then the natural binding sites found on organismal and viral RNAs might represent only a small fraction of the total diversity of sites. In this view, the fact that aminoglycoside-binding sites are found on both ribosomal RNA and the group I intron does not imply that the two molecules have a common ancestor (Fig. 9b).

The fact that numerous, distinct, high-affinity aminoglycoside-binding sites can be derived from random sequence pools suggests that the model described in Figure 9b is more probable than that set out in Figure 9a. Since many different RNA sequences can fold to form many different aminoglycoside-binding sites, the mere presence of aminoglycoside-binding sites on natural RNAs is probably not indicative of common ancestry. The conventional explanation for the presence of LME sites on ribosomes would still appear to be the most plausible: that the 'combinatorial chemistry' programs of aminoglycoside-producing microorganisms successfully discovered compounds that could bind to and inhibit the catalytic core of the ribosome, giving them an advantage over their bacterial competitors.

There are caveats to this analysis. For example, it could be argued that it is the probability of finding a given aminoglycoside-binding site, rather than the raw number of such sites, that makes natural aminoglycoside-binding sites a useful character for determining the evolutionary relatedness of RNAs. In this case, even though there may be millions of different aminoglycoside-binding sites, the likelihood of encountering any given one of them in a pool of random, 30 nucleotide RNAs by chance is roughly 1 in $10^7$. This argument is still not consistent

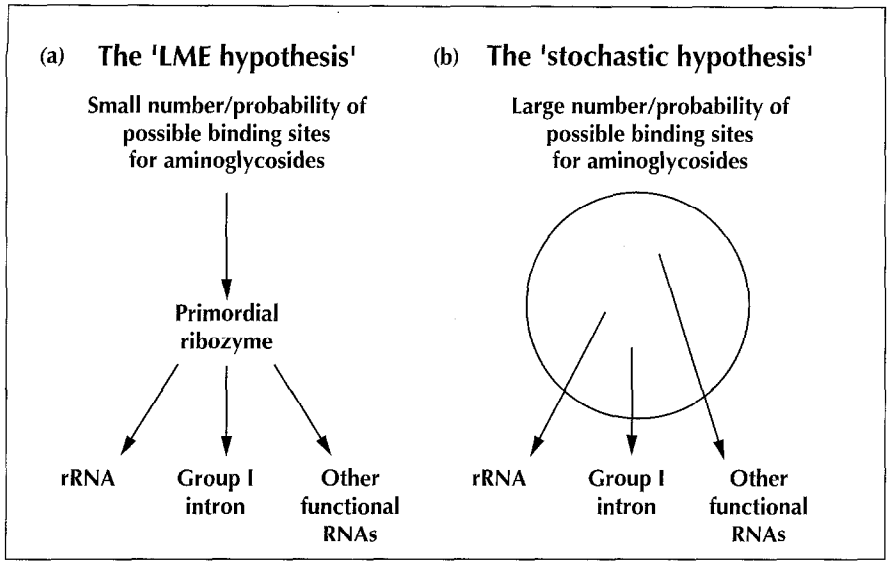


**Fig. 9.** Models for the evolution of aminoglycoside binding sites. Aminoglycoside binding sites can be observed in modern RNAs, and their origins can be interpreted in one of several ways. Two models are shown. **(a)** The 'LME hypothesis' suggests that the functionally similar sites may have a common origin. This view is likely to be correct if there are only a few, rare ways that RNA molecules can form aminoglycoside binding sites. **(b)** The 'stochastic hypothesis' is in essence the null hypothesis to explain the existence of LME sites. In this scenario, RNA molecules can form aminoglycoside-binding sites in many ways, and therefore the presence of such binding sites in modern RNA molecules (or any set of RNA molecules) is to be expected.

with the notion that there was a primordial catalytic precursor to modern ribosomal RNA and group I introns. If the original formation of an LME site was a relatively improbable event, then the sequence or structural divergence of this LME site would also have been relatively improbable. The lack of sequence or structural similarity between selected aptamers suggests that they are distant from one another in 'sequence space'. Thus, they would not only be encountered by chance relatively infrequently, but could have mutated from one to the other relatively infrequently. Thus, any modern LME sites that were derived from a common ancestor should share sequence homology. No sequence or structural similarity has yet been detected between ribosomal RNA and the group I intron, however; they appear to be as different from one another as any one of the selected aptamers is from them.

It should also be noted that, although we have selected RNA species that bind LMEs, natural RNAs not only bind to LMEs but are functionally disrupted by them. It therefore remains possible that the LME-binding sites of rRNA and the group I intron are more unusual than the analysis presented here suggests. For example, many LME-binding sites might have been present on a primordial ribozyme, but only a few of them might have been able to inhibit the function of the ribozyme by a mechanism analogous to the allosteric inhibition of modern enzymes. An unidentified characteristic of this kind might still link aminoglycoside-binding sites on rRNA and the group I intron.

It could also be argued that it may be the three-dimensional structure of natural LME sites, rather than their primary or secondary structural signatures, that has been conserved from their common origin. An examination of the structures of homologous proteins suggests that tertiary structure may be more highly conserved than primary structure. However, as aminoglycoside-binding sites are common in unrelated aptamers, it is likely that there are many tertiary structural folds that can bind aminoglycosides. Ribosomal RNA and group I introns have no obvious similarity at the level of secondary structure, and there is no reason to believe that their tertiary folds are similar.

This study cannot definitively show that rRNA and the group I intron are not related. However, our results show that the fact that they both bind to antibiotics does not in itself imply common ancestry. Our observation that RNAs that bind to antibiotics are common makes the prediction that, if these two RNAs did indeed have a common ancestor, they will be found to be similar either in their three-dimensional structures or in the mechanisms by which they are inhibited by antibiotic binding.

If modern aminoglycosides are not exploiting conserved pre-existing LME-binding sites, then they must be natural compounds that have evolved the ability to recognize RNAs specifically, much as distamycin and netropsin can recognize particular DNA sequences. Indeed, the ability of aminoglycosides to act as 'lexitropsins' (sequence-reading compounds) by distinguishing between different RNAs compares favorably with the ability of DNA-binding compounds to distinguish between different sites on double-stranded DNAs [24]. Just as DNA-binding drugs have proven to be useful lead compounds for engineering new sequence specificities, the aminoglycosides may prove to be similarly interesting as starting points for RNA recognition.

## Significance

**The hypothesis that aminoglycoside-binding sites on functional RNAs such as rRNA and the group I self-splicing intron are historically related is predicated on the notion that these sites must be relatively unusual. We have selected aptamers from a random-sequence pool that can bind tightly and specifically to two aminoglycosides, lividomycin and kanamycin A, and find**

**that there are thousands to millions of distinct aminoglycoside binding sites within the pool. The artificially evolved aminoglycoside-binding sites are functionally similar to aminoglycoside-binding sites found on rRNA and the group I intron, but bear no obvious sequence or structural resemblance to these natural sites. These results cast doubt on the hypothesis that the aminoglycoside-binding sites of natural RNAs share a common evolutionary history.**

**Despite their diversity, the selected RNA populations bind aminoglycosides as tightly as a number of previously selected aptamers that bind ligands of similar size and complexity. This result emphasizes that oligosaccharides, particularly those containing amino sugars, should be excellent targets for the *in vitro* selection of RNA 'lectins'. Moreover, since the Darwinian selection of molecules can almost always reduce random-sequence populations to a relatively few motifs with high affinities and specificities, the prospect now exists that aminoglycoside-binding motifs will be extracted that have $K_d$ values in the low nanomolar range.**

**These results emphasize that aminoglycoside antibiotics and their derivatives may have evolved (and can continue to be used) as natural lexitropsins for the sequence- and structure-specific recognition of RNAs. Although aminoglycosides cannot recognize every sequence, a large subset of RNA sequences can be recognized with high affinity. By examining these sequences and structures, it may be possible to find new targets for aminoglycosides among natural RNAs**

## Materials and methods

### *Materials*

All aminoglycosides were obtained from Sigma (St. Louis, MO) as their sulfate salts. Epoxy-activated affinity resin was obtained from Pierce (Rockford, IL).

### *Affinity-resin synthesis*

Aminoglycosides (100 μmoles) or glycine (200 μmoles) were dissolved in 0.1 M sodium carbonate, pH 10.0 (10 ml), mixed with column resin (2.0 g), and allowed to couple for 16 h at 37° C. The resin was then drained and washed with 3 column volumes of 1x PBS (2.7 mM KCl, 140 mM, NaCl, 9.9 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4). The pH of the final eluate was 7.4. Residual reactive epoxy residues were then blocked with glycine (200 μmoles) in 0.1 M sodium carbonate, pH 8.5 for an additional 10 h. The final columns were again washed with 1x PBS, and column resins were stored in 1x PBS at 4° C between experiments. To estimate the approximate concentration of the antibiotic on the affinity resin, the number of reactive epoxy groups was determined by coupling a chromophore (4-aminophenylacetic acid or tyrosine) in parallel. For example, the columns used for selection were calculated to contain 25 μM kanamycin A or lividomycin. All selections were carried out using this batch of affinity resin.

Kanamycin A and lividomycin contain several primary and secondary amines and could be tethered to the epoxy-activated resin in multiple ways. However, the use of affinity elution for the selection step should have negated any effects of column ligand heterogeneity, since isolated aptamers would preferentially bind the solution conformation of free aminoglycoside.

### *Pool synthesis*

The N30 pool (Fig. 2) was constructed using standard methods for automated DNA synthesis with nucleoside phosphoramidites. A stochastically random sequence core was obtained by mixing the four phosphoramidites in a 3:3:2:2 A:C:G:T ratio [25]. Crude pool (5 μg; ~$10^{14}$ sequences) was amplified using the polymerase chain reaction (PCR). Twenty-five tubes that each contained 0.2 μg of crude DNA in 200 μl of PCR reaction mix (50 mM KCl, 10 mM Tris.Cl, pH 8.3, 1.5 mM $MgCl_2$, 80 μM each deoxynucleotide triphosphate, 0.4 μM of the primers 41.30 and 24.30 (Fig. 2), 2.5 units Taq polymerase) were carried through five thermal cycles of 94° C, 45 s; 45° C, 75 s; 72° C, 120 s. Double-stranded DNAs were purified by ethanol precipitation in the presence of 1 M ammonium acetate, pH 7.4.

The RNA pool was transcribed from double-stranded DNA (1 μg; 2x $10^{13}$ sequences) using T7 RNA polymerase in an Ampliscribe kit (Epicentre Technologies, Madison, WI). The kit was used according to the manufacturer's directions, except that 40 nmoles of α-$^{32}$P UTP (3000 Ci mmol$^{-1}$) was added to a 20-μl reaction. The RNA pool was gel-purified on a 10 % denaturing polyacrylamide gel as previously described [26].

### In vitro *selection*

For a given cycle of selection, RNA in 1x binding buffer (500 mM NaCl, 50 mM Tris.Cl, pH 7.6) was heated to 75° C for 3 min and allowed to cool to room temperature to equilibrate conformers. The RNA solution (15 μg per 100 μl in the first round, 10 μg per 100 μl in subsequent rounds) was then loaded onto a glycine pre-column (1.0 ml in the first round, 0.75 ml in subsequent rounds) that had been pre-equilibrated with 1x binding buffer. The glycine pre-column was washed with 1 column volume of 1x binding buffer and the sample immediately applied to aminoglycoside affinity columns (1.0 ml) that had been pre-equilibrated in 1x binding buffer. The resin and RNA were mixed for 1 h at room temperature. The columnns were developed with 6 column volumes (6.0 ml) of 1x binding buffer, and bound RNAs were progressively eluted with 3 volumes each of 5 mM, 15 mM and 25 mM aminoglycoside in 1x binding buffer. At this stage, all fractions were counted without scintillation fluid. Only RNAs from the 25 mM affinity elution were ethanol precipitated and used for further selections. To remove aminoglycoside antibiotic that precipitated along with the nucleic acid, RNAs were resuspended in 100 μl of water and put through a G-50 Sephadex spin column (Boehringer, Indianapolis, IN). The flow-through was re-precipitated in the presence of glycogen (20 μg).

Following affinity elution in cycles 3 and 4, the aminoglycoside columns were also eluted with 3 column volumes of 5 M NaCl and 3 column volumes of 4 M guanidinium thiocyanate (GuSCN) in 10 mM TrisCl, pH 8.0. These high-salt fractions were combined, ethanol precipitated, and the RNAs amplified

in parallel with those derived from the 25 mM aminoglycoside affinity elution. High-salt-eluted and affinity-eluted RNAs from round 3 were separately selected in round 4.

Two-thirds of the RNA recovered from either high-salt or affinity elutions was used to prepare cDNA as previously described [27]. One-half of the cDNA (1/3 of the total RNA recovered in each cycle) was then amplified using PCR. RNAs for a new cycle of selection were prepared by *in vitro* transcription.

### *Sequence analysis*

Following the fourth cycle of selection, DNA recovered from the PCR amplification reaction was directly cloned into a TA cloning vector (InVitrogen, San Diego, CA). Aptamer sequences were derived from individual plasmid DNAs using standard dideoxy sequencing methods.

The approximate number of aptamers remaining in the selected population was calculated by assuming that the percent of RNA that was carried from one cycle to the next represented a dilution factor for the complexity of the previous population; that is, if 1% of RNAs were selected, then the complexity of the population was assumed to decrease by a factor of 100 (the 'selection coefficient' for that cycle; [11]). As discussed in the text, this estimation is unrealistic because it assumes that every species goes to extinction or fixation during the selection. However, it should be accurate as a lower bound for the total number of species that remain in the population.

Multiple alignments and pairwise sequence comparisons were carried out using the MEGALIGN package (DNA*, Madison, WI), which includes the CLUSTAL alignment method [27]. Secondary structure predictions were made using the program MULFOLD [28].

### *Binding assays*

Bounds on values for the dissociation constants of RNA–aminoglycoside complexes were determined using a modification of the method described by Ellington and Szostak [11]. Since the selected populations bound so tightly to their cognate columns, it would have required a prohibitive number of buffer washes to remove the majority of the RNA. Therefore, upper bounds on the aggregate dissociation constants were established by demonstrating that a large volume of 1x binding buffer could not remove RNAs from the column, while a small volume of free ligand could. RNAs were prepared and applied to affinity columns as described above. The columns were developed with up to 60 column volumes of 1x binding buffer and then RNAs were progressively eluted with 6 column volumes of 5 mM antibiotic in 1x binding buffer and 3 column volumes each of 15 mM, 20 mM and 25 mM antibiotic in 1x binding buffer, followed by high-salt washes with 3 column volumes each of 5 M NaCl and 4 M GuSCN in 10 mM Tris.Cl, pH 8.0. The number of counts in each fraction was determined, and the percent of counts in each fraction was normalized to the total number of counts eluted. Dissociation constants were calculated according to the equation [29]:

$$K_d = [\text{ligand}]_{\text{column}} \times [(\text{column volume} - \text{void volume}) \div (\text{elution volume} - \text{void volume})]$$

The specificity of binding was determined in two ways. First, RNAs selected to bind to one aminoglycoside (for example, kanamycin A) were assayed on a column containing the other aminoglycoside (for example, lividomycin). This procedure was similar to that used for selection, except that only 5 μg of RNA was used and the columns (500 μl bed volume) were washed with 12 column volumes of 1x binding buffer prior to affinity and high-salt elution. The number of counts in each fraction was determined, and the percentage of counts in each fraction was normalized to the total number of counts eluted (Fig. 7). Second, RNA populations selected to bind to one aminoglycoside were eluted from their cognate column with non-cognate aminoglycoside antibiotics. For example, a population of kanamycin-binding RNAs was eluted from a kanamycin column with lividomycin. This procedure was similar to that used for selection, except that only 0.5 μg of RNA was used and the columns (100 μl bed volume) were developed with only 6 column volumes of 1x binding buffer and 3 column volumes each of 5 mM and 25 mM aminoglycoside antibiotic. The number of counts in each eluted fraction was determined and normalized relative to the number of counts that had bound to the column (Table 2).

*Acknowledgements:* This work was supported by a National Science Foundation National Young Investigator Award (A.E.), a Scholar Award from The American Foundation for AIDS Research (A.E.), and The Pew Scholar Awards in the Biomedical Sciences (A.E.).

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Received: **18 Apr 1995**; revisions requested: **2 May 1995**; revisions received: **3 May 1995**. Accepted **5 May 1995**.