Targeting RNA with Small Molecules

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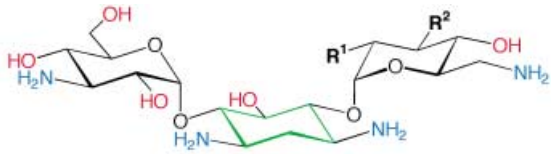
1. Introduction

Remarkable findings have cemented the central dogma of biology, where the transfer of genetic information from a sequence of nucleotides in DNA to a string of amino acids in proteins is mediated through RNA.[1] Although RNA was recognized as playing a key role in this incredible process, for decades it was considered as a passive carrier of DNA's sequence information. The discovery of ribozymes has changed this view and facilitated a major paradigm shift.[2, 3] RNA is now regarded as a respectable functional biomolecule with impressive catalytic potential.[4] RNA has continued to fascinate and surprise the scientific community with its multifaceted roles in cell biology. The recent high-resolution structures of ribosomes show RNA to be the key component responsible for peptide-bond formation in protein biosynthesis.[5–8] The discovery of RNA interference, a cellular response to double-stranded RNA that leads to sequence-specific gene silencing, reveals a new and unexpected role for small RNA molecules in gene regulation.[9] Recent findings demonstrating specific interactions between low-molecular-weight metabolites and messenger RNAs (mRNAs) related to their biosynthetic pathways illustrate exciting new regulatory mechanisms at the RNA level.[10, 11] Thus, another paradigm shift is emerging.

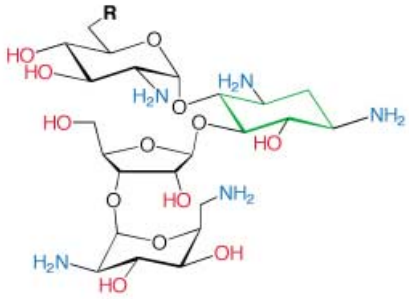
The capability of RNA to specifically communicate with large and small molecules is central to its diverse biological functions. Revealing the structural and dynamic features of RNA–ligand recognition events will have a direct impact on our ability to ultimately control cell function at the RNA level.[12] It will also open up new opportunities to combat pathogens by specifically targeting their RNA or RNA–protein complexes. With this in mind, we initiated, about a decade ago, a research program aimed at unraveling the fundamentals of RNA–ligand interactions and advancing RNA as a drug target. The purpose of this article is to highlight the inspiration for our program and its evolution. There is remarkable interest in this young and rapidly growing field, and this minireview is not intended to be comprehensive. The interested reader is referred to excellent review articles that summarize advances in this and related fields.[13–22]

2. Aminoglycoside Antibiotics and RNA

Our search for small molecules as selective RNA binders was inspired by early observations that demonstrated the ability of certain antibiotics, particularly the aminoglycosides, to interfere with protein biosynthesis. Selected examples of this intriguing family of naturally occurring pseudo-oligosaccharides are shown in Figure 1. The common core of most aminoglycosides is 2-deoxystreptamine (2-DOS), a highly functionalized aminocyclitol. Glycosylation of the 2-DOS core, typically at the 4- and 5-, or 4- and 6-positions, characterizes most aminoglycosides.[23] Interestingly, decades after their discovery, aminoglycoside antibiotics remain clinically useful and enigmatic.[24]



	R^1	R^2	[Self-Sp.]	[Rev-RRE]
Kanamycin A	OH	OH	>2000	> 100
Kanamycin B	NH_2	OH	10	100
Tobramycin	NH_2	H	0.5	10



	R	[Self-Sp.]	[Rev-RRE]
Paromomycin	OH	100	> 100
Neomycin B	NH_2	0.5	1

Figure 1. *Representative examples of aminoglycoside antibiotics and their structure–activity relationship. Inhibition of group I intron's self-splicing (Self-Sp.) is given as IC_{50} values in μM.[28] Inhibition of Rev–RRE binding (Rev–RRE) is given as IC_{90} values in μM.[29] The amino and hydroxy groups as well as the 2-deoxystreptamine core (2-DOS) are colored.*

Twenty years after the discovery by Waksman and co-workers of streptomycin,[25] the first aminoglycoside antibiotic to be isolated, a plausible mode of action was proposed.[26] By using an in vitro translation system, Davies et al. concluded in 1964 that streptomycin interferes with protein synthesis at the level of the "ribosome–messenger RNA–sRNA complex".[26] In a seminal paper published in 1987, Moazed and Noller showed that, indeed, aminoglycoside antibiotics interact with specific sites on

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16S rRNA.[27] The renaissance aminoglycosides have experienced in recent years was prompted by the report by Schroeder and co-workers in 1991 which demonstrated that these antibiotics can also inhibit splicing of group I introns (a prototypical large ribozyme).[28] Green and co-workers, in an intriguing paper published in 1993, demonstrated the capability of the same natural products to inhibit the interaction between the HIV-1 Rev protein and its RNA target, the Rev response element (RRE).[29]

These key discoveries, which accentuated aminoglycoside antibiotics as a family of RNA binders, triggered our interest and stimulated a pursuit for a fundamental understanding of RNA – aminoglycoside interactions. Examination of the data presented in Figure 1 reveals some rudimentary structure – activity relationships. The importance of amino groups for RNA binding and inhibition is apparent. Changing an amino group in kanamycin B to a hydroxy group in kanamycin A practically abolishes inhibitory activity in the self-splicing and Rev-RRE assays (Figure 1). A similar trend is observed for neomycin B versus paromomycin. Electrostatic interactions are therefore a major contributor to RNA affinity. Yet, this binding phenomenon must be more complex. Other aminoglycosides (for example, apramycin), simple polyamines (for example, spermine), and other structurally unrelated antibiotics (for example, viomycin) that possess a comparable number of amino groups are not as active. As evident from the data shown in Figure 1, the hydroxy groups also influence RNA affinity. Kanamycin B, for example, is twenty-fold less active than tobramycin (its 3′-deoxy derivative) in inhibiting self-splicing of group I introns, and the trend holds for other RNA targets. Stimulated by these intriguing observations, we have embarked on a research program aimed at the fundamental understanding of RNA – aminoglycoside interactions.[30]

3. Systematically Modified Aminoglycosides Support a Unique Binding Model

3.1 Key Questions

The early observations discussed above and the lack of a harmonious binding model evoked several key questions: a) What is the role of electrostatic interactions in RNA – aminoglycoside binding? b) Can a general recognition model for RNA – aminoglycoside binding be formulated? c) What is the RNA selectivity of aminoglycoside antibiotics? Do they bind other RNA targets? d) Can multiple binding sites for aminoglycosides be found on RNA molecules? To address these questions, we have selected RNA targets and examined their interactions with systematically modified aminoglycoside derivatives.[31]

3.2 The Hammerhead Ribozyme: A Model RNA Target

RNA enzymes (ribozymes) provide unique opportunities to correlate RNA structure and function. Valuable information regarding RNA – ligand interactions can be obtained from analyzing ribozyme inhibition by exogenous molecules. These considerations prompted us to choose the hammerhead ribozyme (HH), one of the best-characterized RNA enzymes,[32–34] as our first RNA target (Figure 2). A wealth of information regarding its conserved base requirements, kinetics, mechanisms, and structure was available.[35–38] Additionally, Uhlenbeck and co-workers showed that aminoglycoside antibiotics interact preferentially with the enzyme – substrate complex of the ribozyme and inhibit the cleavage step.[39, 40] This turned the HH ribozyme into a useful assay in our hands, as RNA affinity could be deduced by measuring the effect of synthetic ligands on the ribozyme's cleavage kinetics.[41]

3.3 Deoxygenated Tobramycin Derivatives and Electrostatic Interactions

The recurrent higher RNA affinity of tobramycin relative to kanamycin B attracted our attention. To shed light on this observation we systematically deoxygenated tobramycin and evaluated the effect of the modified analogues on the inhibition of the hammerhead ribozyme (Figure 3 a).[42] Certain deoxygenated derivatives (for example, 4′-, 2″-, and 4″-deoxytobramycin) are better HH inhibitors than tobramycin. In contrast, the 6″-deoxy derivative is a poorer HH inhibitor. These results indicate that higher inhibitory activity is observed when a hydroxy group proximal to an amine group is removed. We have

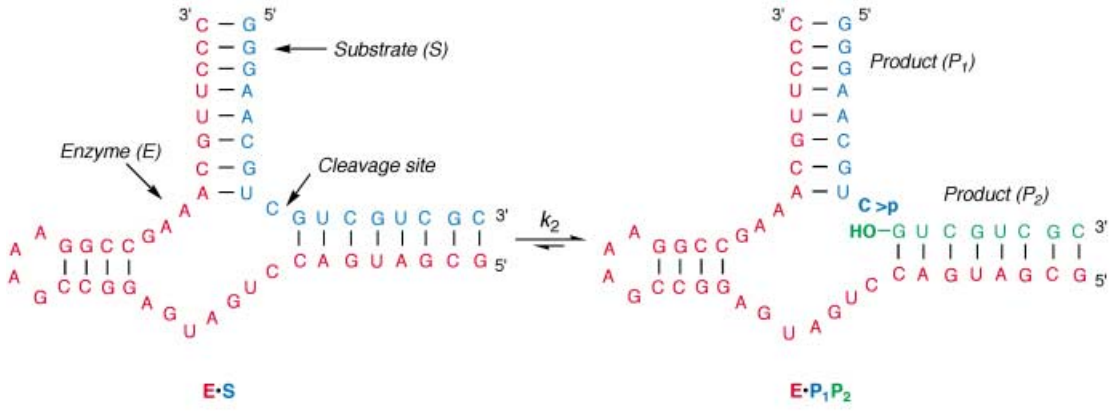
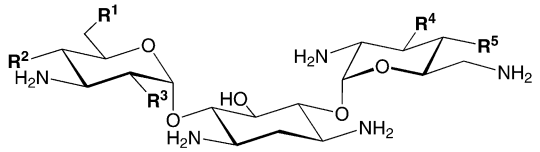


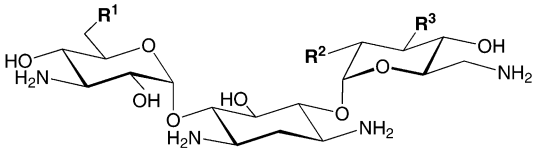
Figure 2. *The hammerhead ribozyme and its cleavage reaction.*

a)



Aminoglycoside	R^1	R^2	R^3	R^4	R^5	Relative Ribozyme Cleavage Rate
Kanamycin B	OH	OH	OH	OH	OH	1.5
Tobramycin	OH	OH	OH	**H**	OH	1.0
4'-Deoxytobramycin	OH	OH	OH	**H**	**H**	0.33
6''-Deoxytobramycin	**H**	OH	OH	**H**	OH	1.4
4''-Deoxytobramycin	OH	**H**	OH	**H**	OH	0.33
2''-Deoxytobramycin	OH	OH	**H**	**H**	OH	0.17

b)



Aminoglycoside	R^1	R^2	R^3	Relative Ribozyme Cleavage Rate
Kanamycin A	OH	OH	OH	4.8
6''-amino-Kan A	**NH_2**	OH	OH	1.5
Kanamycin B	OH	**NH_2**	OH	1.5
Tobramycin	OH	NH_2	H	1.0

Figure 3. *Structure and hammerhead inhibitory activity of: a) deoxytobramycin derivatives[42] and b) selected examples of amino-aminoglycosides.[45] Relative cleavage rates (normalized to that induced by tobramycin) are given. Note that higher numbers reflect lower inhibitory effect.*

proposed that altering the basicity of the amino groups by the removal of their neighboring hydroxy group affects the overall charge of the modified analogues and their RNA binding capability (for example, the pK_a value of $CH_3CH_2NH_3^+$ is 10.7 while that of $HOCH_2CH_2NH_3^+$ is 9.5).[42] Thus, the appropriately deoxygenated aminoglycoside derivatives possess a higher overall positive charge at a given pH value relative to their parent natural product. These data provided strong experimental support for the critical role of electrostatic interactions in RNA – aminoglycoside binding. This study also revealed the interplay between hydroxy and their neighboring ammonium groups, and clarified previously unexplained trends among the naturally occurring aminoglycosides in their ability to bind various RNA targets.

Ribozyme-mediated cleavage experiments are typically carried out at neutral to slightly basic pH values. One may wonder why modulating the pK_a value of the ammonium groups by even one pK_a unit should matter under such conditions. It is important to recognize that the ammonium groups present on the antibiotics possess a wide range of pK_a values (from 5.7 to about 8.8).[43] While the aminoglycosides are predominantly protonated at physiological pH values, the abundance of ammonium groups with pK_a values close to 7.0 implies that RNA binding of such derivatives is heavily dependent on the pH value.[44] Fine modulation of the basicity of the ammonium groups can therefore have a significant impact on the overall charge of an aminoglycoside derivative, and hence on its RNA affinity.

3.4 "Amino-aminoglycosides" Suggest a Binding Model

To further clarify the key role of electrostatic interactions in RNA – aminoglycoside binding, we designed and synthesized a series of "amino-aminoglycosides", in which selected hydroxy groups were converted into amino groups.[45] We demonstrated that adding an amino group to aminoglycosides dramatically increases their RNA binding affinity (Figure 3 b). This observation led to the first aminoglycoside derivatives with hammerhead ribozyme inhibitory activity that surpasses any natural product.

While the enhanced RNA affinity of the amino-aminoglycosides is, in retrospect, not too surprising, the observation that 6''-amino-6''-deoxykanamycin A and kanamycin B have identical ribozyme inhibitory activities was instrumental in advancing a binding model. These isomeric derivatives differ only in the nature of their substituents at positions 2′ and 6''. The synthetic derivative carries an amino and hydroxy group at positions 6'' and 2′, respectively, while in kanamycin B the two groups are interchanged. Although the identical RNA affinity of these derivatives may be coincidental, we felt these results held important clues to the modes in which aminoglycosides bind RNA. The study of models indicated that the positive charges presented by the two isomeric aminoglycosides can nearly be superimposed after rotation around an imaginary pseudo-C_2 axis going through positions 2 and 5 in the central 2-deoxystreptamine ring (Figure 4). Both derivatives can thus display a similar three-dimensional array of ammonium groups and charge densities toward the HH ribozyme.[45]

3.5 Surface Electrostatic Complementarity

This simple model building experiment led us to propose a preliminary binding model in which defined, yet adaptable, three-dimensional projection of positively charged ammonium groups toward the negatively charged RNA surface is employed. The high charge density of the aminoglycosides, together with their unique structural features (namely, conformationally "fixed" six-membered rings that can rotate around "flexible" glycosidic bonds) and the geometrical degeneracy of ammonium groups, allow these compounds to favorably model themselves to match the electrostatic requirements of the RNA surface.[45]

At about the same time, molecular dynamics simulations by Hermann and Westhof uncovered striking complementarity between interionic Mg^{2+}-Mg^{2+} distances in the HH ribozyme structure and the intramolecular distances between the charged ammonium groups on aminoglycosides.[46] Numerous solution conformations of different aminoglycosides could successfully be docked into the hammerhead fold in such a way that ammonium groups of aminoglycosides occupied the Mg^{2+}-binding sites found in the RNA crystal structure. The covalently linked array of ammonium groups thus complements the negative electrostatic potential created by the RNA fold (Figure 5). The model emphasizes a three-dimensional electrostatic complementarity rather than highly specific contacts between

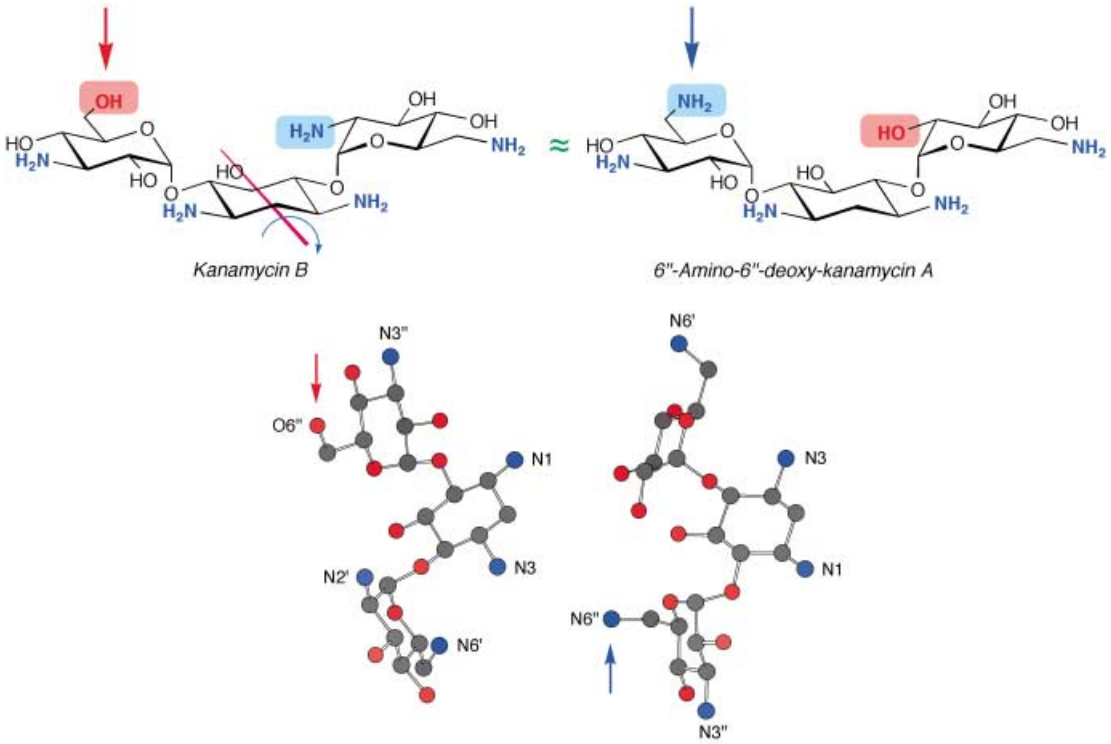


Figure 4. *The identical inhibitory activity of 6′′-amino-6′′-deoxykanamycin A and kanamycin B suggested a possible RNA binding mode.[45] Minimized conformations (Spartan) of the fully protonated 6′′-amino-6′′-deoxykanamycin A (right) and the rotated kanamycin B (left) show the similar position in three dimensions occupied by the ammonium groups. Highlighted are the 6′′- and 2′-positions in both aminoglycosides. Colored arrows provide a point of references to the 6′′-position in both derivatives.*

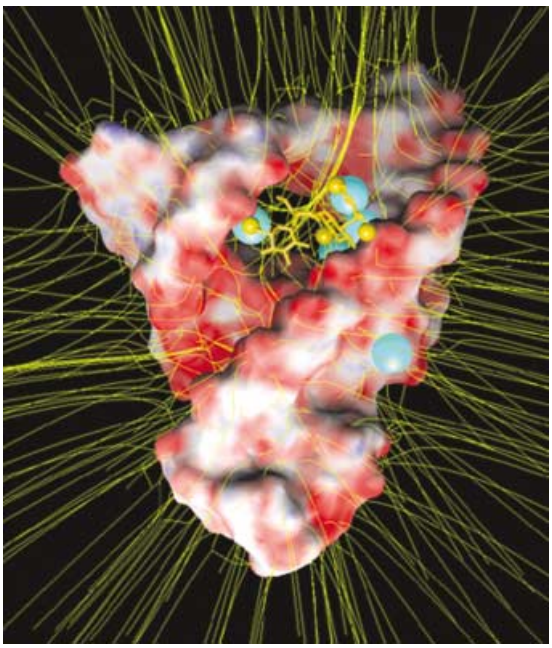


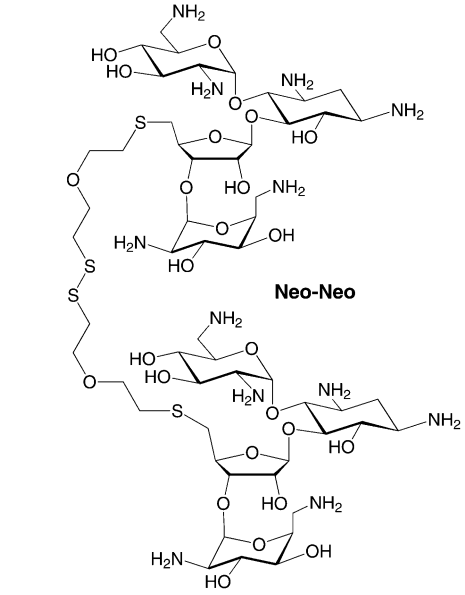
Figure 5. *Structural electrostatic complementarity. Shown is a model of the hammerhead – neomycin B complex obtained by docking a solution conformation of the aminoglycoside with the crystal structure of the ribozyme. Light blue spheres show the position of the Mg^{2+} ions in the crystal structure and yellow spheres depict the ammonium groups. A projection of charge densities (negative in red, positive in blue) and field lines (yellow), which illustrate the gradient of the electrostatic field, are also shown.[47]*

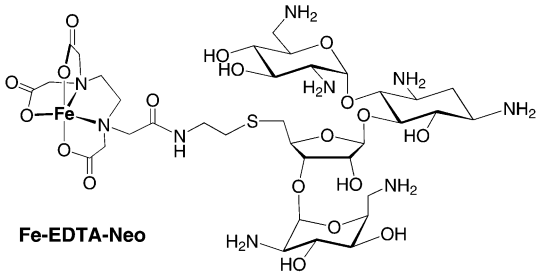
aminoglycoside antibiotics and their RNA hosts. This model, which deviates from the favorable "lock-and-key" recognition so common in biology, matched our experimental observations and showed the aminoglycosides in a new light.[47]

3.6 Aminoglycosides: Promiscuous RNA Binders

While the work described so far has dealt with the binding of aminoglycosides to the HH ribozyme, the recognition model evoking surface electrostatic complementarity has much broader implications.[48] Most importantly, it identifies aminoglycosides as potentially promiscuous RNA binders as a result of their remarkable ability to remodel themselves to fit the RNA fold. This prompted us to explore the binding of aminoglycosides to other RNA targets and to design dimeric aminoglycosides (for example, Neo – Neo) and aminoglycoside conjugates (for example, Fe-EDTA-Neo) to probe the existence of multiple binding sites on common RNA targets.[49, 50]

Since 10 – 15 % of the total cellular RNA is present as soluble transfer RNA (tRNA) molecules, this family represents the most abundant RNA competitor inside the cell. We therefore examined the binding of aminoglycoside antibiotics to $tRNA^{Phe}$, one of the best characterized tRNAs.[51] By using thermal denaturation studies, inhibition of Pb^{2+}-mediated cleavage, gel-mobility shift assays, fluorescence spectroscopy, as well as chemical and enzymatic footprinting we have established that aminoglycosides do interact with yeast $tRNA^{Phe}$. We identified putative





binding sites on tRNA^{Phe} and concluded that these antibiotics are likely to induce a conformational change in the biomolecule.[52] Interestingly, a crystal structure determined several years following our work confirmed our observations and revealed a binding site for neomycin on tRNA^{Phe}.[53]

By studying novel dimeric aminoglycosides we demonstrated that RNA molecules are likely to have more than one binding site for aminoglycosides. By using footprinting and affinity cleaving techniques we were able to identify two putative aminoglycoside binding sites on the hammerhead ribozyme.[54] A thorough biophysical study of the interactions between natural and synthetic aminoglycoside derivatives with a truncated tetrahymena ribozyme (a 388-nucleotide-long catalytically active RNA molecule) has revealed the presence of multiple high-affinity binding sites.[55]

Taken together, these studies have taught us that the promiscuity of the aminoglycosides transpires at different levels. These antibiotics can bind numerous RNA targets with similar, albeit modest, affinity. Additionally, multiple binding sites on RNA molecules are likely to coexist. These selectivity issues are particularly important where future therapeutic applications of RNA ligands are concerned. High concentrations of competing RNA targets might scavenge potential RNA binders thus altering their therapeutic window and causing adverse effects.

4. Beyond Aminoglycoside Antibiotics and Ribozymes

4.1 Targeting Viral RNA Sequences

While aminoglycoside antibiotics bind RNA preferentially over DNA and remain a useful lead for the design of new RNA binders, they suffer from two major shortcomings: moderate affinity and inadequate specificity. Their promiscuous binding to numerous RNA targets raises concerns regarding their potential utility. We therefore felt it was necessary to significantly expand the basic empirical knowledge base of RNA – ligand interactions. Effective ways for the rapid screening of small molecules for their RNA affinity and selectivity became a necessity. Additionally, our research program was ready for a switch from investigating "model" RNA molecules to targeting RNA sequences of potential therapeutic utility. Since the replication of various retroviruses requires an ordered pattern of viral gene expression, we felt that targeting viral RNA sites might lead to novel antiretroviral therapy.

4.2 The HIV-1 Replication Cycle

The successful replication of HIV is an elaborate process that involves the host's own cellular transcription and translation machineries. A small number of protein – RNA interactions that are unique to HIV provide intriguing targets for potential therapeutic intervention. To illustrate the key features, the lifecycle of the virus is schematically shown in Figure 6 and briefly summarized.[56, 57] Following cell adhesion, fusion, and partial loss of capsid, the viral single-stranded RNA is reverse-transcribed to the corresponding DNA and then integrated into the host chromosome. For the infected cell to produce new HIV particles, the viral DNA needs to be transcribed back into the RNA genome, and the newly synthesized RNA must be effectively exported from the nucleus into the cytoplasm. Transcription is rather ineffective during the early stages of replication and short viral RNA transcripts are formed. Additionally, longer RNA transcripts become highly spliced. These relatively short RNA sequences escape the nucleus and are responsible for the expression of a number of viral regulatory proteins, including Tat and Rev (Figure 6).[58] Tat translocates back into the nucleus and binds to an HIV RNA site termed TAR, which is located at the 5′ end of the nascent viral transcripts. This key event enhances the processivity of the polymerization reaction and significantly increases the formation of the full-length viral RNA.

HIV's late replication phase is initiated by the Rev protein and its binding to the HIV RNA site termed the Rev response element (RRE, Figure 7).[57, 59] The binding of Rev to a single high-affinity site on stem II B of the RRE is followed by association of additional Rev molecules on the entire RRE. This key protein – RNA aggregation facilitates the export of HIV RNA out of the host nucleus, while protecting it from the cell's splicing machinery (Figure 6). The presence of viral RNA in the cytoplasm is, of course, essential for the biosynthesis of the necessary viral proteins as well as for packaging the complete viral genome in

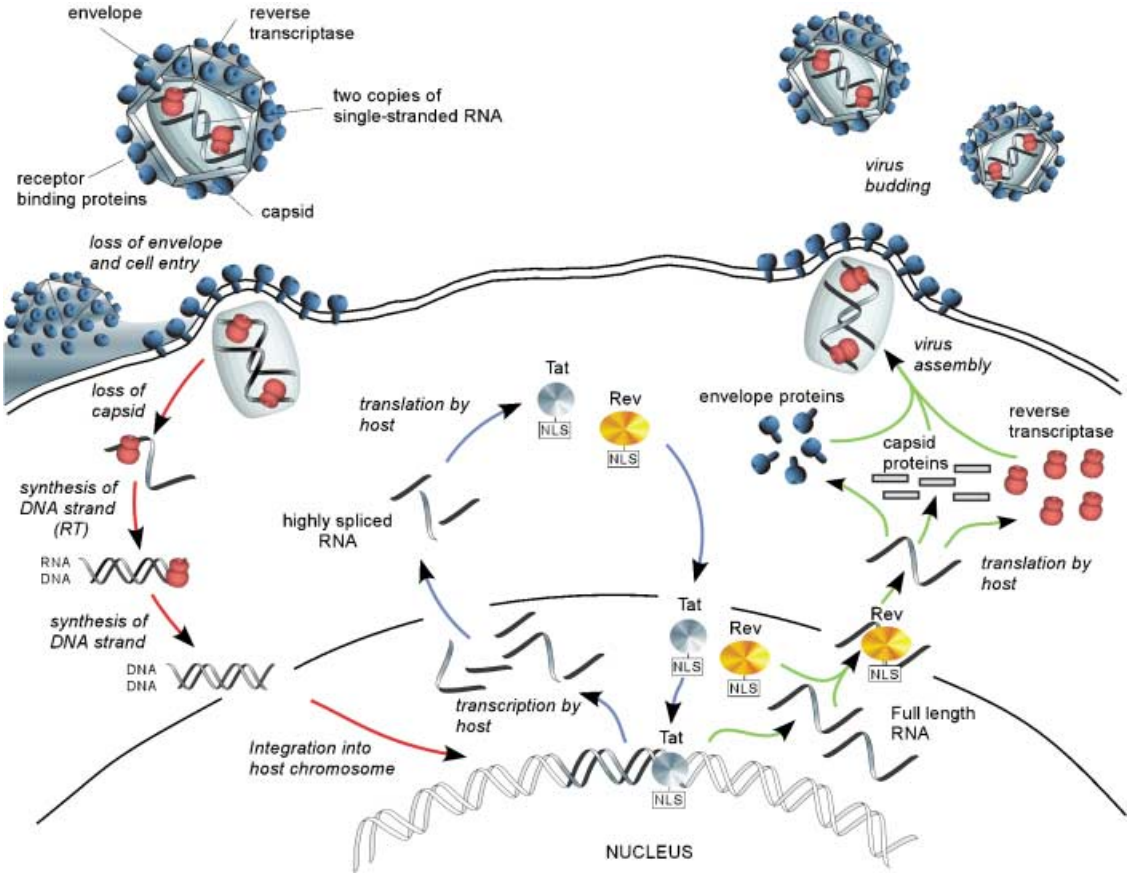


Figure 6. *The life cycle of HIV-1. Following adhesion, fusion, and partial loss of capsid, the viral single-stranded RNA genome is reverse-transcribed to the corresponding double-stranded DNA that is then integrated into the host chromosome (red arrows). In early stages of viral replication, transcription is rather ineffective and short viral RNA transcripts are formed. Additionally, longer RNA transcripts become highly spliced. These relatively short RNA sequences escape the nucleus and are responsible for the expression of a number of viral regulatory proteins, including Tat and Rev (blue arrows). Note that both Tat and Rev possess nuclear localization signals (NLS). Tat translocates into the nucleus and significantly increases the formation of the full-length viral RNA (blue arrows). HIV's late replication phase is initiated by the Rev protein and its binding to the RRE HIV (green arrows). This key protein – RNA recognition facilitates the export of HIV RNA out of the host nucleus, while protecting it from the cell's splicing machinery. The presence of viral RNA in the cytoplasm facilitates the biosynthesis of the necessary viral proteins as well as the packaging of the complete viral genomes in the new HIV particles (green arrows). (Figure courtesy of Dr. Weizman).*

the new viroids. Successful inhibition of Rev – RRE binding should therefore halt HIV replication and prevent the production of new viral particles. Such viral-specific RNA sequences represent attractive, yet under-utilized, targets for drug design. Since the RRE also serves as part of the coding sequence of the envelope protein, its dual function is particularly appealing since the development of drug-resistant variants may be prevented or impeded.

4.3 A Novel Solid-Phase Assembly for Identifying Potent and Selective RRE Ligands

To discover and analyze potent and specific Rev – RRE inhibitors, we developed a novel solid-phase RNA – protein assay.[60] It is comprised of a biotinylated RRE that binds to beaded streptavidin, and a fluorescently tagged Rev peptide (RevFl) that binds to its high affinity site on the RRE (Figure 8). Importantly, RevFl binds to the immobilized RRE with the same affinity as measured in solution by fluorescence anisotropy ($K_d \sim 3$ nM). Challenging the immobilized fluorescent Rev – RRE complex with potential inhibitors and determining the amount of RevFl displaced generates the necessary information regarding a ligand's affinity (Figure 8a).[61] The strengths of this assay are its robustness and reliable performance in the presence of large amounts of competing nucleic acids. Re-evaluating the activity of each inhibitor under such stringent conditions yields essential information regarding the ligand's selectivity (Figure 8b).[61]

As part of the assay's validation process, we demonstrated its capability to faithfully reproduce the known affinity and selectivity trends among aminoglycoside antibiotics.[60] We also illustrated the ability of this novel assembly to readily handle binders that interfere with common biophysical assays (such as fluorescence anisotropy). We have also established the potential of the assay to assist in the discovery of new inhibitors of Rev – RRE binding. For example, backbone-cyclized peptides designed to mimic the Arg-rich motif of the HIV-1 Tat protein have been found to be potent RRE binders.[62] Eilatin-containing octahedral Ru^{II} complexes have also been identified by the assay to be

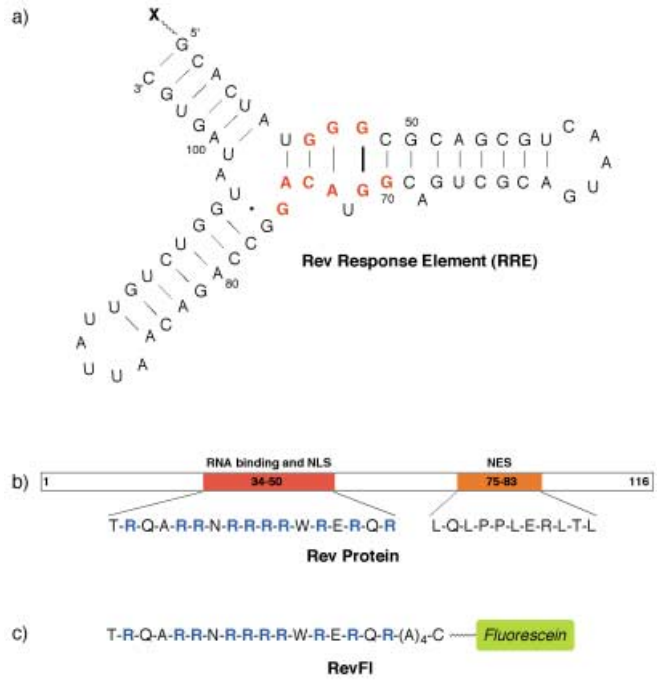


Figure 7. *a) The proposed secondary structure of the 66-nucleotide RRE core of HIV-1 (the actual RRE is larger than 300 nucleotides long). The red nucleotides represent the high affinity Rev recognition element. X represents either a phosphate group (for binding studies in solution) or a biotin – streptavidin linkage to insoluble beads for a solid-phase assay (see Figure 8). b) Schematic representation of the 116 amino acid Rev protein illustrating the relative location of the nuclear localization signal (NLS) and nuclear export signal (NES) domains. c) The fluorescently tagged RevFl utilized for evaluating Rev – RRE binding (see Figure 8).*

competitive inhibitors of Rev – RRE binding.[60] Experiments in cells infected with HIV-1 demonstrated the ability of eilatin-containing complexes to inhibit viral replication with IC_{50} values of about 1 μM.[63] $[Ru(bpy)_2$("pre-eilatin")$]^{2+}$, a complex containing a nonplanar analogue of eilatin, and other metal complexes lacking eilatin, display 10- to 100-fold lower anti-HIV activity. A clear correlation has been observed between affinity and selectivity to the RNA site of RRE with anti-HIV activity of the metal complexes,[63] although the actual in vivo target has not yet been identified.

4.4 RRE Binders with Improved Affinity and Selectivity

The NMR structure of the arginine-rich domain of Rev (Rev_{34-50}) bound to a minimalized RRE construct revealed purine – purine pairing (G48-G71 and G47-A73) and a bulged-out U72 residue (see Figure 7).[64] As large purine – purine surfaces and bulged-out bases may constitute favored intercalation sites, we postulated that appending an intercalator to aminoglycosides might lead to novel binders with higher RRE binding affinity. Indeed, potent RRE binders can be created by covalently linking an intercalator to aminoglycosides. A neomycin – acridine conjugate, trivially named neo-S-acridine, has an apparent K_i value of 3 nM, which is approximately the same affinity as that of the Rev peptide.[65] This conjugate is one of the strongest competitive inhibitors of Rev – RRE binding reported to date. Such conjugates demonstrate that: a) small molecules can effectively interfere with protein – RNA interactions, b) synthetic ligands can achieve very high RNA affinity, approaching that of the natural RNA-binding domains on proteins, and c) the combination of different binding modes (for example, ionic and intercalation) is a powerful approach for enhancing the RNA affinity of synthetic binders.

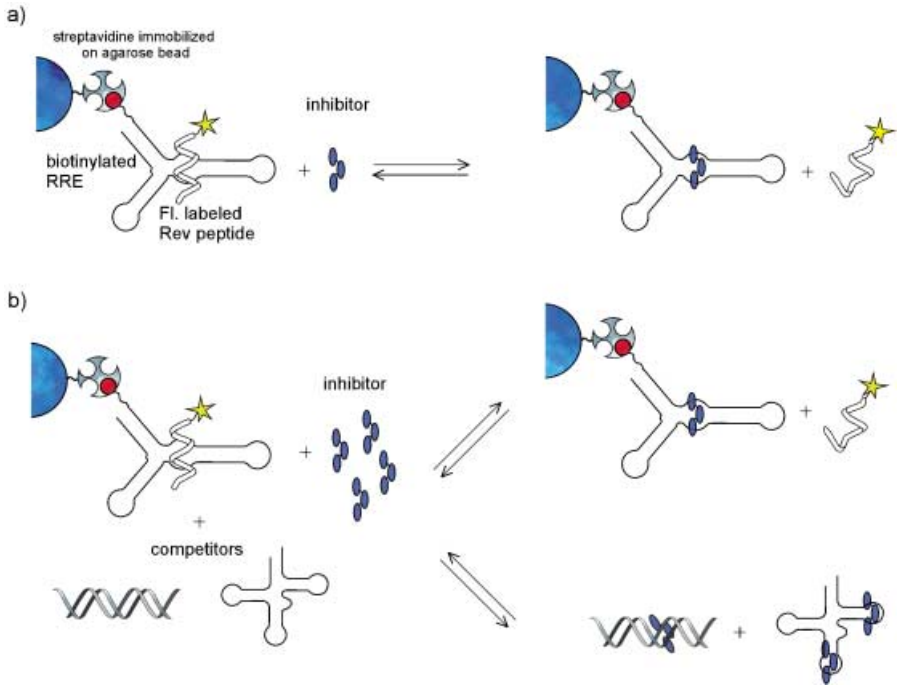
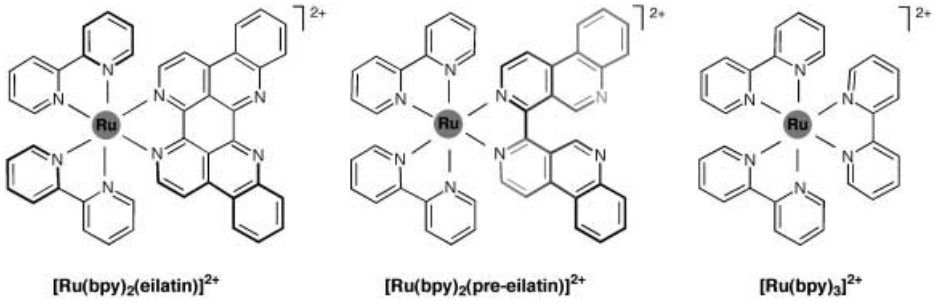
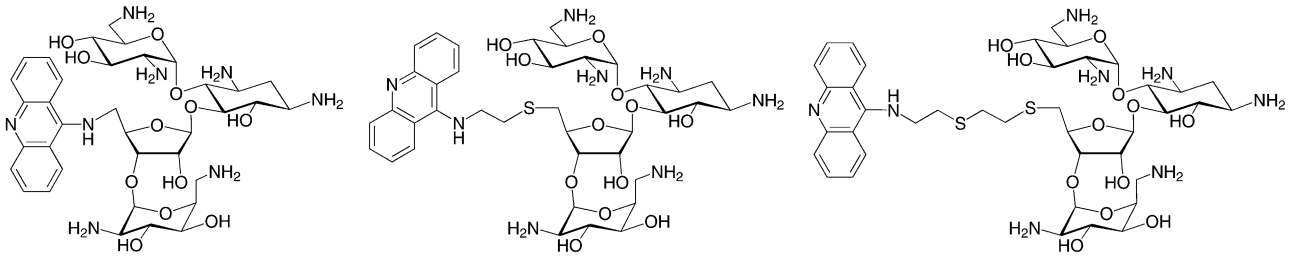


Figure 8. *A novel solid-phase assay for the discovery and study of Rev – RRE inhibitors.[60] a) "Affinity assay": ligands that compete with RevFl for RRE binding displace RevFl into solution. The amount of RevFl in solution (or that remained bound to the solid support) is quantified. b) "Selectivity assay": challenging the mixture with potential competitors (such as tRNA or DNA) facilitates the assessment of ligand selectivity.*

Neo-S-acridine avidly binds tRNA and DNA and thus, as might be expected, displays poor RRE selectivity.[66] Neo-N-acridine, a neomycin – acridine conjugate with a shorter linker between the aminoglycoside and intercalator, has a much better RRE selectivity.[66] Compared to neomycin B, acridine conjugates based on tobramycin and kanamycin A have slightly lower RRE affinity, but improved RRE specificity. Modulating the selectivity of aminoglycoside – intercalator conjugates can therefore be accomplished by varying the length of the linker and the identity of the antibiotic.[66]

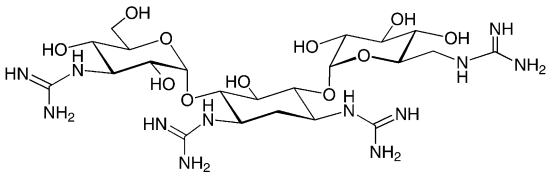
While slight structural alterations can, in certain cases, improve RRE selectivity, a better RNA-selective scaffold has become a necessity. Inspiration came from nature. Highly basic arginine-rich motifs are key to the recognition and binding of RNA by proteins.[67] Unlike ammonium

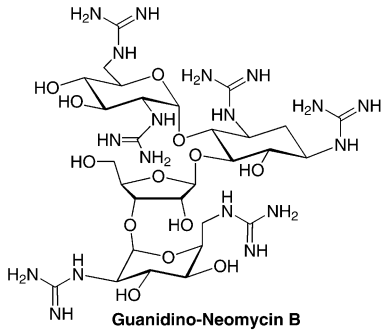




groups, guanidinium groups are highly basic and planar, and are capable of stacking as well as directed H-bonding interactions. We hypothesized that RNA affinity and selectivity of aminoglycoside-based ligands could be enhanced by replacing ammonium with guanidinium groups. A new family of RNA ligands, coined "guanidinoglycosides", in which all the ammonium groups of the natural aminoglycoside antibiotics were converted into guanidinium groups, was then synthesized.[68, 69]

A substantial increase in RRE affinity was observed for all the synthesized glycosides upon guanidinylation.[68] In general, RNA specificity was significantly improved although it is dependent on the total number of basic groups.[68] For example, guanidinylation of paromomycin (five basic groups) increases its selectivity for the RRE, while guanidinylation of neomycin (six basic groups) diminishes its target selectivity. This observation suggests that increasing the affinity above a certain threshold may become detrimental to the ligand's RRE selectivity.[66] Interestingly, guanidinylation does not change the specificity of the glycosides for RNA over DNA.[68] This result implies that structural features, rather than the identity of the basic group, are the dominant factors in governing the preferential binding of aminoglycosides to RNA.





A comparison of the RRE affinity of aminoglycosides to the affinity exhibited by their guanidinylated analogues is enlightening.[68] For example, kanamycin B displays a lower RRE affinity compared to tobramycin (its 3′-deoxy derivative) although the two antibiotics differ only by a single hydroxy group (see Section 3.3). This finding is consistent with the ability of hydroxy groups to lower the basicity of their neighboring amines, thus, conferring a lower overall charge upon kanamycin B. In contrast, the difference between kanamycin B and tobramycin is abolished upon guanidinylation.[68] Since the pK_a values of guanidinium groups are substantially higher and far less plastic than those of the ammonium groups found in aminoglycosides, the neighboring hydroxy groups have minimal effect on the overall charge possessed by the guanidinoglycosides at relevant pH values.

Guanidinoglycosides may have potential applications as new antiviral agents. Preliminary tests in HIV-infected cells have demonstrated that guanidinoglycosides inhibit HIV replication 100-fold more effectively than their parent aminoglycosides.[69] The antiviral activity of the guanidinylated derivatives may therefore be related to their ability to interfere with the formation of the essential Rev – RRE complex and viral replication, however, this has not yet been verified. The higher antiviral activity of the guanidinoglycosides may also be the result of better cellular uptake and localization. Fluorescence-activated cell sorting (FACS) and microscopy experiments show substantial cellular uptake of labeled guanidinoglycosides but not of the corresponding labeled aminoglycosides.[70] Interestingly, com-

petition experiments between fluorescently tagged poly-Arg peptides and guanidinoglycosides suggest a common pathway responsible for the uptake of both families of guanidinium-rich compounds.[70]

5. Summary and Outlook

The well-being of every cell is dependent upon carefully regulated cellular processes. Proteins, the workhorse functional molecules of the cell, efficiently execute most of these life-maintaining events. Not unexpectedly, medicinal chemists have traditionally targeted these biomolecules, typically with small organic molecules capable of modulating their function.[71] As our understanding of the multifaceted behavior of macromolecular communities within the cell advances, other biomolecules are emerging as feasible therapeutic targets. Among them, RNA is assuming a central place.

RNA molecules play key roles in numerous biological processes. Far from being a passive carrier of hereditary information, the activity of RNA spans a broad spectrum, from regulating gene expression to protein synthesis. In addition, RNA serves as a primary genome of many pathogenic viruses. Small molecules that specifically bind unique RNA sites and prevent the formation of key RNA – protein complexes can modulate cell functions and are likely to be of therapeutic potential.[72] RNA is, however, a very dynamic and challenging target. Much remains to be revealed as RNA slowly gains acceptance as a valid therapeutic objective. Future advances in our understanding of RNA folding, structure, and recognition as well as better experimental and theoretical tools will assist our progress towards deciphering the recognition of RNA by small molecules. There is little doubt that, as RNA continues to reveal more of its mysteries, new targets of therapeutic potential will emerge.

Aminoglycoside antibiotics have served the RNA community initially as tools for studying the intricacies of ribosomal function,[73, 74] and in more recent years as "generic" RNA binders.[30] Systematic synthetic modifications have taught us a great deal about the nature of their interactions with RNA. Such modifications have also illustrated how the affinity and selectivity of ligands toward selected targets, some of pharmaceutical importance, can be tuned. Future use of rapid screening assays to analyze modified aminoglycosides, aminoglycoside mimetics, as well as new natural and diverse "unnatural" compounds, will continue to clarify the factors that govern RNA – ligand interactions.

This minireview was written following the first scientific meeting solely dedicated to RNA targeting.[75] In looking to the future, it is intriguing to look back at previous conferences in related fields. More than 35 years ago, Gale, in summarizing the 16th Symposium of the Society for General Microbiology at the Royal Institution in London, stated: "Antibiotics are selective agents provided by nature for our enlightenment and we can hope, from the study of their actions, not only to improve our knowledge of the application of known principles, but also *to find new principles*."[76] Gale's prophecy has materialized.

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Keywords: aminoglycosides · antibiotics · HIV · RNA · RNA recognition

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