Inhibition of bacterial RNase P by aminoglycoside–arginine conjugates

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Abstract The potential of RNAs and RNA–protein (RNP) complexes as drug targets is currently being explored in various investigations. For example, a hexa-arginine derivative of neomycin (NeoR) and a tri-arginine derivative of gentamicin (R3G) were recently shown to disrupt essential RNP interactions between the *trans*-activator protein (Tat) and the Tat-responsive RNA (*trans*-activating region) in the human immunodeficiency virus (HIV) and also inhibit HIV replication in cell culture. Based on certain structural similarities, we postulated that NeoR and R3G might also be effective in disrupting RNP interactions and thereby inhibiting bacterial RNase P, an essential RNP complex involved in tRNA maturation. Our results indicate that indeed both NeoR and R3G inhibit RNase P activity from evolutionarily divergent pathogenic bacteria and do so more effectively than they inhibit partially purified human RNase P activity.

Key words: Aminoglycoside–arginine conjugate; RNase P; Antibacterial compound

1. Introduction

The recent increase in drug-resistant strains and consequent pathogenic bacterial infections lends urgency to the design of novel antibacterial compounds [1]. RNAs and RNA–protein (RNP) complexes are appealing targets for new drugs because of their diverse functional roles in various cellular processes [2]. While the choice of new targets will be primarily influenced by the objective to effectively disrupt an essential pathway in bacteria, specificity aspects are also major considerations since differences between prokaryotic and eukaryotic macromolecules (or their assemblages) are critical determinants in the design of a successful antibacterial compound. Indeed, this is borne out by the currently used drugs that specifically target bacterial cell wall biosynthesis, protein synthesis, or DNA replication and repair [1]. In the search for new targets, bacterial RNase P, an endonuclease, merits consideration since it is both essential and structurally distinct from its eukaryotic counterpart [3–5].

RNase P is a ubiquitous and essential enzyme responsible for processing the 5′ termini of precursor tRNAs (ptRNAs) and some other cellular RNAs (e.g. p4.5S RNA) involved in protein biosynthesis [3–5]. The bacterial RNase P holoenzyme is composed of a catalytic RNA moiety (~350–400 nucleotides) and a protein cofactor (~110–150 amino acid residues). Although the RNA subunit can catalyze the ptRNA-processing reaction in vitro under non-physiological conditions [6], the protein subunit, probably due to its role in substrate binding and catalysis, is absolutely required for RNase P activity in vivo [7,8]. Therefore, inhibition of bacterial RNase P activity could be accomplished with a compound that either interacts directly with the catalytic core in the RNA subunit or disrupts RNP interactions essential for assembly of a functional RNase P holoenzyme.

Aminoglycosides (e.g. neomycin, kanamycin) are small, polycationic molecules which possess a linked ring system consisting of aminosugars and an aminocyclitol [9]. These compounds have found clinical use as antibacterial agents owing to their ability to bind specifically to prokaryotic rRNA and inhibit bacterial protein synthesis [10]. Recently, it has become evident that aminoglycosides as well as their derivatives bind mRNAs [11], tRNAs [12], viral RNAs [13,14] and catalytic RNAs [15,16], and that the binding of these ligands could alter the function of the target RNA. For example, neomycin B is a potent inhibitor of ribozymes such as RNase P and the self-splicing group I intron likely due to its ability to interfere with the binding of divalent metal ions essential for RNA catalysis [15,16]. In this study, we have examined if modified aminoglycosides (such as the arginine derivatives of aminoglycosides, NeoR and R3G; Fig. 1) can inhibit the activity of RNase P derived from certain pathogenic bacteria and also discriminate between bacterial and human RNase P.

2. Materials and methods

2.1. Reagents

The procedures for synthesis of NeoR and R3G are described elsewhere [17–20].

All oligonucleotides used for PCR were synthesized at the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine, New Haven, CT, USA. The 18-mer poly(A) oligoribonucleotide was synthesized by Dharmacon Research, Inc., Boulder, CO, USA. Restriction and modifying enzymes were obtained from either New England Biolabs, Beverly, MA, USA or Gibco Life Technologies, Rockville, MD, USA. T7 RNA polymerase and RNasin were purchased from Promega, Madison, WI, USA. HiTrap (cation exchange and metal-chelating) columns and [α-^{32}P]GTP were obtained from Amersham Pharmacia Biotech, Piscataway, NJ, USA. All other reagents used were purchased from either Sigma-Aldrich, St. Louis, MO, USA or Fisher Biotech, Pittsburgh, PA, USA.

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2.2. Preparation of enzyme and substrate

By using the appropriate primers and genomic DNA as the template for PCR-based amplification, we obtained the genes encoding the RNA and protein subunits of *Neisseria gonorrhoeae*, *Porphyromonas gingivalis*, and *Streptococcus pneumoniae* RNase P. The genes encoding the RNA subunits, under the control of a T7 RNA polymerase promoter, were subcloned into pUC19. The resulting clones were used for T7 RNA polymerase-mediated run-off in vitro transcription to generate the respective RNase P RNAs, which were then purified using a Quick Spin column procedure [21]. The genes encoding the protein subunits of *N. gonorrhoeae*, *P. gingivalis*, and *S. pneumoniae* RNase P were subcloned into either pCRT7TOPO or pBAD (Invitrogen, Carlsbad, CA, USA), two different expression vectors. Subsequently, these proteins were overexpressed in *Escherichia coli* as His_6-tagged chimeras and purified to homogeneity using a combination of cation exchange and immobilized metal affinity chromatography.

All the above mentioned clones were verified by DNA sequencing and the molecular masses of the various purified proteins were confirmed by electrospray ionization mass spectrometry (data not shown). The cloning, overexpression and purification of these bacterial RNase P RNA and protein subunits will be described elsewhere (Jovanovic, M. and Gopalan, V., unpublished data); however, if required, these details will be made available immediately upon request.

The RNA and protein subunits of *E. coli* RNase P were purified according to established procedures [21,22].

Partially purified human RNase P was a gift from Drs. Taijiao Jiang and Sidney Altman, Yale University, and prepared as described elsewhere [23].

The substrate $ptRNA^{Tyr}su3+$ was used for all the inhibition studies described here and was prepared by in vitro transcription of *Fok*I-digested pUC19TyrT [21].

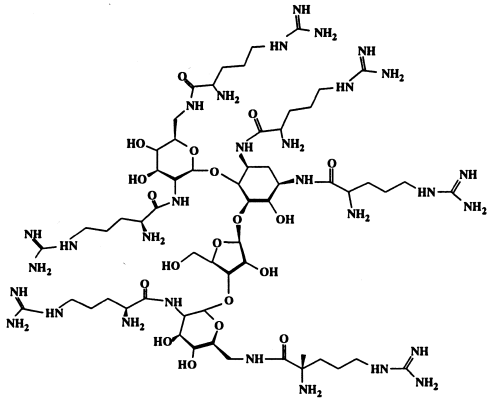
2.3. RNase P assays

RNase P activity was measured at 37°C in the presence or absence of a defined amount of inhibitors in 50 mM Tris–HCl, pH 7.2, 5% (w/v) polyethylene glycol 8000, 1 mM NH_4Cl, 10 mM spermidine, 10 mM $MgCl_2$, 100 nM $ptRNA^{Tyr}su3+$ [15,21,22]. All enzyme assays were performed under multiple-turnover conditions (for example, 100 nM $ptRNA^{Tyr}su3+$ and 0.5 nM *E. coli* RNase P holoenzyme). The inhibitor was added subsequent to holoenzyme assembly and allowed to pre-incubate at 37°C for 5 min prior to addition of the ^{32}P-labeled $ptRNA^{Tyr}su3+$ substrate. After specified incubation periods, the reactions were terminated with quenching dye (7 M urea, 10 mM EDTA, 10% (v/v) phenol, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue) and the products were separated on an 8% polyacrylamide/7 M urea gel. The extent of substrate cleaved was quantitated using a PhosphorImager (Molecular Dynamics) and ImageQuant software. The initial velocity values reported are the averages obtained from two independent experiments and were calculated by measuring the extent of cleavage at four or five different time points in reactions in which $<30\%$ of the substrate was cleaved during the assay. The IC_{50} values (i.e. the concentration of inhibitor required to reduce by 50% the enzymatic activity observed in the absence of the inhibitor) were estimated by plotting cleavage efficiency as a function of inhibitor concentration.

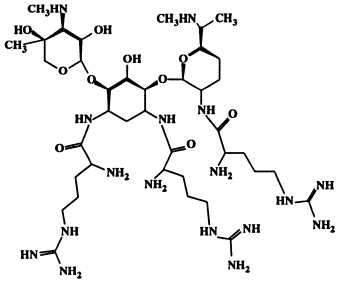
3. Results and discussion

3.1. Rationale

The affinity of aminoglycosides for RNA is attributable in part to their protonated amino groups engaging in electrostatic interactions with the polyanionic target RNAs [24–26]. Structural complementarity between the aminoglycosides and the target RNAs also results in specific hydrogen bonds and van der Waals contacts [24,25]. However, since aminoglycosides bind various unrelated cellular RNAs, their promise as RNA structure-specific drugs will materialize only if the dual criteria of selectivity and high affinity are fulfilled. Towards this goal, various aminoglycoside derivatives displaying structure-specific determinants have been synthesized and tested in Tat–*trans*-activating region (TAR) and Rev–RNA recognition element (RRE), two RNP complexes that are essential for human immunodeficiency virus (HIV) replication [27]. While Tat protein upon binding to its TAR facilitates transcription of the HIV genome, the Rev protein through interactions with its cognate viral RRE aids in nucleocytoplasmic transport and cytoplasmic accumulation of the viral RNAs [27].



NeoR



R3G

Fig. 1. Structures of NeoR and R3G, two different AACs.

Aminoglycosides, with varying efficacy, can competitively block the binding of Tat to TAR and Rev to RRE [13,14,24,25]. Based on this finding and the observation that both Tat and Rev contain arginine-rich RNA-binding domains, a new class of peptidomimetic TAR and Rev RNA binders have been constructed by conjugating arginines or guanidinium moieties to aminoglycosides [17–20,28]. Compared to their unmodified precursors, the aminoglycoside–arginine conjugates (AACs; e.g. NeoR, a hexa-arginine derivative of neomycin; R4K, a tetra-arginine derivative of kanamycin A) display significantly higher and selective affinity to TAR and RRE RNAs. For example, while 100–1000 μM neomycin B is required to non-competitively inhibit the assembly of Tat–TAR complex, the same is accomplished using 150 nM NeoR; under identical experimental conditions, the K_d value for assembly of the Tat–TAR complex is $\sim$50 nM [20]. Similarly, NeoR was 100-fold more effective than neomycin B in displacing the Rev-derived peptide from the Rev–RRE complex (K_d values of 18 nM and 1.8 μM, respectively; Litovchick and Lapidot, unpublished data). NeoR and R4K also caused pronounced inhibition of equine infectious anemia

1. *E. coli*	59	A	H	E	**R**	N	**R**	I	K	**R**	L	T	**R**	E	S	F	**R**	L R	76
2. *C. burnetti*	58	A	V	W	**R**	N	**R**	V	R	**R**	V	V	K	E	A	F	**R**	I R	75
3. *N. gonorrhoeae*	61	A	N	E	**R**	N	Y	M	K	**R**	V	I	**R**	D	W	F	**R**	L N	78
4. *S. bikiniensis*	63	A	V	V	**R**	N	Q	V	K	**R**	R	L	**R**	H	L	V	C	D R	80
5. *B. subtilis*	57	A	V	M	**R**	N	**R**	I	K	**R**	L	I	**R**	Q	A	F	L	E E	74
6. *Synechocystis*	57	A	T	V	**R**	N	**R**	L	K	**R**	Q	I	**R**	A	V	I	N	H F	74
7. *B. burgdorferi*	56	S	V	K	**R**	N	**R**	I	R	**R**	L	F	K	E	A	F	**R**	K R	73
8. *C. pneumoniae*	59	A	H	E	**R**	N	S	F	K	**R**	V	V	**R**	E	V	F	**R**	H V	76
9. *T. maritima*	57	A	T	R	**R**	N	K	L	K	**R**	W	V	**R**	E	I	F	**R**	R N	74
10. *P. gingivalis*	65	A	V	K	**R**	N	**R**	V	K	**R**	L	V	**R**	E	A	Y	**R**	L N	82
11. *D. radiodurans*	55	A	V	K	**R**	N	**R**	A	R	**R**	R	V	**R**	E	A	L	**R**	T M	72
12. *C. tepidum*	48	A	V	D	**R**	N	**R**	I	K	**R**	L	M	**R**	E	A	Y	**R**	L E	65

Fig. 2. Partial sequence alignment of the protein subunit of RNase P from various bacteria to illustrate the conservation of an arginine-rich motif ([22,30]; Jovanovic, M., Braun, E. and Gopalan, V., unpublished observations). The sequences are from *E. coli*, *Coxiella burnetii*, *N. gonorrhoeae*, *Streptomyces bikiniensis*, *Bacillus subtilis*, *Synechocystis* sp. PCC6803, *Borrelia burgdorferi*, *Chlamydia pneumoniae*, *Thermotoga maritima*, *P. gingivalis*, *Deinococcus radiodurans* and *Chlorobium tepidum*.

virus (EIAV) and HIV-1 replication in cell culture, most likely due to their ability to disrupt RNP complexes vital for viral replication [18–20,29]. In contrast to R4K, the conjugate of kanamycin A and γ-guanidino butyric acid (GB4K) binds with very low affinity to TAR RNA and does not inhibit replication of either EIAV or HIV-1 in cell culture [18,20,29]. Therefore, the guanidinium moieties of the arginine side chains are clearly not solely responsible for the vast improvement in the inhibitory potential of AACs compared to their unmodified precursors.

This study on RNase P was prompted by two specific observations in addition to the results cited above. First, several aminoglycosides were recently demonstrated to interact with the RNA subunit of *E. coli* RNase P in vitro and interfere with its ptRNA-processing activity [15]. Of the various compounds tested, neomycin B was determined to be the most potent inhibitor with K_i values of 35 μM and 60 μM for the reactions catalyzed by the RNA subunit and the RNase P holoenzyme, respectively [15]. Second, a sequence alignment of the protein subunit of RNase P from various bacteria reveals an arginine-rich consensus (the 'RNR motif') which is likely to be part of the RNA-binding domain in the RNase P protein cofactor (Fig. 2; [8,22,30]). These findings led us to speculate that AACs (like NeoR and R3G) might be effective inhibitors of bacterial RNase P.

3.2. Inhibition of bacterial and human RNase P by NeoR and R3G

Subsequent to purification of the RNA and protein subunits of RNase P from *E. coli*, *N. gonorrhoeae*, *P. gingivalis* and *S. pneumoniae*, we reconstituted each RNase P holoenzyme in vitro and tested the ability of NeoR and R3G to inhibit the respective RNase P activity. While there is nearly complete inhibition of *E. coli*, *N. gonorrhoeae* and *S. pneumoniae* RNase P activity when either 500 nM NeoR or 1500 nM R3G is included in the assay mixture, these compounds were slightly less effective against *P. gingivalis* RNase P (Fig. 3).

We assayed *E. coli* RNase P activity in the presence of increasing concentrations of either NeoR or R3G to determine their respective IC_{50} values. For these analyses, the initial velocities were calculated at various concentrations of the two inhibitors. NeoR and R3G inhibit *E. coli* RNase P activity with IC_{50} values of ~125 nM and 300 nM, respectively (Fig. 4). Our preliminary screens indicate that the IC_{50} values

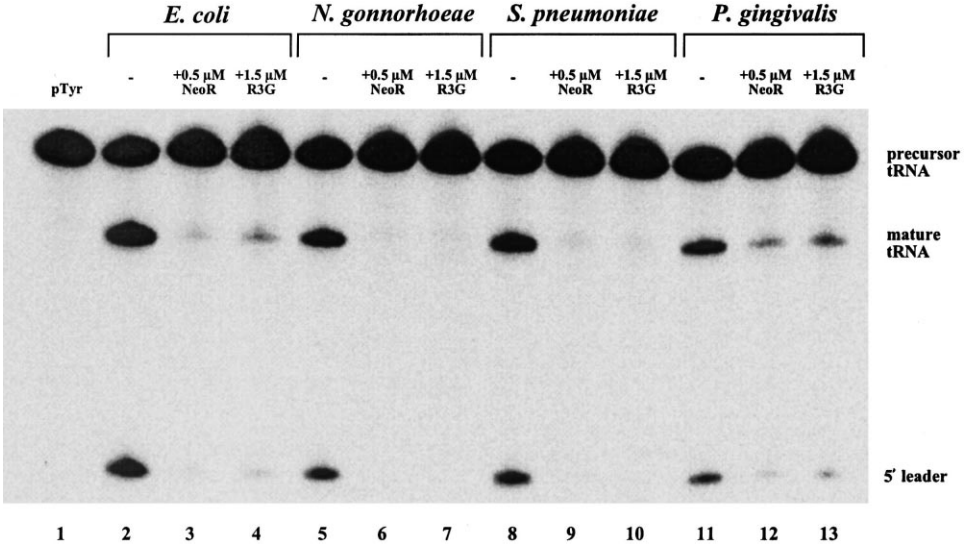


Fig. 3. Inhibition of bacterial RNase P activity by NeoR and R3G. The source of bacterial RNase P and the concentration of inhibitors in the assay are indicated. The lanes labeled 'pTyr' and '−' indicate substrate (without any inhibitor) incubated in the absence and presence of bacterial RNase P, respectively.

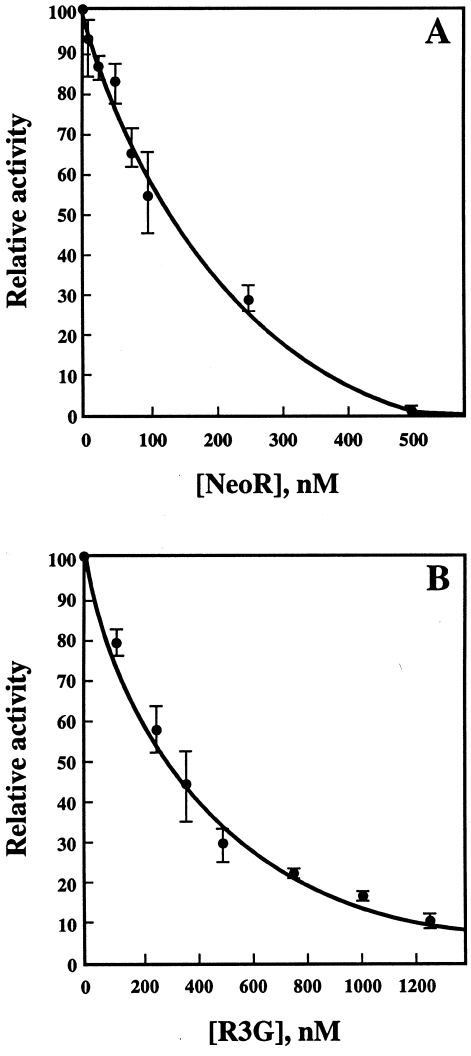


Fig. 4. Determination of IC_{50} values for inhibition of *E. coli* RNase P activity by NeoR and R3G. The activity observed in the presence of varying concentrations of either NeoR or R3G is presented as relative activity compared to that observed with the holoenzyme in the absence of the inhibitor, which is normalized as 100%. The averages from at least two independent experiments were used to obtain the data depicted in this figure.

for NeoR- and R3G-mediated inhibition of different bacterial RNase P activities are also likely to be in the sub-micromolar concentration range (Fig. 3; data not shown).

Although RNase P functions as an RNP complex in all living organisms, there is significant variation in its structure and composition. Compared to the simple composition of the bacterial RNase P holoenzyme (one RNA and one protein subunit), there is a marked increase in the complexity of human nuclear RNase P [3]. In addition to a 340-nucleotide long RNA subunit, at least eight protein subunits ranging in size from 14 to 115 kDa have been found associated with human RNase P [3]. Intriguingly, none of these human RNase P protein subunits possesses the conserved 'RNR motif' found in the bacterial RNase P protein subunit. Moreover, the eukaryotic RNase P RNAs are catalytically inactive in vitro unlike their bacterial counterparts. Despite these differences, it is important to determine if the activity of human RNase P is affected by compounds which inhibit bacterial RNase P activity and could serve as potential antibacterial leads. Although human RNase P activity was largely unaffected at concentrations of NeoR and R3G that are 10-fold greater than the IC_{50} values of NeoR and R3G for *E. coli* RNase P, there was nearly complete inhibition at 7.5 μM NeoR and 20 μM R3G (Fig. 5). Based on our preliminary analysis we expect that the IC_{50} values of NeoR and R3G for human RNase P will be at least 10-fold greater than those calculated for *E. coli* RNase P (data not shown). These inferences are subject to the caveat that only a partially purified human RNase P was used in these experiments.

The polybasic nature of the AACs (Fig. 1) raises the possibility that the inhibition of RNase P by NeoR and R3G could stem from their ability to bind the ptRNA substrate and sequester it from cleavage by RNase P. The following experiment was performed to address this postulate. By including an 18-mer poly(A) RNA in the RNase P assay, we sought to provide a competitor RNA that could bind NeoR or R3G through non-specific, electrostatic interactions and thus reduce their availability for similar interactions with the ptRNA substrate. Our results indicate that 1 μM poly(A) RNA, i.e. a 10-fold molar excess over the ptRNA concentration in the assay, failed to diminish the ability of NeoR or R3G to inhibit *E. coli* RNase P activity (Fig. 6). This is also consistent with our finding that the inhibitory potential of NeoR and R3G does vary based on the source of enzyme even when the same ptRNA substrate and identical assay conditions are employed for the inhibition studies (Figs. 3 and 5). Lastly, the documented observation that a 10-fold excess of tRNA has no effect on the ability of R3G to disrupt the RNP complex formed between HIV TAR and a Tat-derived peptide [20] indicates that the AACs have only a weak affinity to tRNAs. These results cumulatively indicate that the mechanism of inhibition of bacterial RNase P by NeoR and R3G is not due to their direct sequestration of the ptRNA substrate by non-specific interactions.

A few other findings warrant mention since they relate to the mechanism of inhibition of RNase P by NeoR and R3G. First, 1 mM L-arginine does not inhibit *E. coli* RNase P (Fig. 6). Therefore, the aminoglycoside backbone to which the arginine residues are conjugated in NeoR and R3G must contribute significantly to their inhibitory potential. Second, the inhibition experiments with NeoR and R3G were performed by adding the inhibitors subsequent to assembly of the respective bacterial RNase P holoenzymes. However, if the order of addition was altered such that the inhibitor was added to the RNA subunit prior to inclusion of the protein cofactor in the assay reaction, there was no change in the inhibitory potential of NeoR and R3G (data not shown). Also, both NeoR and R3G inhibit the ptRNA-processing reaction catalyzed solely by the RNA subunit of *E. coli* RNase P in the absence of its protein cofactor (data not shown). Even though the mechanism of inhibition of the RNase P holoenzyme- and catalytic RNA-mediated reactions might be different, these results suggest that the mode of action of NeoR and R3G might involve their direct binding to the RNA subunit of RNase P. Lastly, because identical assay conditions were employed for the inhibition studies performed with bacterial and human RNase P, the difference in inhibitory potential of NeoR and R3G could presumably stem from structural variations in these two enzymes and the weaker binding affinities of human RNase P for NeoR and R3G compared to bacterial RNase P.

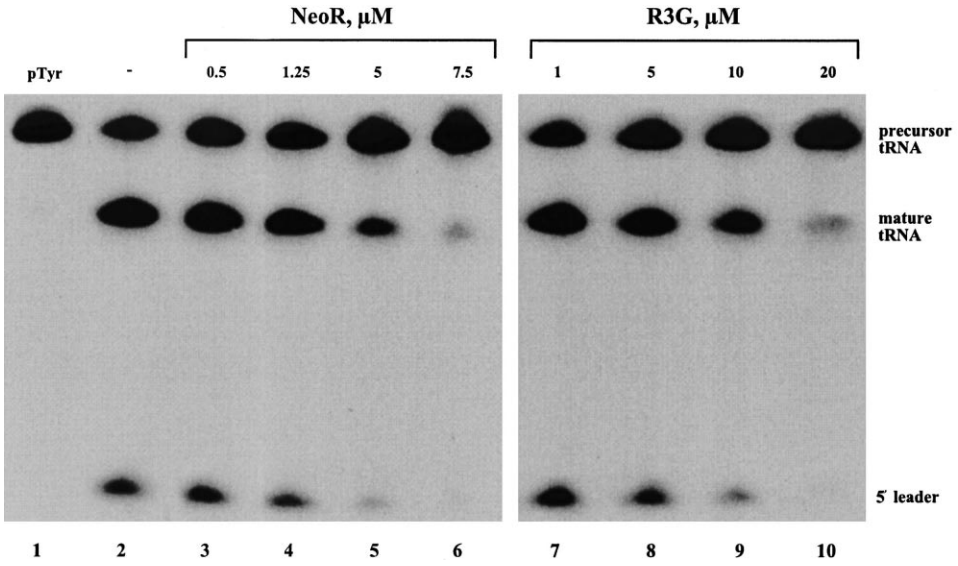


Fig. 5. Inhibition of human RNase P activity by NeoR and R3G. The concentrations of each inhibitor in the assay are indicated. The lanes labeled 'pTyr' and '—' indicate substrate (without any inhibitor) incubated in the absence and presence of human RNase P, respectively.

3.3. Conclusions

The findings that *E. coli* RNase P activity is inhibited with IC_{50} values of 60 μM and 125 nM by neomycin B and NeoR, respectively, suggest that the conjugation of arginine residues to neomycin B results in a nearly 500-fold more effective inhibitor of *E. coli* RNase P (Fig. 4; [15]). This finding together with the observation that *E. coli* RNase P activity is unaffected in the presence of even 1 mM L-arginine (Fig. 6) illustrates the premise that the affinity of a ligand for a target RNA can be enhanced synergistically by fusing two different functionalities capable of RNA-binding (i.e. aminoglycosides and arginine-rich peptides). Moreover, the finding that both NeoR and R3G are better inhibitors of bacterial RNase P compared to human RNase P activity (Figs. 4 and 5) validates the idea that bacterial RNase P-specific inhibitors could either be rationally designed or identified from a combinatorial library of small molecules.

3.4. Speculation

We are currently examining the possibilities that the mechanism of inhibition by NeoR and R3G involves either disruption of RNP interactions in the holoenzyme complex and/or displacement of essential metal ions in the RNA subunit of bacterial RNase P.

NeoR and R3G could serve as peptidomimetics and competitively block the protein cofactor of bacterial RNase P

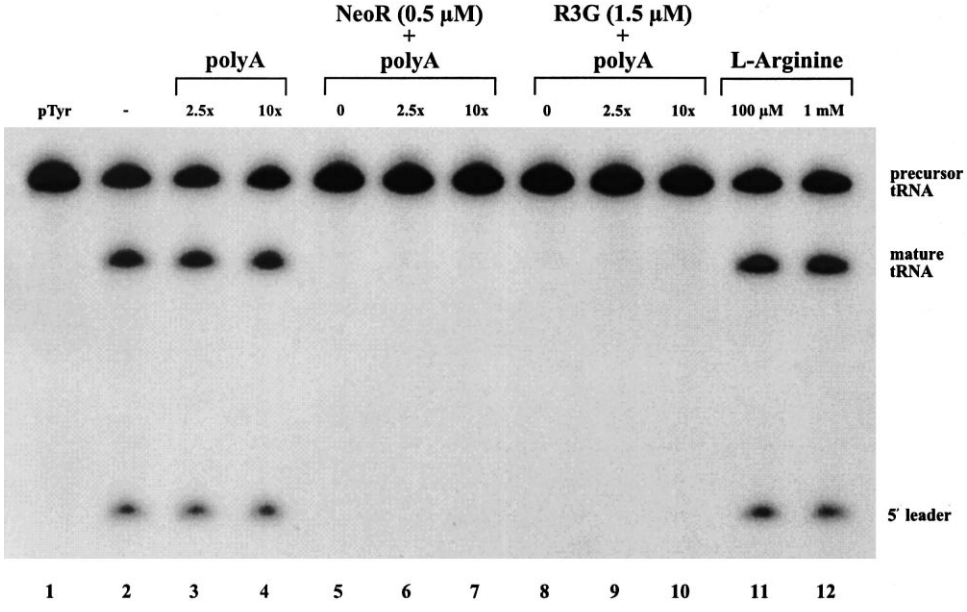


Fig. 6. Effect of poly(A) on the inhibition of *E. coli* RNase P activity by NeoR and R3G. The concentrations of RNase P holoenzyme and ptRNA substrate in the assay were 0.5 nM and 100 nM, respectively. The poly(A) oligoribonucleotide was included in the assay at either 2.5- or 10-fold molar excess over the ptRNA concentration either in the absence of inhibitor (lanes 3 and 4) or in the presence of NeoR or R3G (lanes 6, 7, 9 and 10). The lack of effect of L-arginine on *E. coli* RNase P activity is also depicted (lanes 11 and 12). The lanes labeled 'pTyr' and '—' indicate substrate (without any inhibitor) incubated in the absence and presence of *E. coli* RNase P, respectively.

from binding to the catalytic RNA subunit and thereby disrupt assembly of the RNase P holoenzyme. The proven ability of (i) neomycin B to occupy the Rev-binding site and thereby directly displace the Rev peptide from the Rev–RRE complex [13,14,31], and (ii) NeoR to displace a Tat-derived peptide from the Tat–TAR complex and a Rev-derived peptide from the Rev–RRE complex ([20]; Litovchick and Lapidot, unpublished data) serves as precedents for this mechanism. A variation on this theme is the idea that the binding of NeoR and R3G to the RNA subunit of bacterial RNase P somehow alters the conformation of the protein-binding site, and thereby results in weaker affinity for the protein cofactor and dissociation of the RNP complex. This type of an allosteric mechanism indeed underlies the ability of neomycin B to act as a non-competitive inhibitor capable of binding the Tat–TAR RNP complex and promoting dissociation of the Tat-derived peptide from the Tat–TAR complex [14,32].

An alternative mechanism for inhibition of RNase P by NeoR and R3G would be compatible with the proven ability of aminoglycosides and their derivatives to occupy metal-binding sites in RNAs. For instance, Mikkelsen et al. [15] performed Pb(II) cleavage experiments with the catalytic RNA subunit of *E. coli* RNase P in the absence and presence of aminoglycosides and demonstrated that the inhibition by compounds like neomycin B relies on their ability to interfere with the binding to the catalytic RNA moiety of divalent metal ions (Mg^{2+}) that are critical for the chemical cleavage step.

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