Inhibition of protein synthesis by aminoglycoside–arginine conjugates

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ABSTRACT

Inhibition of translation by small molecule ligands has proven to be a useful tool for understanding this complex cellular mechanism, as well as providing drugs of significant medical importance. Many small molecule ligands inhibit translation by binding to RNA or RNA/protein components of the ribosomal subunits and usurping their function. A class of peptidomimetics [aminoglycoside–arginine conjugates (AAC)] has recently been designed to inhibit HIV TAR/tat interaction and in experiments aimed at assessing the inhibitory effects of AACs on TAR-containing transcripts, we found that AACs are general inhibitors of translation. Experiments reported herein aim at characterizing these novel properties of AACs. We find that AACs are inhibitors of eukaryotic and prokaryotic translation and exert their effects by blocking peptide chain elongation. Structure/activity relationship studies suggest that inhibition of translation by AACs is directly related to the number of arginine groups present on the aminoglycoside backbone and to the nature of the core aminoglycoside. AACs are therefore attractive tools for understanding and probing ribosome function.

Keywords: aminoglycoside–arginine conjugates; peptidyl transferase inhibitor; ribosome; translation inhibition

INTRODUCTION

RNA contains complex and sophisticated higher order structures that are essential for recognition by other macromolecules and/or required for catalytic processes. As such, it offers an interesting target for small molecule ligands and, indeed, the ability of RNA to interact with small molecules has long been recognized. Antibiotics are a chemically and structurally diverse collection of molecules, with some classes capable of interacting with rRNA to exert profound effects on the translation process. Functional insights from structural studies of compounds bound to ribosomal subunits have revealed that rRNA/small molecule ligand recognition is based on a combination of shape recognition, electrostatic, and hydrogen-bonding interactions (Broder-

sen et al., 2000; Carter et al., 2000; Pioletti et al., 2001; Schlunzen et al., 2001). Additionally, RNA SELEX (Systematic Evolution of Ligands by Exponential Enrichment) has enabled the identification of minimal nucleic acid recognition motifs for ligand binding, demonstrating that RNA three-dimensional structures can form a large number of highly specific ligand-binding sites (Gold et al., 1995).

The ribosome is the target for many important antibacterial agents; these compounds interfere with essential steps of protein synthesis (Pestka, 1977; Vazquez, 1979; Gale et al., 1981; Noller, 1991). Among these, 2-deoxystreptamine aminoglycosides (small polycationic compounds possessing linked ring systems consisting of aminosugars and an aminocyclitol) cause codon misreading by interfering with the decoding process (Carter et al., 2000). These compounds have found clinical use as antibacterial agents due to their ability to specifically bind bacterial ribosomes (Gale et al., 1981) and are thought to exert their effects by increasing the error rate of the ribosome (Carter et al., 2000). Eukary-

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otic cytoplasmic ribosomes are relatively insensitive to 2-deoxystreptamine aminoglycosides (Kurtz, 1974; Palmer & Wilhelm, 1978; Wilhelm et al., 1978a, 1978b), and it has been suggested that the sensitivity of a ribosomal system to antibiotics is determined by the sequence of its rRNA (Sor & Fukuhara, 1984; Beckers et al., 1995). Indeed, the nephrotoxicity and ototoxicity associated with use of aminoglycosides in the clinical setting may be linked to the susceptibility of mitochondrial ribosomes to these compounds (Bottger et al., 2001).

Aminoglycosides are also capable of binding to and affecting the activity of a large number of other RNAs, including the HIV TAR element (Mei et al., 1997), HIV Rev-responsive element (Zapp et al., 1993), hammerhead ribozymes (Stage et al., 1995; Jenne et al., 2001), hairpin ribozymes (Earnshaw & Gait, 1998), ribonuclease P RNA (Mikkelsen et al., 1999), self-splicing of the T4 phage thymidylate synthase mRNA group I intron (von Ahlsen et al., 1991; Hoch et al., 1998), and the human hepatitis delta virus ribozyme (Rogers et al., 1996). In addition, aminoglycosides are capable of inhibiting aminoacylation of some tRNAs (Walter et al., 2002). The RNA binding abilities of aminoglycosides likely stem from their multiple positive charges that allow them to interact with the negatively charged RNA backbone, their ability to hydrogen bond, and their high flexibility, which accommodates binding to internal loops and bulges within RNA (for reviews, see Walter et al., 1999; Schroeder et al., 2000). In these settings, aminoglycosides may exert their effects by promoting conformational changes, preventing folding of RNA into active tertiary architecture, displacing catalytically active ions, and/or blocking important functional interactions.

Recently, aminoglycoside–arginine conjugates (AAC) have been synthesized as potential antiviral agents to capitalize on the RNA-binding ability of aminoglycosides and the specific binding of arginine to HIV TAR RNA (Tao & Frankel, 1992; Puglisi et al., 1992, 1993). The AACs bind to TAR RNA (Litovchick et al., 1999, 2000), and exhibit antiviral activity (Litovchick et al., 2001; Cabrera et al., 2002). They also antagonize some of the extracellular properties of Tat—such as increased viral production, induction of CXCR4 chemokine receptor expression, suppression of CD3-activated proliferation of lymphocytes, and up-regulation of the CD8 receptor (Litovchick et al., 2001). Additionally, a hexa-arginine derivative of neomycin (NeoR) and a tri-arginine derivative of gentamicin (R3G), have been reported to inhibit bacterial (and to a lesser extent mammalian) RNase P activity (Eubank et al., 2002).

In the current study, we report an additional property of AACs—the capacity to inhibit translation. Unlike their aminoglycoside precursors that show specificity for inhibiting prokaryotic translation, AACs inhibit both eukaryotic and prokaryotic translation with some selectivity

for the eukaryotic process. AACs inhibit translation in a number of eukaryotic translation systems, including wheat germ extracts, rabbit reticulocyte lysates, Krebs extracts, and in vivo in microinjected frog oocytes. The AACs do not affect translation initiation, but rather inhibit peptidyl transferase activity. The extent of inhibition appears related to the number of arginine groups present on the aminoglycoside, as well as on the nature of the core aminoglycoside. These results identify a novel activity for AACs and indicate that they represent a new class of eukaryotic translation inhibitors that may be useful tools for better understanding the complex process of eukaryotic translation.

RESULTS

Experimental rationale

We have recently demonstrated that small molecule ligands can be used to inhibit translation of eukaryotic mRNAs when the ligand binding site is situated within the 5' untranslated region (UTR) of the mRNA (Harvey et al., 2002). Inhibition appears to be the result of a decreased number of 80S initiation complexes formed on the mRNA template. The ability of AACs to bind to the HIV TAR element present within the 5' UTR of HIV transcripts (Litovchick et al., 1999) prompted us to question whether some of the antiviral properties attributed to AACs might be due to inhibition of translation of TAR containing transcripts. In preliminary experiments aimed at assessing this hypothesis, we noticed that the AACs showed a general inhibitory effect on translation of reporter transcripts, regardless of whether or not these contained a TAR element within their 5' UTRs (M. Carriere & J. Pelletier, data not shown). The current study was thus designed to characterize these novel properties of AACs on translation.

Inhibition of eukaryotic and prokaryotic translation systems by AACs

A series of AACs based on the aminoglycosides neomycin, gentamicin, neamine, and paromomycin was used in the current study. NeoR contains six arginine groups conjugated to the three pyranoside rings of neomycin B (Fig. 1A). NeoR1 is 1:1 mixture of two mono-arginine substituted neomycin isoforms, in which either ring I or IV contains an arginine residue (Fig. 1A). NeoR2 is a di-arginine substituted neomycin derivative in which both rings I and IV contain an arginine residues (Fig. 1A). NeaMR1 and NeaMR4 contain a neamine core with either one or four arginine groups, respectively, attached to amino groups present on the two pyranoside rings of this aminoglycoside (Fig. 1A). ParomR1 and ParomR5 contain a paromomycin core with one or five arginine groups, respectively, attached to amino groups

FIGURE 1. Schematic representation of aminoglycoside–arginine conjugates and aminoglycosides used in this study. All AACs were prepared as acetate salts. **A:** NeoR, Hexa-arginine neomycin conjugate; NeoR1, a 1:1 mixture of two mono-arginine neomycin conjugates; NeoR2, a di-arginine neomycin conjugate of neomycin B; NeaMR1, a mono-arginine neamine conjugate; NeaMR4, a tetra-arginine neamine conjugate; ParomR1, a mono-arginine paromomycin conjugates; and ParomR5, a penta-arginine paromomycin conjugate. **B:** R3G, tri-arginine gentamicin C1 conjugate.

present on the three pyranoside rings of paromomycin (Fig. 1A). R3G contains three arginine groups conjugated to two of the three gentamicin C1 pyranoside rings (Fig. 1B). These compounds resemble oligocationic peptides and share features of relative rigidity of the pyranoside sugar rings with relative flexibility of inter-ring and side chain links. The arginine groups are important for high affinity AAC–RNA interaction, at least with respect to interaction with the TAR element (Litovchick et al., 1999).

The AACs described above were tested in three different eukaryotic and one prokaryotic in vitro translation systems (Fig. 2). Titration of NeoR into wheat germ (Fig. 2A) or rabbit reticulocyte (Fig. 2B) extracts programmed with CAT mRNA demonstrated that NeoR is a potent inhibitor of eukaryotic translation. Inhibition is not specific to the CAT mRNA reporter, as a similar dose response was observed when NeoR was titrated into wheat germ extracts programmed with BMV viral RNA (M. Carriere & J. Pelletier, data not shown). NeoR completely inhibited expression of CAT mRNA at 10 μM and displayed a IC_{50} of $\sim 2.5 \times 10^{-6}$ M in both

extracts. One feature that could contribute to the activity of NeoR is the presence of the multiple, positively charged arginine groups on the relatively flexible neomycin backbone (the inter-ring and side chain links). To address this issue, we assessed the activity of two compounds, NeoR1 and NeoR2, that have the same neomycin backbone but carry only one or two arginine residues (Fig. 1A). Both compounds displayed similar activities when tested in either wheat germ (Fig. 2A) or rabbit reticulocyte (Fig. 2B) translation extracts. Neither was as effective as NeoR at inhibiting translation (Fig. 2A,B). Both NeoR1 and NeoR2 inhibited translation ~ 5 – 10 -fold at 50 μM , but only showed a modest effect at 10 μM ($\sim 40\%$ reduction in translation) compared to NeoR at the same concentration (~ 10 -fold inhibition; Fig. 2A,B). The observed inhibition was specific to the AACs, as neither of the AAC building blocks, neomycin or arginine, had a significant effect on translation in these two systems (Fig. 2A,B). These results are consistent with the idea that increasing the number of arginine groups on neomycin produces a more potent inhibitor of eukaryotic translation.

A Wheat Germ

B Rabbit Reticulocyte Lysate

FIGURE 2. Inhibition of translation by aminoglycoside–arginine conjugates. **A:** Titration of AACs in in vitro translations performed in wheat germ extracts. Translations were performed with CAT RNA (20 $\mu\text{g}/\text{mL}$) in the absence or presence of the indicated amounts of AACs, aminoglycosides, or arginine. TCA precipitation of translated products was used to quantitate reactions. Translation efficiencies were standardized to the efficiency obtained with the reporter transcript translated in the absence of AAC, which was set at one. All values represent the average of at least three independent experiments with standard error shown. **B:** Titration of AACs in in vitro translations performed in reticulocyte lysates. **C:** Titration of AACs in in vitro translations performed in Krebs extracts. **D:** Effects of AACs on prokaryotic translation. Translations were performed with *lux* AB RNA (115 $\mu\text{g}/\text{mL}$) in *E. coli* S30 translation extracts in the absence or presence of the indicated amounts of AACs, aminoglycosides, or arginine. The background in translations with prokaryotic extracts was significant due to the presence of endogenous mRNA and is represented by two dashed lines (representing standard error). (Figure continues on facing page.)

On the other hand, 10 μM R3G inhibited translation approximately threefold in both wheat germ extracts and rabbit reticulocyte lysates (Figs. 2A,B). R3G showed an IC_{50} of 10^{-5} M in wheat germ and $\sim 8 \times 10^{-6}$ M in reticulocyte lysates, indicating that R3G is not as potent an inhibitor as NeoR. Gentamicin had no effect on translation of CAT mRNA in the wheat germ extracts or reticulocyte lysates at concentrations as high as 50 μM (Fig. 2A,B). NeaMR1 did not significantly inhibit translation in either wheat germ extracts or reticulocyte lysates at the concentrations tested, whereas NeaMR4 inhibited translation approximately fourfold at 50 μM and only showed slight inhibition at 10 μM ($\sim 25\%$ inhibition; Fig. 2A,B). These results suggest a contribution to inhibition from the core aminoglycoside structure,

as NeaMR4 containing four arginine residues is not as effective an inhibitor as R3G—an AAC containing three arginines.

ParomR1 and ParomR5 are AACs based on the paromomycin backbone and containing either one or five arginine residues, respectively (Fig. 1A). A greater inhibitory effect on translation in wheat germ extracts and reticulocyte lysates is observed with ParomR5 ($\text{IC}_{50} = \sim 10 \mu\text{M}$ in wheat germ; $\text{IC}_{50} = \sim 5 \times 10^{-6}$ in reticulocyte lysate) than with ParomR1 ($\text{IC}_{50} \geq \sim 50 \mu\text{M}$ in wheat germ; $\text{IC}_{50} = 50 \mu\text{M}$ in reticulocyte lysate; Fig. 2A,B).

Unlike rabbit reticulocyte lysate, Krebs extracts show cap-dependent and poly(A⁺)-stimulated translation (Svitkin et al., 1996, 2001). Inhibition of translation in

C Krebs Extracts

D *E. coli* S30

FIGURE 2. Continued.

Krebs extracts by the AACs followed the same general trend as observed in both the wheat germ extracts and reticulocyte lysates (Fig. 2C). NeoR shows a IC_{50} of $\sim 2.5 \times 10^{-6}$ M in Krebs extracts. These studies demonstrate that among the AACs tested, NeoR is the most potent inhibitor in all three eukaryotic in vitro translation systems tested.

We wished to assess whether the AACs under study could also inhibit translation in a prokaryotic translation system. In *Escherichia coli* S30 extracts programmed with *luxAB* mRNA, neomycin was a potent inhibitor of translation, showing an $IC_{50} = \sim 10^{-7}$ M (Fig. 2D). Like neomycin, gentamicin was a potent inhibitor of *luxAB* translation, showing an $IC_{50} = \sim 5 \times 10^{-7}$ M (Fig. 2D). At concentrations equal to or below 10^{-4} M, free arginine showed no significant effect on translation in *E. coli* S30 extracts (Fig. 2D). The two AACs tested in this system, NeoR and R3G, were not as effective as when tested in the eukaryotic extracts. At $10 \mu\text{M}$ NeoR, a

concentration that completely inhibits translation in eukaryotic extracts (Fig. 2A–C), translation of *luxAB* translation was only slightly affected (Fig. 2D). Indeed, the IC_{50} of NeoR in the *E. coli* extracts is ~ 20 times higher ($\geq 50 \mu\text{M}$) than obtained in eukaryotic extracts. R3G was similar to NeoR in its behavior, showing little effect at $10 \mu\text{M}$, and approximately three- to fourfold inhibition at $100 \mu\text{M}$ (Fig. 2D). Unlike the eukaryotic translation systems used, the *E. coli* S30 extracts show a high background of ^{35}S -methionine incorporation ($\sim 22,000$ cpm), due to the presence of endogenous mRNA templates, which could be inhibited by both aminoglycosides and AACs. These results demonstrate that bacterial translation extracts are not as sensitive to the inhibitory effects of NeoR (or R3G) as are eukaryotic extracts.

A distinguishing feature of AACs is their high arginine content. We wished to address whether small molecules containing an equivalent number of arginines

FIGURE 3. Titration of two hexa-arginine peptides in wheat germ and rabbit reticulocyte translation extracts. Translations were performed with CAT RNA (20 μ g/mL) in the absence or presence of the indicated amounts of RRGGRRGGRR or RRGRRGRR. TCA precipitation of translated products was used to quantitate reactions. Translation efficiencies were standardized to the efficiency obtained with CAT mRNA translated in the presence of peptide. All values represent the average of two independent experiments with error range shown. **A:** Titration of hexa-arginine peptides in *in vitro* translations performed in wheat germ extracts. **B:** Titration of hexa-arginine peptides in *in vitro* translations performed in rabbit reticulocyte extracts.

as NeoR, on a flexible backbone, could exert the same effects on translation. To this end, we tested two polypeptides that contained six arginine residues separated by glycine residues (RRGGRRGGRR and RRGRRGRR) for their ability to inhibit translation in wheat germ (Fig. 3A) or rabbit reticulocyte (Fig. 3B) extracts. Neither peptide showed significant influence on translation of the CAT mRNA reporter at concentrations at or below 10 μ M (Fig. 3). Only a very small effect (20% inhibition) was observed at 50 μ M for both peptides in wheat germ extracts (Fig. 3A) and for RRGGRRGGRR in rabbit reticulocyte lysates (Fig. 3B). These results indicate that the juxtaposing of six arginine residues is insufficient to account for the inhibitory effects of NeoR on translation.

Stability of reporter transcripts in the presence of NeoR

One explanation that could account for the observed inhibition of mRNA expression in the presence of some of the AACs would be if these harbored nucleolytic activity—either because of the presence of a contaminating nuclease, activation of an endogenous nuclease, or by promoting degradation upon RNA binding (e.g., changing the structure of the mRNA to make it more susceptible to cleavage). To directly address this, we performed a kinetic analysis of CAT mRNA stability when translated in wheat germ extracts in the absence or presence of NeoR (Fig. 4). Reisolation of radio-labeled CAT transcripts from programmed translation extracts followed by fractionation on formaldehyde agarose gels revealed that the stability of CAT mRNA is

similar in the absence or presence of 10 μ M NeoR during the time course of the experiment (Fig. 4). Thus, the observed effects of NeoR on translation cannot be attributed to the presence of a nucleolytic activity associated with NeoR.

FIGURE 4. Stability of CAT mRNA translated in the presence of NeoR in wheat germ extracts. **A:** 32 P-labeled CAT mRNA was translated in the absence or presence of 10 μ M NeoR. At the indicated time points, an aliquot was removed from the translation reaction, and the RNA isolated from the sample and fractionated on 1.4% agarose/formaldehyde gels. The time at which mRNA was extracted is shown above the panel, as well as whether or not NeoR was present in the translation extract. **B:** Summary of three experiments measuring the relative stability of CAT mRNA in wheat germ extracts at the indicated times. Radioactive mRNA templates obtained in **A** were quantitated on a Fuji BAS2000 with a Fuji imaging screen. Values were set relative to the value obtained at the beginning of the experiment ($T = 0$ min). The time points and the presence or absence of NeoR in the translation reaction is indicated below the panel.

In vivo translation assays

To assess the effects of NeoR on translation *in vivo*, we used the *Xenopus laevis* oocyte system. Injected mRNAs translate very efficiently in *Xenopus* oocytes. The translation efficiency of CAT mRNA, in the presence or absence of NeoR, was determined by analyzing CAT activity after injection of mRNA into oocytes. Extracts from oocytes not injected with mRNA showed no CAT activity (data not shown). In the experiment shown in Figure 5A, injection of CAT mRNA into oocytes yielded an average conversion of 39% in the absence of NeoR (lane 1). Increasing concentrations of NeoR produced an approximately eightfold decrease in CAT expression with 10 μ M NeoR (Fig. 5A, compare lane 3 to lanes 2 and 1) and complete inhibition at 100 μ M and 450 μ M NeoR (Fig. 5A, lanes 4 and 5). To exclude the possibility that degradation of CAT mRNA, in the presence of NeoR, was responsible for the observed differences on CAT expression *in vivo*,

FIGURE 5. Effect of NeoR on *in vivo* translation in *Xenopus laevis* oocytes. **A:** An autoradiograph of a representative TLC for the CAT assays performed is shown. The predicted final concentrations of NeoR is shown below the panel. The percent conversion shown below the panel is the average obtained from two independent experiments. AcC: acetylated forms of chloramphenicol; C: chloramphenicol; O: origin. **B:** Stability of injected mRNA in *X. laevis* oocytes. Following injection of 32 P-mRNA (700 cpm/egg), oocytes were incubated at 22°C for 2 h. Total RNA was harvested utilizing Trizol, as indicated by the manufacturer's recommendations (Invitrogen). Following ethanol precipitation, RNA samples were fractionated on a 1.2% agarose/formaldehyde gel. After staining the gel with SYBR gold (Molecular Probes) to ensure equal recovery of rRNA from the oocytes (data not shown), the gel was dried and exposed to X-OMAT X-ray film (Kodak) at -70°C with an intensifying screen for 6 h. The presence of NeoR coinjected with CAT mRNA is indicated above the panel; the position of migration of CAT mRNA is also indicated.

we analyzed the integrity of CAT mRNA that had been coinjected with NeoR into oocytes. Injected radiolabeled CAT mRNA was reisolated from oocytes and analyzed by fractionation on agarose/formaldehyde gels (Fig. 5B). The results indicate that CAT mRNA stability is not significantly affected by the presence of NeoR in *X. laevis* oocytes (Fig. 5B).

NeoR does not inhibit eukaryotic translation initiation

We wished to determine if NeoR was exerting its effect by preventing translation initiation, as the observed inhibition was not mRNA specific. To assess this, we monitored 43S preinitiation complexes on CAT mRNA, by performing ribosome-binding assays in the presence of GMP-PNP (which inhibits the joining of the 60S ribosome subunit as well as release of eIF2; Fig. 6A). We observed no differences in the amount of 48S initiation complexes formed in the presence or absence of NeoR (Fig. 6A), indicating that binding of 40S ribosomes to the input CAT mRNA template is not affected by the presence of NeoR. To assess if 60S subunit binding was affected by NeoR, we repeated the ribosome binding assays in the presence of cycloheximide, an inhibitor of peptide chain elongation (Fig. 6B). Initiation complex formation (80S) was not affected by the presence of NeoR in the binding reactions (Fig. 6B, compare dashed line to solid line) indicating that NeoR does impair the ability of 80S ribosomes to assemble onto mRNA templates.

Inhibition of elongation by NeoR and R3G

To directly assess whether NeoR was inhibiting peptide chain elongation, we utilized the puromycin assay. In this approach, [35 S]methionine-charged tRNA (Met-tRNA_i) is bound to the P-site of the 80S ribosomal complex. As an analog of the 3' end of a charged tRNA, puromycin is capable of binding in the A-site of the ribosome and reacting with the ester linkage of Met-tRNA_i to produce a [35 S]methionine-puromycin dipeptide. Hence, small molecule effects on peptide chain elongation can be monitored by assessing the rates of [35 S]methionine-puromycin dipeptide formation.

Inhibition of Met-Puro production was observed when NeoR or R3G were included in the puromycin assay (Fig. 7A,B). Varying the concentration of the AACs, neomycin, or gentamycin in the puromycin assay yielded dose-response curves (Fig. 7B), in which the relative extent of inhibition of NeoR and R3G paralleled those observed in the *in vitro* translation systems (Fig. 2). That is, NeoR inhibited Met-Puro production at concentrations lower than required by R3G to achieve the same effect (compare K_i s in Fig. 7C).

A direct testable prediction of these results is that NeoR should be capable of replacing cycloheximide in

FIGURE 6. Effect of NeoR on 40S ribosome binding and 80S assembly in wheat germ extracts. ^{32}P -labeled CAT was assayed in the absence (solid line) or presence of 10 μM NeoR (dashed line). The 48S complexes were visualized by performing binding reactions in the presence of GMP-PNP (**A**) and 80S complexes were visualized by treatment of extracts with cycloheximide (**B**). Fractions from each sucrose gradient were collected using a Brandel Tube Piercer connected to an ISCO fraction collector and individually counted. **A:** Total counts recovered from each gradient and percent mRNA bound to 48S complexes were: CAT mRNA, 52,047 cpm, 10% binding, and CAT mRNA + NeoR, 57,036 cpm, 8% binding. **B:** Total counts recovered from each gradient and percent mRNA bound to 80S complexes were: CAT mRNA, 43,493 cpm, 6% binding, and CAT mRNA + NeoR, 50,805 cpm, 5% binding.

ribosome binding assays and trapping 80S initiation complexes on the mRNA template. To this end, we replaced cycloheximide with NeoR in ribosome binding assays in an attempt to trap 80S complexes on mRNA. We performed ribosome binding assays with ^{32}P -labeled CAT mRNA in the presence of either GMP-PNP (Fig. 8, lane 1), cycloheximide (lane 2), cycloheximide and NeoR (lane 3), or NeoR (lane 4). In this experiment, complexes were resolved by gel electrophoresis and visualized by autoradiography. In the presence of GMP-PNP, a distinct complex was observed that migrated near the bottom of the gel (Fig. 8, lane 1). This complex was not observed in the presence cycloheximide (Fig. 8, compare lane 1 to 2) and is taken to represent 48S

initiation complexes. The complex obtained in the presence of cycloheximide is taken to represent the 80S initiation complex (Fig. 8, lane 2). In the presence of both cycloheximide and NeoR (Fig. 8, lane 3), a single 80S complex is observed, as demonstrated previously by glycerol gradient centrifugation (Fig. 6B). An 80S complex is also observed when the ribosome binding is performed in the presence of only NeoR (Fig. 8, lane 4). A significant amount of radiolabeled mRNA is present in the wells and was also seen when no inhibitor of elongation was added to the binding reaction (data not shown), suggesting that these are mRNA/protein aggregates and not ribosomes (Lorsch & Herschlag, 1999). These results demonstrate that NeoR

FIGURE 7. Effect of AACs on Met-Puro production. **A:** The formation of the [35 S]Met-Puro dipeptide was followed using cation-exchange TLC. A kinetic analysis in which a single concentration of R3G was used in the Met-Puro assay is shown. The positions of migration of [35 S]Met-tRNA_i, free methionine, and the Met-Puro dipeptide product are indicated. **B:** Dose response curve showing the effect of increasing AACs concentration on Met-Puro production. **C:** Apparent K_i s determined for NeoR, Neomycin B, R3G, and Gentamicin C1 in the Met-Puromycin assay.

can inhibit peptide chain elongation and can replace cycloheximide to trap 80S complexes on mRNA templates.

DISCUSSION

Inhibitors of protein synthesis belong to an extremely important class of therapeutic drugs targeting bacterial ribosomes that are used in human and veterinary medicine. Additionally, protein synthesis inhibitors have been useful tools to help assign ribosome functional domains and identify key steps of the translation process. Aminoglycosides are not very effective at inhibiting eukaryotic cytoplasmic ribosomes (Fig. 1A,B; 140 μ M neomycin is required to achieve 60% inhibition of wheat germ ribosomes; Wilhelm et al., 1978a, 1978b). Arginine is also not a very good inhibitor of eukaryotic translation (50% inhibition at 20 mM; Palacian & Vazquez, 1979). However, the conjugation of aminoglycosides with arginine appears to generate a series of peptidomimetic compounds that show good activity in inhibiting eu-

karyotic translation (this study). Given the structural differences between the AACs and the parental compounds from which they are derived, we had no reason to suspect that AACs should have this property. To our surprise, several of the AACs proved to be low micromolar inhibitors of eukaryotic translation (Fig. 2)—a feature not associated with the original parental building blocks.

The eight AACs tested in this report followed very similar inhibition profiles in all three in vitro eukaryotic translation extracts. The rank order of the AACs in the different extracts is very similar, and proceeding from lowest to highest IC_{50} is: (1) NeoR, (2) (ParmR5/R3G/NeoR1/NeoR2), (3) NeaMR4, (4) ParomR1, and (5) NeaMR1. An exception to this order is the behavior of NeaMR4 in Krebs extracts that would place it equivalent to (ParmR5/R3G/NeoR1/NeoR2) (Fig. 2D). In the best characterized systems (wheat germ and reticulocyte lysates), the observed inhibition of translation by AACs correlates with the number of arginine groups linked to the aminoglycoside, as well as the nature of

FIGURE 8. EMSA demonstrating the ability of NeoR to trap 80S complexes on an mRNA template. Ribosome binding assays were performed in wheat germ extracts with ^{32}P -labeled mRNA (50,000 cpm/reaction) in the presence of GMP-PNP (lane 1), cycloheximide (Chx; lane 2), cycloheximide and NeoR (lane 3), and $10\ \mu\text{M}$ NeoR (lane 4). Complexes were resolved by polyacrylamide gel electrophoresis on native gels using conditions previously described (Lorsch & Herschlag, 1999). Following electrophoresis, the gel was dried and exposed to X-OMAT X-ray film (Kodak) at -70°C with an intensifying screen. The positions of migration of the 48S and 80S complexes are indicated by arrows, as is the position of the wells of the gel. The asterisk indicates a complex whose identity is not known (possibly a 40S + 80S complex) and that may correspond to the complexes present in the shoulder observed at fraction #15 of Figure 6A.

core aminoglycoside. NeoR was the most effective inhibitor, showing an $\text{IC}_{50} = \sim 2.5 \times 10^{-6}\ \text{M}$ (Fig. 2A,B). Decreasing the number of arginine groups from six to either one (NeoR1) or two (NeoR2) produced compounds that were not as potent as NeoR (Fig. 2A,B). Comparison of the activity of the neamine and paromomycin derivatives are also consistent with the role of the increased number of arginines, as in both these cases the derivatives with the larger number of arginine residues (NeaMR4 and ParomR5) are more potent than the mono-arginine derivatives (Fig. 2A,B).

The juxtaposing of six arginine residues is insufficient to inhibit translation, because neither RRGGR RGGRR or RRGRGR, showed significant inhibition of translation in wheat germ extracts and rabbit reticulocyte lysates at the concentrations tested (Fig. 3). These results are consistent with a role for the core aminoglycoside in contributing to the properties of the ACCs (in translation inhibition). Indeed, changing the nature of the core aminoglycoside to paromomycin or gentamicin, with either five (ParomR5) or three (R3G) arginine residues produced compounds that were similar in activity to NeoR1 and NeoR2. ParomR1 was less inhibitory than NeoR1 in wheat germ extracts (Fig. 2A) and reticulocyte lysates (Fig. 2B). A neamine derivative

containing one arginine residue (NeaMR1) was not as inhibitory as NeoR1, showing little effect at concentrations as high as $50\ \mu\text{M}$ (Fig. 2A,B). These results suggest that, among the AACs tested, neomycin provides the best core onto which to link arginine groups to produce the most potent AAC. These conclusions assume that all of the AACs being compared inhibit translation through a common mechanism.

The AACs were developed to take advantage of the role of arginine in RNA binding (reviewed in Cheng et al., 2001). The guanidinium groups have the potential to make electrostatic, hydrogen bonding, and cation- π and π -stacking interactions. Tethering of the guanidinium groups to aminoglycosides is expected to produce compounds that are relatively flexible, due to inter-ring and side chain links. Hence, defining the nature of the NeoR binding pocket has the potential to lead to the design of compounds with increased rigidity and higher affinities. The inherent rigidity of parts of the AACs (e.g., the pyranoside rings) place some constraints on distance between some of the side groups, and may also contribute to the overall energetics of binding.

Bacterial extracts are not as sensitive to inhibition by AACs as the eukaryotic translation extracts tested (Fig. 2D). For example, NeoR is approximately 20 times less potent at inhibiting prokaryotic translation (Fig. 2D; $\text{IC}_{50} \geq 50\ \mu\text{M}$), than at inhibiting translation in eukaryotic extracts (Fig. 2A–C; $\text{IC}_{50} = \sim 2.5 \times 10^{-6}\ \text{M}$). The reason for this difference is not known, but may reflect structural differences in the AAC binding site. Alternatively, there is a marked difference in the divalent cation optima between prokaryotic and eukaryotic extracts. Prokaryotic systems have a higher *in vitro* Mg^{2+} optima (~ 10 – $15\ \text{mM}$; Chen & Zubay, 1983), whereas the eukaryotic systems we used have a lower Mg^{2+} optima (2.0 – $3.0\ \text{mM}$ for wheat germ and rabbit reticulocyte; Anderson et al., 1983; Merrick, 1983), which could influence binding of the AACs to their targets or the conformation of the binding site. Of course, we cannot exclude nonspecific effects such as the possibility that macromolecules in the prokaryotic extract bind to and titrate out NeoR, causing an apparent increase in IC_{50} .

The observed inhibition of NeoR on translation is not reporter specific, as very similar inhibition curves were obtained when NeoR was titrated in wheat germ extracts programmed with BMV viral mRNA (data not shown). The effect is also not due to differences in mRNA stability, as we observed no difference in stability between translation extracts containing or lacking NeoR (Fig. 4). The ability to inhibit translation in frog oocytes demonstrated that NeoR is also an effective *in vivo* translation inhibitor (90% inhibition of CAT expression at a final concentration of $10\ \mu\text{M}$ NeoR; Fig. 5).

NeoR and R3G did not inhibit loading of either 40S or 80S ribosomes onto mRNA templates (Fig. 6), indicating that they do not function by inhibiting translation

initiation. These results also indicate that the observed inhibition is not due to nonspecific effects exerted by the AACs on translation. Rather, NeoR and R3G appear to function by inhibiting peptide chain elongation, as both prevented ^{35}S -Met-Puromycin formation in a chain elongation assay (Fig. 7). Consistent with this result is the ability of NeoR to function like cycloheximide and trap 80S initiation complexes on input mRNA templates (Fig. 8).

Aminoglycosides interact in complex ways with the prokaryotic ribosome. They interfere with A-site function and some stimulate misreading of the mRNA template, resulting in the incorporation of the wrong amino acid (Carter et al., 2000). Neomycin and hygromycin have also been reported to block translocation (Hausner et al., 1988) and more recently, neomycin and paromomycin have been shown to inhibit 30S ribosomal subunit assembly (Mehta & Champney, 2002). Given the steric bulk of AACs, as well as the presence of multiple arginine functional groups (compared to aminoglycosides), we suspect that AACs interact with a different site on the ribosome. Our studies have not addressed the nature or location of the AAC binding site, but they could bind to either or both ribosomal subunits. Binding to the 40S subunit could be feasible if induced structural changes altered activity of the peptidyl transferase site of the 60S subunit. It will be interesting to test these possibilities in future experiments.

Additional properties of AACs include inhibition of Tat/Tar interaction (Litovchick et al., 2000), down-regulation of the chemokine CXCR4 receptor type 4 (Litovchick et al., 2001), and inhibition of bacterial RNase P (Eubank et al., 2002), suggesting that AACs (especially NeoR) could be considered for both HIV and bacterial chemotherapy. In this report, we now demonstrate that AACs can inhibit eukaryotic translation. We do not know if these activities of AACs are intimately linked or can be separated into different activities of the AACs. Clearly, in-depth structure-activity relationship studies could help assign the different properties to functional groups.

The characterization of most inhibitors of protein synthesis has focused on small molecules that exert an effect on prokaryotic translation, because of the need for developing drugs that target this essential process. However, eukaryotic translation is also a very important target, as several components of this pathway are misexpressed in human malignancies. For example, eIF4E expression is elevated in human cancers (Nathan et al., 1997, and references therein), eIF4G is over-expressed in squamous cell lung carcinoma (Bauer et al., 2002), and the expression of many ribosomal proteins is altered in cancers (Tarbe et al., 2001). Such genetic changes in transformed cells provide an interesting target for chemotherapy exploitation. AACs thus represent a class of ligands that might be further developed and exploited to validate the hypothesis that compounds targeting eukaryotic translation could be

used to achieve specific growth inhibition of transformed cells.

MATERIALS AND METHODS

Synthesis of aminoglycoside-arginine conjugates

The procedures for the synthesis and purification of NeoR and R3G have been detailed (Litovchick et al., 2000, 2001). Synthesis and chemical characterization of NeoR1, NeoR2, NeaMR1, NeaMR4, ParomoR1, and ParomoR4 will be published elsewhere. Neomycin B was purchased from Fluka. Gentamicin and L-arginine were obtained from Sigma. The peptides RRGGRRGGRR and RRGRGRGR were purchased from American Peptide Company, Inc. (Sunnyvale, California) and characterized by RP-HPLC and mass spectral analysis. Peptides were >97% pure.

In vitro translations and ribosome bindings

The generation of the CAT reporter, pSP/CAT, has been previously described (Harvey et al., 2002) and for in vitro transcription reactions, pSP/CAT was linearized with *Hind*III. In vitro transcriptions, in the presence of m^7GpppG , were performed as previously described (Pelletier & Sonenberg, 1985). RNA transcripts were quantitated by monitoring incorporation of ^3H -CTP (20 Ci/mmol; Perkin Elmer) and the quality of each preparation assessed by fractionation of the RNA on formaldehyde/1.2% agarose gels followed by SYBR gold staining. In vitro translations in wheat germ, rabbit reticulocyte lysates, and in *E. coli* S30 extracts were performed using ^{35}S -methionine as directed by the manufacturer (Promega). Translation reactions utilizing *E. coli* S30 extracts (for linear templates) were programmed with a *lux* AB transcript (Szittner & Meighen, 1990), which had been produced by in vitro transcription reactions from pT7-5 (Dr. Ted Meighen, McGill University) utilizing T7 RNA polymerase, followed by treatment with DNase I to remove any remaining plasmid DNA. Translations in *E. coli* S30 extracts were performed at a final mRNA concentration of 115 $\mu\text{g}/\text{mL}$. Translations in eukaryotic extracts were performed at mRNA concentrations of 20 $\mu\text{g}/\text{mL}$. Krebs translation extracts were prepared and used for in vitro translation reactions as previously described (Svitkin & Agol, 1978). The final KOAc concentration in the wheat germ, rabbit reticulocyte lysate, and Krebs translations was 130 mM. The order of addition of reagents to translation reactions involved the preparation of a master mix at 4 °C containing mRNA template, followed by dispensing into individual Eppendorf tubes, at which point the test compounds (AACs or aminoglycosides) were added. TCA precipitation of translation products was used to determine relative translation efficiencies.

Ribosome binding assays were performed essentially as previously described (Pelletier & Sonenberg, 1985). Briefly, ^{32}P -labeled CAT mRNA was incubated in 25 μL of wheat germ extract alone, or supplemented with 10 μM NeoR, in the presence of 0.6 mM cycloheximide (to monitor 80S assembly) or 1 mM 5'-guanylylimidodiphosphate (GMP-PNP; to monitor 48S assembly; Eckstein et al., 1971; Svitkin et al.,

1996), at 20 °C for 10 min. The final KCl concentration was adjusted to 0.5 M. Initiation complexes formed were analyzed by sedimentation through 10–30% glycerol gradients. Centrifugation was for 4 h at 39,000 rpm at 4 °C in an SW40 rotor. Fractions of ~500 μ L (10 drops) were collected and radioactivity was determined by scintillation counting.

Translations in *Xenopus* oocytes

Translations in *Xenopus* oocytes consisted of microinjecting 10 oocytes with 50 nL containing [3 H]-labeled mRNA (1.75 ng) alone or in combination with NeoR. Given an average oocyte diameter of 1.2 mm (~0.7 μ L; Colman, 1984), we aimed to achieve a final concentration of 2.5 μ g/mL mRNA and the indicated amounts of NeoR (Fig. 4A). Oocytes were incubated at 20 °C for 2 h, homogenized in lysis buffer (20 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), centrifuged in a microfuge for 5 min at $14,000 \times g$, and the recovered supernatant used to measure CAT activity (Gorman, 1985). Quantitations were performed on a Fuji BAS2000 with a Fuji imaging screen. Because each injection was spiked with a known amount of [3 H]-CTP, portions of the homogenate were counted in a scintillation counter to normalize for variations in microinjection and sample preparation.

RNA stability assays

To determine mRNA stability in the presence or absence of NeoR, wheat germ translation extracts were programmed with 32 P-labeled mRNA, except that 35 S-methionine was replaced with 80 μ M unlabeled methionine. At various time points, an aliquot was removed (10 μ L), incubated with 50 μ g proteinase K at 37 °C for 15 min, and the sample was then phenol/chloroform extracted. Following ethanol precipitation, RNA samples were fractionated on a 1.4% agarose/formaldehyde gel. Gels were stained with SYBR gold (Molecular Probes) to ensure equal recovery of rRNA from the translation extracts (data not shown), and then dried and exposed to X-OMAT film (Kodak) at –70 °C with an intensifying screen. Quantitations were performed on a Fuji BAS2000 with a Fuji imaging screen.

Met-Puromycin dipeptide assays

Met-puromycin dipeptide assays were performed as previously described (Lorsch & Herschlag, 1999). Briefly, reactions included 10 nM ribosomes, 2 nM 35 S-methionyl tRNA, 400 μ M puromycin, and 1 μ M model mRNA, in a buffer containing 500 μ M GTP and 1.75 mM magnesium acetate, and other components as previously described (Lorsch & Herschlag, 1999). 80S complexes were preformed prior to addition of puromycin and inhibitor, as described (Lorsch & Herschlag, 1999). Reactions proceeded at 26 °C for 12 min, with aliquots removed periodically and quenched in 3 M sodium acetate, pH 5.1. Quenched samples were resolved by cation-exchange TLC, and quantitated by comparing 35 S-Met-puromycin product to 35 S-Met tRNA + 35 S-Met-puromycin. Observed rate constants were calculated by plotting these

data and fitting to a single-exponential curve. To calculate K_i , the observed rate constant at several concentrations of inhibitor was measured. K_{obs} for each reaction was normalized to a positive control reaction performed in the absence of inhibitor, and the data fit with a binding isotherm (Fersht, 1999).

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