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Inhibition of Bacterial IF2 Binding to FMet-tRNA$^{(fMet)}$ by Aminoglycosides

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Abstract—Screening for inhibitors of bacterial protein synthesis Initiation Factor 2 (IF2) binding to *N*-formyl-Methionyl-transfer RNA (fMet-tRNA$^{(fMet)}$) identified a series of aminoglycosides, that included amikacin and kanamycin A1, as inhibitors of this interaction. Subsequent testing revealed that aminoglycosides displayed a wide range of inhibitory activity. However, the failure of these compounds to completely inhibit binding of IF2 to fMet-tRNA$^{(fMet)}$, the known ability of aminoglycosides to bind RNA, and the ability of the aminoglycosides to displace PicoGreen bound to fMet-tRNA$^{(fMet)}$ suggest these compounds act by binding fMet-tRNA$^{(fMet)}$. This hypothesis is further supported by isothermal denaturation experiments that failed to show any interaction between the IF2 protein and the aminoglycosides.

Initiation Factor 2 (IF2) is an essential component of bacterial protein translation delivering formyl-Methionyl-transfer RNA (fMet-tRNA$^{(fMet)}$) to the P-site of the ribosome during initiation of translation. IF2 may be a suitable target for the discovery of novel antibacterial compounds because it has been shown to be essential in *Escherichia coli*,[1] and has been shown to be conserved within bacterial species.[2] IF2 is the largest of three bacterial initiation factors. IF1 facilitates the association/dissociation of the ribosomal subunits and aids binding of IF-2 to the 30S subunit. IF2 stimulates the binding of fMet-tRNA$^{(fMet)}$ to the P-site of the ribosome, and IF3 acts as a fidelity factor stabilizing 30S initiation factors containing the correct initiation codon and fMet-tRNA$^{(fMet)}$. The specificity for initiating protein translation with fMet-tRNA$^{(fMet)}$ is unique to bacterial translation, and IF2 has been shown to specifically recognize the formyl group of the initiation fMet-tRNA$^{(fMet)}$.[3] The IF2 protein is composed of three domains. The N-terminal domain is poorly conserved amongst bacterial species and, at least in *E. coli,* is not required for viability.[1] The central portion of the protein contains a GTP binding domain (the G-domain), and the C-terminal domain contains the fMet-tRNA$^{(fMet)}$ binding domain.[4] The gene encoding IF2 is well conserved amongst bacteria with the majority of this conservation lying within the G-domain and to a lesser extent the C-terminal domain. This pattern of conservation is also seen between bacterial IF2s and the human mitochondrial IF2 homologue. However, the level of conservation is much lower in the C-terminal domain (the fMet-tRNA$^{(fMet)}$ binding domain) than the G-domain (ranging between 30–40% compared to 60–70% identity).[5]

Aminoglycosides are well known antibacterial agents whose mechanism of action have been studied extensively, their major target being bacterial 16S ribosomal RNA. Binding results in inhibition of translation and amino acid mis-incorporation.[6] However, aminoglycosides have also been reported to bind a number of different RNA molecules including the HIV RRE and TAR RNA regions, and RNA aptamers that bind aminoglycosides have been reported.[7] Aminoglycosides have been shown to inhibit the action of certain ribozymes and even a number of enzymes, including RNase P cleavage of RNA and aminoacylation of yeast tRNA$^{(Asp)}$ by aspartyl-tRNA synthetase.[8]

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A program of screening for inhibitors of fMet-tRNA$^{(fMet)}$ binding to recombinant *Staphylococcus aureus* IF2, tagged at its amino terminus with a six histidine extension to aid purification (His6IF2), has been undertaken. Initially, the assay was characterized by screening a small, diverse library of known pharmacologically active compounds (the Genesis Library of compounds from Microsource) followed by further testing of compounds of similar structure. This led to the identification of seven aminoglycosides including amikacin and kanamycin, and subsequently other aminoglycosides, as inhibitors of fMet-tRNA$^{(fMet)}$ binding to IF2.

A filter binding assay to monitor *S. aureus* IF2 binding to fMet-tRNA$^{(fMet)}$ was developed to allow high throughput screening, using recombinant *S. aureus* IF2.[9] The assay was derived from that originally described by Sundari et al., and then optimized for use in a 96-well filter plate format.[10] Buffer and assay conditions were optimized, and the system gave an estimated *K*d for fMet-tRNA$^{(fMet)}$ binding to the recombinant *S. aureus* His6IF2 of 40±4 nM. This estimate of the dissociation constant is consistent with the value previously reported for IF2 from *Bacillus stearothermophilus* (Fig. 1).[10]

Screening identified a number of possible inhibitors of fMet-tRNA binding. As with most screens, many of these represent assay artifacts such as compounds that compete for binding to the filter or that color quench. However, of these hits, amikacin, gentamycin, kanamycin A1, neomycin and sisomycin were valid inhibitors that did not exhibit these assay artifacts. Selection and testing of a number of different aminoglycosides identified additional aminoglycosides (i.e., hygromycin and streptomycin) shown in Figure 2, with a wide range of potencies and efficacies (Table 1).

These results show that a number of different aminoglycosides are capable of inhibiting IF2 binding to fMet-tRNA$^{(fMet)}$ with a broad range of potency. The most likely explanations for the observed effect are interaction of the aminoglycosides with the IF2 protein or fMet-tRNA$^{(fMet)}$.

Isothermal denaturation experiments were then conducted to determine if the inhibition was due to binding

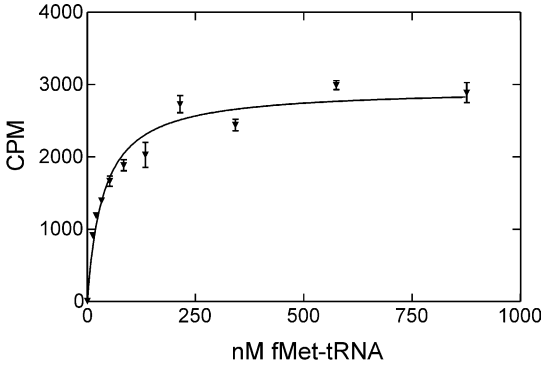

Figure 1. Binding response of recombinant IF-2 binding fMet-tRNA$^{(fMet)}$ The data shown are the average of six replicates of the dilution series with error bars showing one S.D.

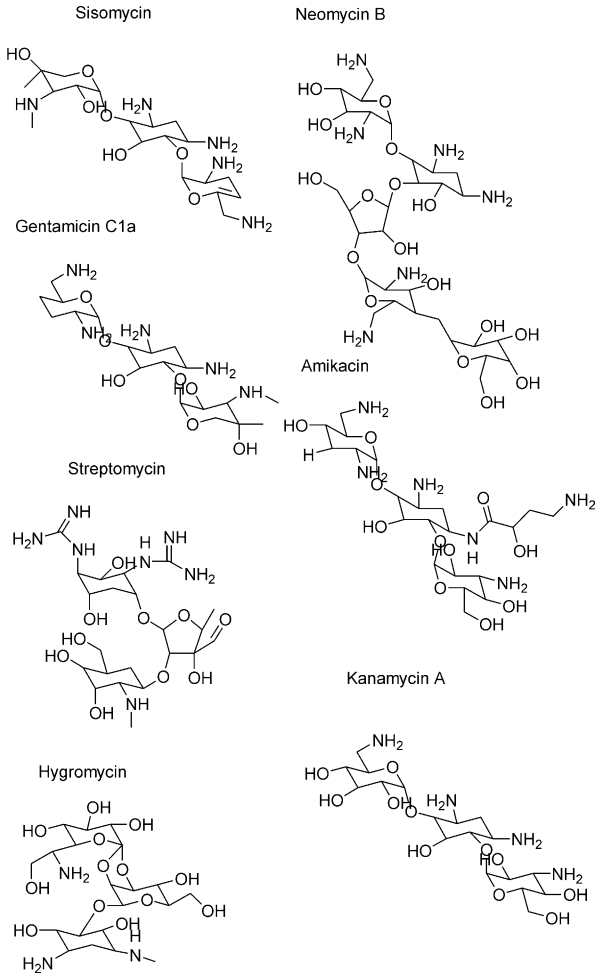

Figure 2. Aminoglycosides identified as inhibiting recombinant IF-2 binding fMet-tRNA$^{(fMet)}$.

of the aminoglycosides to the IF2 protein. Figure 3 shows IF2 isothermal denaturation profile in the absence and presence of two different inhibitors. IF2 exhibits an isothermal denaturation rate constant of 0.12 min^{-1} and this was not significantly altered by any of the aminoglycosides (rate constants listed in Table 2). This result is consistent with aminoglycosides not binding to IF2.

Experiments were then conducted to determine if the aminoglycosides could bind to the fMet-tRNA$^{(fMet)}$ by

Table 1. Characterization of the inhibitory effects of aminoglycosides on fMet-tRNA$^{(fMet)}$ binding

	IC_{50}, μM[a,b]	Maximal inhibition	Slope
Sisomycin	0.0007	75%	0.32 (±0.6)
Neomycin	0.05	76%	1.8 (±0.7)
Gentamycin	0.45	66%	1.74 (±1.6)
Amikacin	1.02	43%	2.8 (±2.1)
Streptomycin	1.49	43%	0.82 (±0.8)
Kanamycin A1	17.8	61%	1.3 (±1.0)
Hygromycin	66	84%	1.4 (±0.76)

[a]Values are means repeated in duplicate; standard deviations are not shown but were less than 10% of the calculated IC_{50}.
[b]IC_{50}, maximal inhibition and slope were calculated using The GraphPad Prizm software.

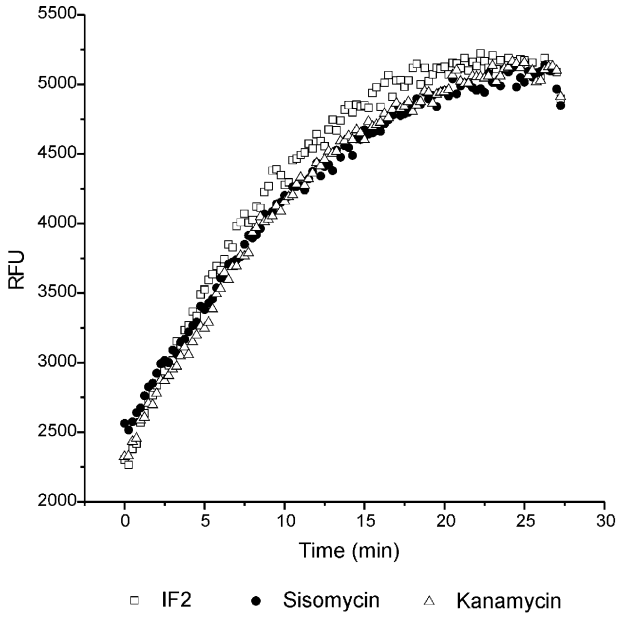

Figure 3. Isothermal denaturation response of Recombinant IF-2 in the presence of two different aminoglycosides. These experiments were conducted under standard IF2 binding conditions in a similar manner described by Sarver, et al., 2002.[11] Experiments were then conducted to determine if the aminoglycosides could bind to the fMet-tRNA$^{(fMet)}$ by monitoring RNA hyperchromicity (data not shown) or the displacement of PicoGreen from the RNA, Figure 4.

Table 2. Characterization of the displacement of PicoGreen from fMet-tRNA$^{(fMet)}$ by aminoglycosides and denaturation of IF2 in the presence of aminoglycosides[12]

	IC_{50}, μM[a,b]	Maximal inhibition (%)	Slope (S.D.)	Denaturation rate constant (min^{-1})
Sisomycin	0.007	100	−1.2 (0.1)	0.087
Neomycin	0.009	100	−1.6 (0.13)	0.095
Amikacin	0.16	85	−0.36 (0.16)	0.093
Streptomycin	1.79	95	−1.01 (0.284)	0.094
Kanamycin A1	0.024	92	−0.36 (0.16)	0.092
Hygromycin	0.278	81	−0.64 (0.13)	0.096

[a]Values are means of two experiments were each data point repeated in duplicate, standard deviations were less than 10% of each value.
[b]IC_{50}, maximal inhibition and slope calculated using the GraphPad Prizm software.

monitoring RNA hyperchromicity (data not shown) or the displacement of PicoGreen from the RNA (Fig. 4).

These experiments are consistent with aminoglycosides inhibiting IF2 binding to fMet-tRNA$^{(fMet)}$ by binding to the RNA portion of the molecule. Consistent with this was the observation that displacement of PicoGreen from yeast Phe-tRNA by these aminoglycosides, under the same conditions, gave similar results (data not shown). While the potency of the compounds to displace PicoGreen from the fMet-tRNA is not statistically correlated with their ability to block IF2 binding, these results are consistent with the aminoglycosides binding the tRNA portion of the molecule. The correlation between RNA binding and inhibition of IF2 binding may not be exact, as binding of the aminoglycoside to the tRNA alone may not be sufficient to inhibit IF2 binding, some other effect on tRNA conformation or

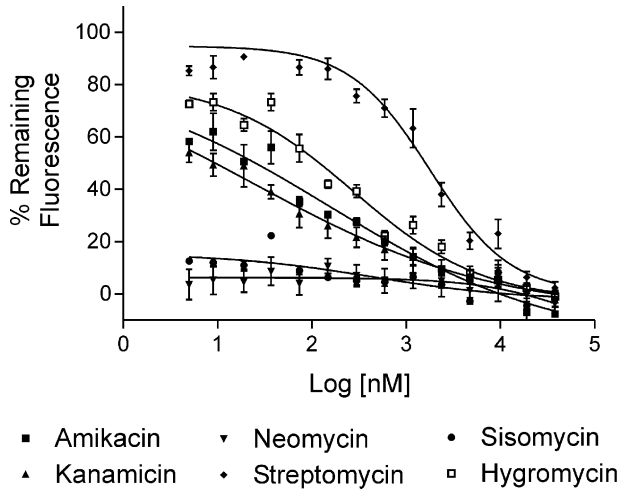

Figure 4. Displacement of PicoGreen from fMet-tRNA$^{(fMet)}$ under standard binding conditions.

steric hindrance may be needed to inhibit IF2 binding. These results are in general agreement with those recently reported by Walter et al.,[13] in which tobramycin inhibits aminoacylation of yeast tRNA Asp by binding to and causing a conformational change in the tRNA such that it can no longer act as a substrate for aminoacyl tRNA synthetase. This observation is also in agreement with an earlier report of the inhibition of *E. coli* phenylalanyl-tRNA-synthetase by aminoglycosides.[14]

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9. The *infB* gene encoding IF2 with an engineered N-terminal Met-His_6-Ala_2 tag was PCR amplified from *S. aureus ATCC* 9218. Expression was induced with Isopropylthiogalactoside from a T7 promoter in *E. coli* BL21(DE3)pLysS (Novagen). Cells were lysed via French Press, and *S. aureus* His6-IF2 was purified from the soluble lysate via immobilized-affinity chromatography (IMAC) using Ni-NTA Superflow (Qiagen). Material was eluted via a shallow linear imidazole gradient and was >99% pure as judged by SDS-PAGE. Protein was dialyzed into storage buffer (30 mM Tris pH 8.0, 140 mM NaCl, 1 mM DTT) at 4 °C before use.
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All data were analyzed using a custom data analysis package. Controls included: (a) DMSO alone that acts as a no inhibition control and (b) 10 M *E. coli* tRNA that acts as a 100% inhibition control. The% inhibition was then calculated as follows:% Inhibition = ((DMSO Control-Sample)/(DMSO Control-*E. coli* tRNA Control))×100%. Using the% inhibition at each concentration IC_{50}s were calculated using an equation that fits to a sigmoidal dose-response with a variable slope, using the GraphPad software package.
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12. Binding of aminoglycosides to fMet-tRNA was monitored by measuring the displacement of PicoGreen, (Molecular Probes, Oregon) diluted 1: 16 250 from 4.5 nM of the fMet-tRNA under the binding conditions described above. This concentration of PicoGreen is half of the concentration that generates a maximal signal (non-linear modeling of the data did not fit either a single site binding model possibly due to the presence of multiple PicoGreen binding sites on the fMet-$tRNA^{(fMet)}$). Fluorescence was measured using a Spectramax Gemini at 480 nm excitation and 530nm emission. Binding values were then converted to percentage bound by correction to a no compound control.
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