Rescue of the streptomycin antibiotic activity by using streptidine as a “decoy acceptor” for the aminoglycoside inactivating enzyme adenylation transferase

Montserrat Latorre, Pablo Peñalver, Julia Revuelta, Juan Luis Asensio, Eduardo García-Junceda* and Agatha Bastida*

The use of streptidine as a “decoy acceptor” allows the antibiotic activity of streptomycin to recover against the E. coli strain overexpressing the aminoglycoside-modifying enzyme 6-O-adenyl transferase.

Emergence of bacterial resistance for all classes of antipathogenic agents has become a serious problem over recent years. Aminoglycosides were one of the first groups of antibiotics to meet the challenge of resistance. Acquired resistance to aminoglycoside antibiotics can occur via three different mechanisms: Mutation of the ribosomal target, reduced permeability for the antibiotics and enzymatic modification of the drugs leading to inactivation. The most prevalent source of clinically relevant resistance is conferred by the third mechanism, the enzymatic inactivation of the drugs. There are well over 50 aminoglycoside-modifying enzymes (AME) that have been characterized at gene level. Genes encoding for these enzymes can be found on the bacterial chromosome, on broad-host-range plasmids or integrated into transposons. These characteristics facilitate the quick dissemination of these genes. The AME can be classified as N-acetyltransferases (AACs), O-adenyl transferases (ANTs) and O-phosphotransferases (APHs). In each of these families are several enzymes that catalyze the reactions with different regioselectivity and substrate specificity. There is strong evidence that inhibitors of AME have the potential to meet the challenge of resistance. A deeper knowledge of the molecular mechanism of the AME and of their structures and interactions with the drugs, is needed to facilitate the design either of effective and potent inhibitors, or of novel aminoglycosides, not susceptible to modification by these enzymes.

ANT family is the smallest of the three groups with only 10 enzymes identified to date, including enzymes that regioselectively adenylate the 6 and 3′′ positions of the streptomycin (1) and the 9 and 3′′ positions of the spectinomycin. Up to now, only the 3D structure of the Staphylococcus aureus ANT(4′), has been determined.

In our group, we are involved in the overexpression and physico-chemical characterization of aminoglycoside modifying enzymes. For this reason, we have overexpressed the aadk gene from Bacillus subtilis by cloning it in the pET28-b(+) vector. The vector pET-aadk-his6 was transformed in the E. coli BL21 (DE3) strain and the recombinant protein purified by Ni2+ affinity column11. The activity of the pure ANT(6) (Scheme 1) was followed by HPLC and the identity of the adenylated product (2) was confirmed by 1H-NMR12.

Here, we report the use of streptidine (3) as a “decoy acceptor” of the ANT(6) to rescue the antibiotic activity of streptomycin (1) against bacterial strains overexpressing this aminoglycoside-modifying enzyme.

![Scheme 1](image-url)

**Scheme 1** Recombinant ANT(6) catalyze adenylation of streptomycin (1). Typical reaction conditions are ATP (10 mM), Streptomycin (10 mM), MgCl2 (10 mM), ANT(6) (10μM) in Tris-HCl buffer (50 mM, pH 7.5).

In our previous paper,12 the antibiotic recognition epitope was determined by STD-NMR (Saturation Transfer Spectroscopy) experiments. The data obtained indicates that positions 1 and 6 in the streptidine moiety are in close contact with the protein binding site. In order to investigate if the streptidine (3) could be O-adenylated by the recombinant enzyme, we obtained the streptidine (3) by acid methanolysis of the streptomycin (1) with H2SO4/MeOH13 (Scheme 2). The streptidine was subjected to...
Scheme 2: Acid methanolysis of streptomycin (1) gives streptidine (3) and methyl dihydrostreptobiosaminidine (4). a) 0.5 g of 1 was dissolved in 4 mL of MeOH containing 0.15 mL of H$_2$SO$_4$. After 48-72 h at room temperature, streptidine (3) appears as a white precipitate.

enzymatic adenylation with ATP and recombinant ANT(6). Formation of the adenylated product (AMP-streptidine) was confirmed by $^1$H-NMR, $^{13}$C-NMR and (ES+) m/z,\textsuperscript{14} The most relevant kinetic parameters for the recombinant ANT(6) are summarized in Table 1. This data strongly suggests that the enzyme presents higher affinity for streptomycin than for streptidine (the two $K_M$ values differ in one order of magnitude) which was expected.

**Table 1. Kinetic parameters of the ANT(6) from **Bacillus subtilis**.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$ (μmol/min/mg)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>0.06</td>
<td>0.04</td>
<td>9.2x$10^6$</td>
</tr>
<tr>
<td>Streptidine</td>
<td>0.0012</td>
<td>0.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

All enzymatic reactions were carried out at: pH=7.5; 25 ºC and 10 μM of enzyme. The concentration of ATP and MgCl$_2$ was fixed at 10 mM when the concentration of streptomycin and streptidine was modified.

In view of this result, we decided to explore if streptidine could act in vivo as a “decoy acceptor” of the ANT(6) allowing the streptocin activity to recover (Table 2).

Streptomycin is a powerful antibiotic with a MIC\textsuperscript{3} of 5 μg/mL\textsuperscript{8} for the *E. coli* BL21(DE3) strain. However, as expected, when the aminoglycoside-modifying enzyme ANT(6) was overexpressed streptomycin completely lost its antibiotic activity (MIC >200 μg/mL). On the other hand, streptidine did not show any antibiotic activity either in presence or absence of ANT(6) (MIC >400 μg/mL). When streptomycin and streptidine were co-administered to the BL21(DE3)/pET-aaadk-his$_5$ strain, a significant decrease in the streptomycin MIC value was observed (Table 2). Thus, when the antibiotic was administered with 50 μg/mL of streptidine, its MIC value dropped to 50 μg/mL, but if the concentration of streptidine was increased to 400 μg/mL the MIC for streptomycin lowered to 10 μg/mL, recovering its antibiotic activity to a great extent. The streptidine/streptocin ratio of 40/1 needed to recover the antibiotic activity of the streptomycin in vivo, is lower than the difference between the $k_{cat}/K_M$ of ANT(6) with both substrates. This could be due to the fact that the simultaneous presence of streptidine and/or AMP-streptidine can limited the overall rate of the reaction with streptomycin.

In conclusion, we have shown that the streptidine moiety of streptomycin, is a substrate for the aminoglycoside inactivating enzyme ANT(6). The addition of this molecule in cell culture restores the activity of the streptomycin antibiotic normally inactivated by the ANT(6) enzyme, because the streptidine competes with the streptomycin acting as a “decoy acceptor” of the ANT(6). Thus, streptidine can be a good starting compound for the design of more efficient “decoy acceptor” of aminoglycoside-modifying enzymes.

This work was supported by the Spanish Ministerio de Educación y Ciencia (Grant CTQ2004-03523/BQU). M.L. thanks to regional government of Madrid for the award of a predoctoral fellowship. The authors acknowledge Dr. P. Armisen for the Ni\textsuperscript{2+}-IDA-agarose gift.

**Notes and references**

\textsuperscript{1} C. A. Smith and E. N. Baker, *Current Drug Targets - Infectious Disorders*, 2002, 2, 143.

**Table 2: In vivo activity\textsuperscript{a}** of streptomycin (1) alone or in combination with streptidine (3) against *E. coli* strains expressing or not the ANT(6).

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>3</th>
<th>1+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>5</td>
<td>&gt;400</td>
<td>5</td>
</tr>
<tr>
<td>BL21(DE3)/pET-aaadk-his$_5$ (ANT(6))</td>
<td>&gt;200</td>
<td>&gt;400</td>
<td>50 (10)$^b$</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Activity of the aminoglycoside antibiotics is expressed by the MIC value (μg/mL). \textsuperscript{b}MIC values for streptomycin in presence of 50 or 400 μg/mL of streptidine.

A complete account of the cloning, over expression and biochemical characterization of the recombinant ANT(6), will be published elsewhere.


AMP-streptidine: ¹H-NMR (D₂O at 500 MHz, pH=3.0), δ=1.06 (t, 1H, J=7.1Hz) 3.45 (m, 5H) 3.90 (dd, 1H, J=9.3Hz, J=18.8Hz) 4.09 (m, 1H) 4.18 (d, 1H, J=1.6Hz) 4.27 (m, 1H) 4.31 (s, 1H) 4.39 (t, 1H, J=4.5Hz ) 4.45 (t, 1H, J=5.4Hz) 6.07 (d, 1H, J=5.2Hz) 8.34 (d, 1H, J=4.5Hz) 8.52 (t, 1H, J=5.7Hz)). ¹³C NMR (D₂O at 125Hz,: 15.3, 58.2, 59.0, 65.5, 70.2, 70.6, 71.6, 74.3, 77.1, 84.3, 88.2, 145.3, 158.0, 158.3). MS (ES⁺) m/z (%): 592.2 [M+H⁺].