# Rescue of the streptomycin antibiotic activity by using streptidine as a "decoy acceptor" for the aminoglycoside-inactivating enzyme adenyl transferase

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

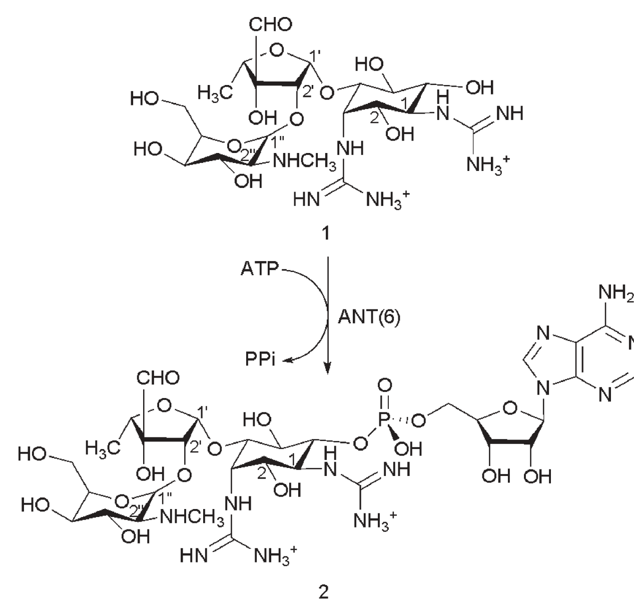
Emergence of bacterial resistance for all classes of antipathogenic agents has become a serious problem over recent years.[1] Aminoglycosides were one of the first groups of antibiotics to meet the challenge of resistance.[2] Acquired resistance to aminoglycoside antibiotics can occur *via* three different mechanisms:[3] 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.[4] There are well over 50 aminoglycoside-modifying enzymes (AME) that have been characterized at the gene level.[5] 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.[6] 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 reverse resistance and rescue antibiotic activity[7] if they are administered together with existing aminoglycosides, following a methodology well established for β-lactam antibiotics.[8] However, 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.[9]

The 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 streptomycin **1** and the 9 and 3″ positions of spectinomycin.[7] Up to now, the 3D structure of only the *Staphylococcus aureus* ANT(4′), has been determined.[10]

In our group, we are involved in the overexpression and physicochemical 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-his*$_6$ was transformed in the *E. coli* BL21 (DE3) strain and the recombinant protein purified using a $Ni^{2+}$ affinity column.[11] The activity of the pure ANT(6) (Scheme 1) was followed by HPLC and the identity of the adenylated product **2** was confirmed by $^1$H-NMR spectroscopy.[12]

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

In our previous paper,[12] the antibiotic recognition epitope was determined by STD-NMR (saturation transfer spectroscopy) experiments. The data obtained indicate that positions 1 and 6 in the streptidine moiety are in close contact with the protein binding site. In order to investigate if streptidine **3** could be O-adenylated by the recombinant enzyme, we obtained streptidine **3** by acid methanolysis of the streptomycin **1** with $H_2SO_4$–MeOH[13] (Scheme 2). The streptidine was subjected to 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* spectroscopies.[14] The most




**Scheme 1** Recombinant ANT(6) catalyzes adenylation of streptomycin **1**. Typical reaction conditions are ATP (10 mM), streptomycin (10 mM), $MgCl_2$ (10 mM), ANT(6) (10 μM) in Tris-HCl buffer (50 mM, pH 7.5).

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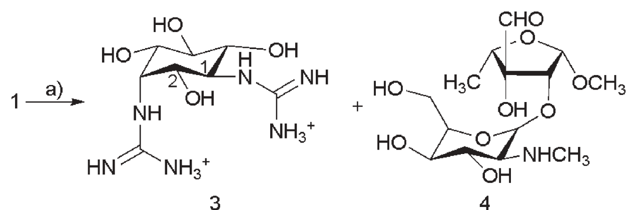


**Scheme 2** Acid methanolysis of streptomycin **1** gives streptidine **3** and methyl dihydrostreptobiosaminidine (**4**). (a) 0.5 g of **1** was dissolved in 4 ml of MeOH containing 0.15 ml of $H_2SO_4$. After 48–72 h at room temperature, streptidine **3** appears as a white precipitate.

relevant kinetic parameters for the recombinant ANT(6) are summarized in Table 1. These data strongly suggest that the enzyme presents higher affinity for streptomycin than for streptidine (the two $K_M$ values differ by one order of magnitude) which was expected.

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

Streptomycin is a powerful antibiotic with an MIC† of 5 μg $ml^{-1}$ 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 $ml^{-1}$). On the other hand, streptidine did not show any antibiotic activity either in presence or absence of ANT(6) (MIC > 400 μg $ml^{-1}$). When streptomycin and streptidine were co-administered to the BL21(DE3)/pET-*aadk-his*$_6$ strain, a significant decrease in the streptomycin MIC value was observed (Table 2). Thus, when the antibiotic was administered with 50 μg $ml^{-1}$ of streptidine, its MIC value dropped to 50 μg $ml^{-1}$, but if the concentration of streptidine was increased to 400 μg $ml^{-1}$ the MIC for streptomycin lowered to 10 μg $ml^{-1}$, recovering its antibiotic activity to a great extent. The streptidine : streptomycin 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 limit 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 could be a good starting compound for the design of more efficient "decoy acceptors" of aminoglycoside-modifying enzymes.

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

| Substrate | $V_{max}$/μmol $min^{-1}$ $mg^{-1}$ | $K_M$/mM | $(k_{cat}/K_M)/s^{-1}$ $M^{-1}$ |
|---|---|---|---|
| Streptomycin | 0.06 | 0.04 | $9.2 \times 10^2$ |
| Streptidine | 0.0012 | 0.6 | 1.2 |

[a] All enzymatic reactions were carried out at: pH = 7.5; 25 °C and 10 μM of enzyme. The concentrations of ATP and $MgCl_2$ were fixed at 10 mM when the concentration of streptomycin and streptidine was modified.

**Table 2** *In vivo* activity[a] of streptomycin **1** alone or in combination with streptidine **3** against *E. coli* strains expressing, or not, ANT(6)

| Strain | 1 | 3 | 1 + 3 |
|---|---|---|---|
| BL21(DE3) | 5 | >400 | 5 |
| BL21(DE3)/pET-*aadk-his*$_6$ (ANT(6)) | >200 | >400 | 50 (10)[b] |

[a] Activity of the aminoglycoside antibiotics is expressed by the MIC value (μg $ml^{-1}$). [b] MIC values for streptomycin in the presence of 50 (or 400) μg $ml^{-1}$ of streptidine.

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## Notes and references

† Minimal inhibitory concentrations (MIC) were measured according to a published method.[15] *E. coli* BL21(DE3) was grown in 1 ml of Mueller–Hinton broth to an optical density ($OD_{600}$) of 0.5 units. The desired concentrations of antibiotic were added from stock solutions. After incubation at 37 °C for 24 h, the $OD_{600}$ of each sample was read. In the case of the recombinant strain BL21(DE3)/pET-*aadk-his*$_6$, the described protocol was slightly modified. The bacteria were grown in 1 ml of Mueller–Hinton broth to an $OD_{600}$ of 0.3 units. Then, temperature was switched to 30 °C, and the culture was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). When the $OD_{600}$ reached 0.5 units, the desired concentrations of antibiotic were added. The samples were incubated at 30 °C for 24 h. In both cases, the MIC was taken as the lowest antibiotic concentration inhibiting bacterial growth by greater than 90%.

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14 AMP–streptidine: $^{1}$H-NMR ($D_2O$ at 500 MHz, pH = 3.0) $\delta$ = 1.06 (t, 1H, $J$ = 7.1 Hz), 3.45 (m, 5H), 3.90 (dd, 1H, $J$ = 9.3, 18.8 Hz), 4.09 (m, 1H), 4.18 (d, 1H, $J$ = 1.6 Hz), 4.27 (m, 1H), 4.31 (s, 1H), 4.39 (t, 1H, $J$ = 4.56 Hz), 4.45 (t, 1H, $J$ = 5.4 Hz), 6.07 (d, 1H, $J$ = 5.2 Hz), 8.34 (d, 1H, $J$ = 4.5 Hz), 8.52 (t, 1H, $J$ = 5.7 Hz); $^{13}$C-NMR ($D_2O$ at 125 Hz) $\delta$ = 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 $[M + H]^{+}$.

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