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

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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.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 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 activity⁷ 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

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.⁷ Up to now, only the 3D structure of the *Staphylococcus aureus* ANT(4'), has been determined.¹⁰

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 Recombinant ANT(6) catalyze adenylation of streptomycin (1). Typical reaction conditions are ATP (10 mM), Streptomycin (10 mM), MgCl₂ (10 mM), ANT(6) (10 μ M) 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

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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 H₂SO₄. 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 adenylation product (AMP-streptidine) was confirmed by ¹H-NMR, ¹³C-NMR and (ES+) *m/z*.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*.

Substrate	<i>V_{max}</i> (μmol/min/mg)	<i>K_M</i> (mM)	<i>k_{cat}/K_M</i> (s ⁻¹ × M ⁻¹)
Streptomycin	0,06	0,04	9,2 × 10 ²
Streptidine	0,0012	0,6	1,2

All enzymatic reactions were carried out at: pH=7.5; 25 °C and 10 μM of enzyme. The concentration of ATP and MgCl₂ 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 streptomycin activity to recover (Table 2).

Streptomycin is a powerful antibiotic with a MIC[†] of 5 μg/mL 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-*aadk-his₆* 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/streptomycin

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

Strain	1	3	1+3
BL21(DE3)	5	>400	5
BL21(DE3)/pET- <i>aadk-his₆</i> (ANT(6))	>200	>400	50 (10) ^b

^aActivity of the aminoglycoside antibiotics is expressed by the MIC value (μg/mL). ^bMIC values for streptomycin in presence of 50 or 400 μg/mL of streptidine.

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.

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Notes and references

- † Minimal inhibitory concentration (MIC) was measured according to a published method.¹⁵ *E. coli* BL21(DE3) was grown in 1 mL of Mueller-Hinton broth to an optical density (OD₆₀₀) of 0.5 units. The desired concentrations of antibiotic were added from stock solutions. After incubation at 37 °C for 24 h. the OD₆₀₀ of each sample was read. In the case of the recombinant strain BL21(DE3)/pET-*aadk-his₆*, the described protocol was slightly modified. The bacteria was grown in 1 mL of Mueller-Hinton broth to an OD₆₀₀ of 0.3 units. Then, temperature was switched to 30 °C, and the culture was induced with 0.5 mM IPTG. When the OD₆₀₀ 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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- 11 A complete account of the cloning, over expression and biochemical characterization of the recombinant ANT(6), will be published elsewhere.
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- 13 R. L. Peck, R. P. Graber, A. Walti, E. W. Peel, C. E. Hoffhine, K. Folkers, *J. Am. Chem. Soc.*, 1946, **68**, 29. Streptidine; ¹H-NMR (500 MHz, D₂O, pH=3,0 δ 3.25-3.45 m, 6H) and MS (ES+) *m/z* 263 [M+H]⁺.
- 165 14 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=4.56Hz) 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]⁺.
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