EFFECTS OF *N*-ALKYLATION AND *N*-ACYLATION ON TOBRAMYCIN ACTIVITY

S. Sicsic, J. F. Le Bigot, C. Vincent, C. Cerceau and F. Le Goffic

C.N.R.S.-C.E.R.C.O.A. (laboratoire associé à l'E.N.S.C.P.)
2 à 8, rue Henry Dunant 94320, Thiais, France

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The activities of tobramycin derivatives acetylated and ethylated on the 6′-*N*, 2′-*N* and 3-*N* positions were examined. The MICs of these derivatives against tobramycin sensitive strains indicated that 2′-*N*-ethylated and 6′-*N*-ethylated derivatives have a fairly good activity, and confirmed that the 3-*N* position is the most important one for antibiotic activity since 3-*N* derivatives were less active. The MICs of these derivatives against tobramycin resistant strains, and their inactivation by tobramycin modifying enzymes were examined. These results showed that 2′-*N* or 6′-*N* ethylation protects the drug against inactivation by AAC(2′) or AAC(6′), respectively, and 2′-*N*-ethyltobramycin and 6′-*N*-ethyltobramycin were active against strains containing these modifying enzymes. On the other hand, 3-*N* ethylation protects the drug against inactivation by AAC(3) but 3-*N*-ethyl tobramycin does not inhibit strains containing this enzyme.

The number and the positions of amino functions in the aminoglycoside antibiotics are the most important parameters accounting for their powerful antibiotic activity[1,2]. In the deoxystreptamine group of these drugs, the relative importance of the amino functions has been evaluated and classified as follows: 3-amino>6′-amino>2′-amino in terms of their relative activities on protein synthesis[3]. The relative importance of amino groups has also been evaluated from MIC values[4].

These amino moieties are also involved in the resistance of microorganisms to the drugs, since they are modified by *N*-acetylation catalyzed by plasmid mediated enzymes. They are also involved in drug transport mechanisms which are closely related to ribosomal binding[5].

In this work, we have compared the activities of some *N*-substituted derivatives of tobramycin on sensitive and resistant strains. The initial assumption was that if the substitution of one amino group protects this site against further acetylation and if the resistance of strains harboring AAC enzymes is due solely to this acetylation, then this *N*-substituted tobramycin should have the same activity against the resistant strain as against the sensitive one. We should keep in mind that this comparison is strictly valid only if the sensitive strains are isogenic with their resistant counterparts.

In order to discuss our results in terms of resistance mechanisms, we have measured the ability of these compounds to be substrates of some aminoglycoside modifying enzymes.

Materials and Methods

Tobramycin Acetylated Derivatives

2′-*N*-Acetyltobramycin (2′-*N*-AcTo), 6′-*N*-acetyltobramycin (6′-*N*-AcTo), 3-*N*-acetyltobramycin (3-*N*-AcTo) were enzymatically synthesized, 2′,6′-*N*,*N*′-diacetyltobramycin (2′,6′-*N*,*N*′-diAcTo) was chemically synthesized as previously described[6].

Tobramycin Ethylated Derivatives

All the *N*-ethylated derivatives were obtained by reduction of the acetylated corresponding molecule

by diborane. The general procedure used was as follows: the *N*-acetylated derivative (20~100 mg) was neutralized by 50 mM CF_3COOH. The solution thus obtained was evaporated to dryness under vacuum, and the residue dissolved in 30 ml of a 1 M solution of diborane in THF. It was then refluxed for 12 hours. Then, 5 ml of water was added slowly, the pH of the solution was brought to 10.5 and the solution was evaporated to dryness under vacuum. The residue was then dissolved in 5 ml of water and applied to a CG-50 cation exchange column (NH_4^+ form, 100~200 mesh, 30×2 cm). Elution was performed with a gradient of ammonia from 0.1 to 1 M. The purity of the derivatives obtained was checked by TLC on silica gel plates (MeOH - 20% NH_4OH, 4: 1) and their structure was determined by ^{13}C NMR spectroscopy in D_2O at pH 1 with dioxane as internal standard. Compared with the spectra of the *N*-acetyl derivatives[6], those of the reduced products show the disappearance of the C signals of the acetyl groups and the appearance of the C signals of the ethyl groups as follows: 6′-*N*-ethyltobramycin (6′-*N*-EtTo), δ=47.3 ppm (C of CH_2) and δ=10.2 ppm (C of CH_3); 2′-*N*-ethyltobramycin (2′-*N*-EtTo), δ=40.9 ppm (C of CH_2) and δ=10.4 ppm (C of CH_3); 3-*N*-ethyltobramycin (3-*N*-EtTo), δ=41.2 ppm (C of CH_2) and δ=10.5 ppm (C of CH_3); 2′,6′-*N*,*N*′-diethyltobramycin (2′,6′-*N*,*N*′-diEtTo), δ=40.8 ppm and 47.4 ppm (C of the two CH_2) and δ=9.6 ppm and 10.3 ppm (C of the two CH_3).

Bacterial Strains

The MRE600 *E. coli* came from the Microbial Research Establishment (England). The other bacterial isolates had been collected over the past 7 years.

Susceptibility Tests

An inoculum of 10^5 colony forming units was used. Minimal inhibitory concentrations (MICs) were determined by two fold dilution of the antibiotics on MUELLER-HINTON agar (Baltimore Biological Laboratory, BBL) by using a multiple inoculating device (multipoint inoculator-Denley Tech., England).

In Vitro Poly U Directed Polyphenylalanine Biosynthesis

The effect of tobramycin derivatives on polyphenylalanine biosynthesis was carried out as described previously[7]. Factors contained in an S_{150} ultracentrifugation fraction[8] and 70 S "tight" ribosomes were prepared as described earlier[9].

Misreading Induction Evaluation in Polyphenylalanine Biosynthesis

Polyphenylalanine synthesis was carried out with unlabelled phenylalanine in the presence of 10 μl of 0.5 mM (^{14}C) L-leucine (specific activity 12.5 Ci/mole).

Modification of Tobramycin Derivatives by Aminoglycoside Modifying Enzymes

The enzymes used in the assays have been isolated as described earlier[10] from appropriate strains. The assays were carried out by measuring initial rates at pH values varying from 5.5 to 8.5. The amount of modified aminoglycoside antibiotic was determined by the phosphocellulose paper binding assay[2] using appropriate radiolabelled cofactors.

Results

Evaluation of the Antibacterial Activity of Tobramycin Derivatives against Tobramycin-sensitive Strains

Table 1 summarizes the activities of the synthesized molecules against different tobramycin sensitive strains. The Table also indicates relative activities with tobramycin activity as a reference. As previously described[3], monoacetylation results in a decrease in antibiotic activity of the drugs, the importance of this effect decreasing in the order 3-*N*>6′-*N*>2′-*N*. Monoethylation results also in a decrease of antibiotic activity with the same order but the effect is clearly weaker. Disubstitution on 2′-*N* and 6′-*N* produces effects which are the addition of the effects observed separately on each position.

Effects of the Tobramycin Derivatives on "*In Vitro*" Polyphenylalanine Synthesis and on Misreading Induction of Poly U

Table 1. MICs and relative activities (in parentheses) of the tobramycin derivatives against tobramycin sensitive strains.

Strain	Tobramycin	2′-*N*-AcTo	2′-*N*-EtTo	6′-*N*-AcTo	6′-*N*-EtTo	3-*N*-AcTo	3-*N*-EtTo	2′,6′-*N*,*N*′-diAcTo	2′,6′-*N*,*N*′-diEtTo
E. coli [a]	0.5 (1)	32 (64)	2 (4)	128 (256)	4 (8)	256 (512)	8 (16)	>2000 (>4000)	16 (32)
Staphylococcus aureus [b]	0.125 (1)	8 (64)	0.5 (4)	32 (256)	1 (8)	64 (512)	2 (16)	>500 (>4000)	4 (32)
Pseudomonas aeruginosa [b]	0.5 (1)	[c]	4 (8)	[c]	6 (8)	[c]	8 (16)	[c]	32 (64)
Serratia marcescens	2 (1)	512 (256)	4 (2)	512 (256)	4 (2)	1500 (750)	32 (16)	>8000	8 (4)
Proteus inconstans	4 (1)	[c]	8 (2)	[c]	16 (4)	[c]	64 (16)	[c]	64 (16)

[a] Mean of the results of 5 strains.
[b] Mean of the results of 2 strains.
[c] These assays were not run for lack of *N*-acetyl derivatives.

As one of the most important effects of the aminoglycoside antibiotics is inhibition of protein synthesis, we have assayed the effect of tobramycin derivatives on *in vitro* polyphenylalanine synthesis using *E. coli* ribosomes. Drug concentrations necessary to induce 50% inhibition of protein synthesis (IC_{50}) are listed in Table 2. Drug concentrations necessary to induce maximum misreading of poly U (C_M) are also listed. This Table shows that *N*-substitution produces parallel effects at ribosomal and intact cell levels.

Table 2. Inhibition of the "*in vitro*" polyphenylalanine biosynthesis, induction of misreading of poly U by the tobramycin derivatives and MICs (μg/ml) found with *E. coli* strains.

Antibiotic	IC_{50}(μM)	C_M(μM)	MIC (μg/ml)
Tobramycin	64	10	0.5
2′-*N*-EtTo	68	18	2
6′-*N*-EtTo	80	62	4
3-*N*-EtTo	100	190	8
2′,6′-*N*,*N*′-diEtTo	200	510	16
2′-*N*-AcTo	200	$>10^3$	32
6′-*N*-AcTo	280	$>10^3$	128
3-*N*-AcTo	360	$>10^3$	256
2′,6′-*N*,*N*′-diAcTo	$>10^3$	$>10^3$	>2000

Activity of Tobramycin Derivatives against Tobramycin Resistant Strains

Table 3 reports the MICs of the tobramycin derivatives against tobramycin resistant strains producing aminoglycoside modifying enzymes. In each case, an index termed I was calculated (I=MIC against resistant strain/MIC against sensitive strain) which expresses the loss of activity of a derivative as compared with its activity against a corresponding sensitive strain. A high value for I indicates a dramatic loss of antibiotic activity. Conversely, an I value of about one indicates no inactivation of the derivative by the resistant strain. As indicated, 6′-*N* derivatives are not inactivated by *E. coli* R_5 harboring an AAC(6′) and 2′-*N*-ethyl derivatives are not inactivated by *Proteus inconstans* PV 164 harboring an AAC(2′), but 3-*N* derivatives have weaker activities against strains harboring an AAC(3) than against strains which do not possess this enzyme.

Inactivation of Tobramycin Derivatives with Aminoglycoside Modifying Enzymes

Table 4 indicates the susceptibility of the derivatives to be substrates of AAC and ANT enzymes at the optimum pH in each case.

Table 3. MICs and I[a] values (in parentheses) of the tobramycin derivatives against tobramycin resistant strains.

Strain[b]	Tobra-mycin	2'-*N*-AcTo	2'-*N*-EtTo	6'-*N*-AcTo	6'-*N*-EtTo	3-*N*-AcTo	3-*N*-EtTo	2',6'-*N*,*N* diEtTo
E. coli R_{176} AAC (3)	8 (16)	512 (16)	16 (8)	1024 (8)	32 (8)	*>4000 (>15)*	*128 (16)*	64 (4)
Serratia marcescens 22522 AAC(3)	128 (64)	d)	128 (32)	d)	128 (32)	*>4000 (>5)*	*2048 (64)*	126 (16)
E. coli R_5 AAC(6')I	32 (64)	512 (16)	128 (64)	*128 (1)*	*4 (1)*	>2048 (>8)	256 (32)	*8 (0.5)*
P. inconstans PV 164 AAC(2')	64 (16)	>2048	*16 (2)*	>2048	2048 (126)	>4000	2048 (32)	*64 (1)*
E. coli R_{55} ANT(2'')	32 (64)	512 (16)	32 (16)	2048 (16)	32 (8)	>4000 (>15)	1024 (128)	16 (1)
Staphylococcus aureus[c] ANT(4')	256 (2048)	512 (64)	170 (340)	512 (16)	64 (64)	>2048 (>32)	4096 (2048)	128 (32)

a) I values are calculated as $I = \frac{\text{MIC against resistant strain}}{\text{MIC against sensitive strain}}$

b) The type of aminoglycoside modifying enzymes produced by each strain is indicated.

c) Mean of the results of 3 resistant strains.

d) These assays were not run for lack of *N*-acetyl derivatives.

Table 4. Modification of tobramycin derivatives by aminoglycoside modifying enzymes.

Aminoglycoside modifying enzyme (strain)	Tobra-mycin	2'-*N*-AcTo	2'-*N*-EtTo	6'-*N*-AcTo	6'-*N*-EtTo	3-*N*-AcTo	3-*N*-EtTo	2',6'-*N*,*N*' diEtTo
AAC (3)[a]	100	30	40	0	25	0	10	15
AAC (6')[b] I	100	80	90	0	0	0	25	0
AAC (2')[c]	100	0	25	10	15	not run		35
ANT (2'')[d]	100	120	125	50	30	90	110	45
ANT (4')[e]	100	15	100	0	75	0	35	75

Percentage of modification was calculated by comparison with tobramycin. The experiments were performed so that initial rates were measured.

a) From *E. coli* R_{176}; b) from *E. coli* R_5 and *Staphylococcus* Palm; c) from *Proteus inconstans* PV164; d) from *E. coli* R_{55}; e) from *Staphylococcus* Riv, Steph and APO1.

Discussion

The aim of the present study was to evaluate the effect of *N*-substitution on the biological properties of tobramycin and to discuss the results thus gathered in terms of resistance phenomena. In this respect, we have assayed two types of *N*-substituted derivatives: those where the substituent is an electron withdrawing group (acetyl residue) and those where the substituent does not modify the nucleophilic character of the molecule, but introduces a steric hindrance on the nitrogen atom (ethyl residue).

From Table 1, it can be seen that, in terms of antibiotic activity, the decreasing importance of amino groups which was previously suggested[8], 3-*N*>6'-*N*>2'-*N*, is valid from an electronic as well as a steric point of view. It is also obvious that the electronic effect produced by a substituent is much more important than its steric hindrance, since the *N*-ethyl derivatives are 16 to 32 times more active than the corresponding *N*-acetyl derivatives, and the ethyl monosubstitution induces only 2 to 16 fold decrease of activity.

The activities of the tobramycin derivatives (expressed in Table 2) on ribosomal function as inhibition of polyphenylalanine biosynthesis and induction of poly U misreading follow the same order as that observed on their MICs.

All these results are consistent with those previously described about the effects of *N*-acetylation on the activity of gentamicin on bacterial ribosomes[3] and the effects of *N*-ethylation on the antibiotic activity of kanamycin A and B[4].

The expected behavior of the tobramycin derivatives against sensitive strains and on *E. coli* ribosomes allowed us to assay these derivatives against resistant strains with the assumption described in the introduction. It was expected that the 2′-*N*-ethyl derivative should have about the same activity on sensitive *Proteus inconstans* and on resistant *Proteus inconstans* PV164 harboring an AAC (2′). 6′-*N* Derivatives would have about the same activities on sensitive *E. coli* and on resistant *E. coli* R_5 harboring an AAC (6′). 3-*N* Derivatives would have about the same activities on sensitive *E. coli* and *Serratia* and on *E. coli* R_{176} and *Serratia* 22522 respectively harboring an AAC (3).

Table 3 shows that this assumption is fully verified for 2′-*N*-ethyl and 6′-*N*-derivatives, but not for 3-*N* derivatives. Now, let us compare these results with those described in Table 4: 6′-*N* derivatives are not acetylated "*in vitro*" by AAC (6′) I and their MICs against resistant *E. coli* R_5 and against sensitive *E. coli* are identical. These results strongly suggest that the resistance of *E. coli* R_5 is essentially due to the presence and the activity of AAC(6′) toward tobramycin. 2′-*N*-Ethyl derivative is a poor substrate of AAC(2′), though its MICs against resistant *Proteus inconstans* PV164 and against sensitive *Proteus inconstans* are identical. A possible explanation of this discrepancy given previously by many workers[5] is that the rate of drug transport is higher than its rate of enzymatic modification. Here also our results suggest that the resistance of *Proteus inconstans* PV164 is essentially due to the presence and the activity of AAC(2′) toward tobramycin. Conversely, our assumption is not verified for the 3-*N* derivatives, since Table 3 shows that the MICs of 3-*N*-ethyltobramycin which are 8 and 32 against sensitive *E. coli* and *Serratia marcescens* respectively (Table 1), increase to 128 and 2048 against *E. coli* R_{176} and *Serratia marcescens* 22522 harboring an AAC(3) (Table 3). The same phenomenon can be pointed out for 3-*N*-acetyltobramycin. However, Table 4 clearly indicates that 3-*N* derivatives are not substrates for AAC(3). These results suggest strongly that the presence and the activity of AAC(3) in *E. coli* R_{176} and *Serratia marcescens* 22522 are not sufficient to explain the resistance of these bacteria to tobramycin. As previously reported[11,12], it is likely that the presence of the R plasmid coding for AAC(3), in *E. coli* R_{176} and in *Serratia marcescens* 22522 affects directly the cell envelope and as a consequence modifies drug transport. The comparison of other results can be explained if we consider the presence and the activity of modifying enzymes, the drug transport, and also the competition between modification rate and transport rate of the drug[5,13].

Moreover, 3-*N*-ethyltobramycin has a singular behavior: it is modified by ANT(4′) and AAC(6′) less than 2′-*N*-ethyltobramycin and tobramycin; yet, I values are never weaker towards the former compound. These results suggest that 3-*N*-ethyltobramycin is more sensitive than 2′-*N*-ethyltobramycin and tobramycin to modification of drug transport.

If we now consider the effect of *N*-substitution on the efficiency of aminoglycoside modifying enzymes, it can be seen that 6′-*N*- and 3-*N* positions are more important than 2′-*N* position since 2′-*N*-ethyltobramycin is a substrate of almost all enzymes and 2′-*N*-acetyltobramycin a substrate of AAC(6′)I, ANT (2″) and AAC(3)IV. 6′-*N*-Acetyltobramycin, 3-*N*-acetyltobramycin and 3-*N*-ethyltobramycin are only modified by ANT (2″) and 6′-*N*-ethyltobramycin is a substrate of AAC(2′) and ANT(4′).

It is noteworthy that ANT(2″) is little or not influenced by monosubstitution at 3-*N*, 6′-*N* and 2′-*N* positions, since even the acetylated derivatives are substrates; and also that 2′,6′-*N*,*N*′-diethyltobramycin is the only derivative to retain a fairly good antibiotic activity on *E. coli* R_{55}.

In conclusion, the present work provides some additional arguments that 3-*N*>6′-*N*>2′-*N* is the decreasing order of importance of the amino groups of aminoglycosides in the expression of antibiotic activity, effectiveness at ribosomal level, and susceptibility of the drug to be transformed by plasmid mediated modifying enzymes. It also provides additional arguments that in some cases, the presence and activity of modifying enzyme is sufficient to explain strain resistance, but that in other cases we must put forward a modification of drug transport which makes it particularly sensitive to modifications on the 3-*N* position.

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