HAVE DEOXYSTREPTAMINE AMINOGLYCOSIDE ANTIBIOTICS THE SAME BINDING SITE ON BACTERIAL RIBOSOMES ?

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(^{3}H) Tobramycin was used as a probe to determine the relationship between the structure of aminoglycoside antibiotics and their ability to remove this drug from its higher affinity binding site on the ribosome.   The dissacharide moieties (neamine, tobramine, gentamine) appeared to have a common binding site, whereas the kanosamine, garosamine and ribose moieties determined the specificity of this binding.   Amikacin and butikacin behaved in an anomalous manner in spite of their close structural relationship to tobramycin.

Biochemical experiments have recently demonstrated that those aminoglycoside antibiotics with deoxystreptamine and kanosamine moieties possess two types of binding sites on the bacterial ribosome.[1,2]   When the binding experiments were carried out with the ribosomal subunits two types of binding sites were also found on the 50 S subunit whereas only one type of binding site was located on the 30 S particle.[3]

The question then arises as to whether all aminoglycoside antibiotics possessing a deoxystreptamine moiety glycosidically bound to other aminosugar residues have the same receptor site.   The present study tries to answer this important question.

Materials and Methods

Chemicals: (^{3}H) Tobramycin was synthesized as described previously[3] and had a specific radioactivity of 5,000 Ci/mole.   Putrescine and spermidine were from Sigma.

Antibiotics were furnished thanks to the following laboratories: tobramycin, apramycin (Eli Lilly), kanamycin A, kanamycin B, kanamycin C, amikacin (Bristol Myers), dibekacin (Roger Bellon), neamine, neomycin, streptomycin (Roussel-Uclaf), gentamicin C_{1A} (Unilabo), ribostamycin (Delalande), butikacin (Pfizer), fortimicin A, sorbistin, seldomycin (Abbott).

Biological material: Tight and run-off ribosomes were prepared by zonal centrifugation as described previously[3] from an S_{30} fraction of Escherichia coli MRE 600 strain.

Methods: The binding experiments were conducted by equilibrium dialysis[2,3] with a dianorm apparatus (rotating cells) using total volume of 500 μl.   Two compartments (250 μl each) were formed by a semi-permeable membrane (Visking) which had been pre-soaked in the following buffer: 20 mM Tris-HCl, pH 7.8, 10 mM Mg $(OAc)_2$, 100 mM NH_4Cl.   200 μl of the ribosomal preparation were introduced in one compartment and 200 μl of the antibiotics ((^{3}H) tobramycin+drug assayed) in the other.

When equilibrium was reached (6 hours, 20°C) 100 μl were removed from each compartment and the radioactivity determined by liquid scintillation in 10 ml of Bray's solution.   The isotopic dilution method[3] was used; the experiment was performed with constant concentrations of ribosomes (4.6×10^{-7} M) and (^{3}H) tobramycin (2×10^{-8} M) and increasing concentrations (from 0 to 10^{-3} M) of the drug chosen for the assay.

The expression $(D_R - D_L/D_R + D_L) = f(\text{drug})$ is used to represent the results obtained where D_R and D_L are the d.p.m. in the ribosomal and antibiotic compartments.[3]   These curves allow us to determine

EC 50 (the quantity of drug required to remove 50% of (^{3}H) tobramycin bound to the ribosome).

Results

A sample of (^{3}H) tobramycin of high specific radioactivity (5,000 Ci/mole) has been used as a probe to classify the aminoglycoside antibiotics with respect to their ability to remove this drug from its higher affinity binding site on bacterial ribosomes.

A small quantity of (^{3}H) tobramycin (1/10 K_D for the higher affinity group of binding site) has been employed to ensure that almost all the radioactive drug binds specially to the high affinity ribosomal site. The results of the competition experiments, using different unlabelled antibiotics are shown on Fig. 1.

Three different groups of molecules have been detected, with respect to their behaviour in competition. The first includes molecules which are closely related to tobramycin, such as kanamycin A (Fig. 1A), kanamycin B, dibekacin. They are able to remove bound tobramycin from the bacterial ribosome with high efficiency.

The second group of molecules includes kanamycin C, neamine, the gentamicins (Fig. 1B), seldomycin, ribostamycin, neomycin and butikacin (Fig. 1C). These antibiotics are able to remove bound tobramycin from the bacterial ribosome to a moderate extent. The third group of drugs studied includes

Fig. 1. General profiles of the results gathered during competition experiments (see Materials and Methods and Reference[3] for experimental procedures).
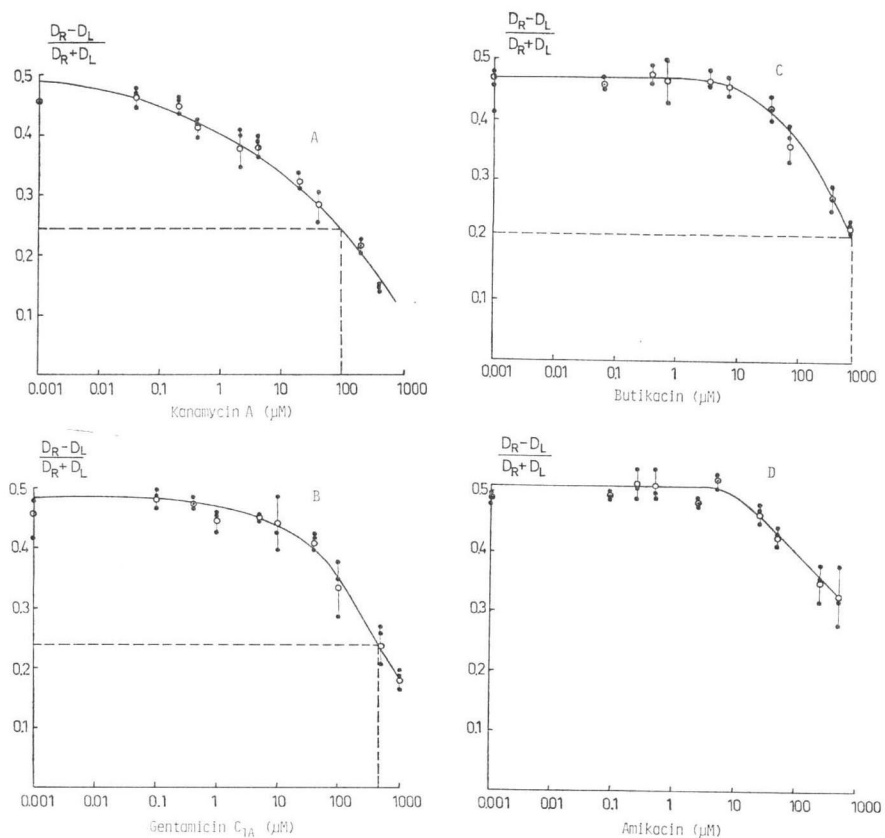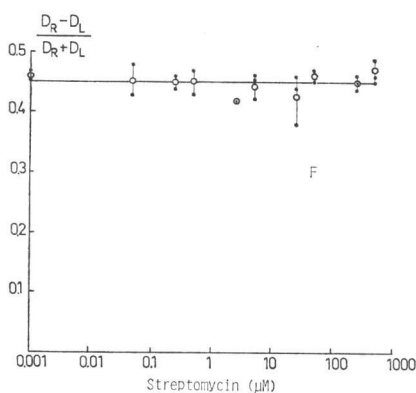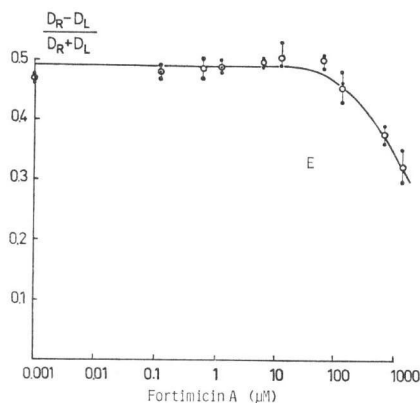
Fig. 1. (continued)

streptomycin (Fig. 1F), amikacin (Fig. 1D), the sorbistins, fortimicin (Fig. 1E) and the poly-amines, putrescine and spermidine. These molecules are ineffective at removing tobramycin (if at all) from its high affinity binding sites on the ribosome.

These results have been summarized in Table 1 in which their efficiency in competing with (^{3}H) tobramycin binding on the ribosome is expressed as EC 50 in micromoles.

Discussion

We have already demonstrated that high specific activity radioactively labelled tobramycin can bind to the bacterial ribosome in two ways. This in turn uncovered two groups of binding sites: a single high-affinity binding site with the following characteristics (K_D: 0.2×10^{-8} M, n: 1) and a secondary group of binding sites which has the following properties (K_D: 10^{-5} M, n: 30). We have now used traditional isotopic dilution methods as a means of classifying a variety of different aminoglycoside antibiotics in terms of their ability to remove tobramycin from its primary binding site on the ribosome. A concentration of this drug (1/10 K_D: 2×10^{-8} M) was

Fig. 2. The structures of the aminoglycoside antibiotics.

1: those having a kanosamine moiety. $R_1=H$, $R_2=NH_2$, $R_3=H$, $R_4=OH$: tobramycin; $R_1=H$, $R_2=R_3=R_4=OH$: kanamycin A; $R_1=H$, $R_2=NH_2$, $R_3=R_4=OH$: kanamycin B; $R_1=H$, $R_2=NH_2$, $R_3=R_4=H$: dibekacin; $R_1=HABA$, $R_2=R_3=R_4=OH$: amikacin; $R_1=-CH_2-CH-CH_2-CH_2NH_2$, $R_2=R_3=R_4=OH$: butikacin.
$\overset{|}{OH}$

2: gentamicin C_{1A}.

3: ribostamycin.

The arrows show how an amino sugar residue can rotate around a glycosidic linkage.
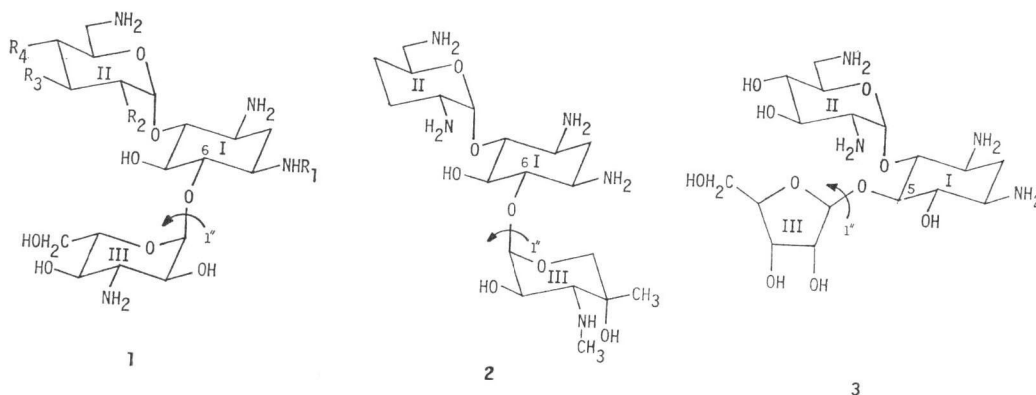
Table 1. Classification of the aminoglycoside antibiotics in relation to their ability to remove (^{3}H) tobramycin from its primary binding site. EC 50 (μ moles) is the concentration of drug necessary to remove half of the radioactivity bound.

Group I	EC 50 μM	Group II	EC 50 μM	Group III	EC 50 μM
Tobramycin	50	Neamine	250	Amikacin	>1,000
Kanamycin B	50	Kanamycin C	400	Fortimicin A	>1,000
Dibekacin	50	Gentamicin C_{1A}	400	Sorbistin	>1,000
Kanamycin A	100	Ribostamycin	600	Streptomycin	∞
		Seldomycin	400	Putrescine	>1,000
		Butikacin	900	Spermidine	∞
		Neomycin	500		
		Apramycin	800		

chosen to make sure that only strongly bound tobramycin would be removed from its target and these experiments have allowed us to classify three different groups of aminoglycosides depending on their ability to remove this molecule from its target site on the ribosome.

In the first group, are molecules related in structure to tobramycin like kanamycin B, dibekacin or kanamycin A. We should mention that the presence of hydroxyls on the 3' or 4' positions is of little importance with respect to competition. On the contrary, the number of amino groups present on the aminosugar residues of these molecules is very important. Therefore, kanamycin A, which possesses only four amino functions, is a worse competitor than kanamycin B or dibekacin.

In the second group of drugs, are found molecules as different as neamine, kanamycin C, gentamicin C_{1A} (Fig. 1B), ribostamycin, neomycin, butikacin (Fig. 1C), apramycin or seldomycin; these molecules can remove half the radioactivity in tobramycin from the bacterial ribosome at concentrations from 250 to 900 μM.

Neamine, with its four amino groups, allows us to evaluate the contribution of the bicyclic system to this competition. Kanamycin C, which has only three amino functions, behaves poorly in eliminating radioactive tobramycin. Gentamicin C_{1A} is an even worse competitor than neamine. This fact means that, given the contribution of the garosamine moiety to be zero in this competition, it can also prevent binding to some extent. It is interesting to compare dibekacin and gentamicin C_{1A} in terms of competitive power. Both molecules have a common bicyclic system (gentamine moiety); even so, there is a dramatic difference in their behaviour. This allows the relative contribution of the kanosamine and garosamine moieties to be evaluated in this competition experiment. The conclusion to be drawn is that both molecules probably have part of their binding site (the gentamine moiety) in common on the bacterial ribosome. In addition, dibekacin and its congeners have a specific ribosomal target for their kanosamine residue, whereas the garosamine moiety of the gentamicin components is located elsewhere on the ribosome (Fig. 2). The other gentamicins (data not shown) behave as gentamicin C_{1A}.

Ribostamycin and neomycin are also poor competitors for tobramycin binding as shown by their EC 50. This means that, in spite of their neamine moieties which they share in common with kanamycins A and B, the ribose residues glycosidically linked to the 5 hydroxyl group of deoxystreptamine must interfere with their ability to remove (^{3}H) tobramycin from the ribosome to a large extent, even more so than the garosamine moiety does in gentamicin C_{1A}. This implies that a sugar residue, covalently bound on the 5 hydroxyl group of neamine, can prevent either the recognition or the entry of the drug residue in the ribosome binding site of tobramycin. In spite of the fact that it has 5 amino functions, butikacin has a poor ability to remove (^{3}H) tobramycin. This property could perhaps be explained by the following reason: Its side arm, which is a hydroxy-2 putrescine moiety would modify the overall conformation of the molecule, due to yet unknown interactions. Finally, neither putrescine nor spermidine has shown any removal property in the experiments described here.

The behaviour of amikacin was unexpected. This molecule was synthesized with the objective of preventing the enzymatic inactivation of kanamycin A by aminoglycoside inactivating enzymes, and due

to its close relationship with kanamycin A or tobramycin, amikacin was expected to remove tobramycin from its binding site as well as, if not better than, kanamycin A, because amikacin's structure has five amino groups, whereas kanamycin A has only four.   This unexpected result leads us to conclude that amikacin, in spite or because of its HABA chain on the 1-amino group could have, like butikacin, a binding site on the ribosome which does not fit with that of the other deoxystreptamine aminoglycoside antibiotics.   Binding experiments with radioactive amikacin are necessary to verify this assumption.

Fortimicin A and sorbistin as well as apramycin are far in structural terms from tobramycin, and are unable to remove tobramycin from its specific binding site.   Streptomycin is also unable to remove tobramycin from the ribosome.   Based on previous results, this comes as no surprise for FLAKS[4] and LANDO[5] have already shown that this drug will bind to the 30 S subunit and we ourselves have demonstrated[3] that tobramycin binds to 50 S subunit better than to 30 S particle.

Conclusion

With the exception of amikacin and butikacin, we conclude from these experiments that the aminoglycoside antibiotics possessing a deoxystreptamine moiety probably have part of their binding site in common: the neamine, tobramine, gentamine, paromamine residues.   This binding is almost certainly determined by the number of amino groups present.   Four amino functions provide tight binding whereas three amino functions give these molecules a lower binding capacity.   The recognition property is probably determined by the third amino sugar residue of these tricyclic antibiotics.   Thus, we can imagine that the kanosamine, the garosamine or the ribose residues rotate around either the $6-1''$ or the $5-1''$ linkage as shown in Fig. 2.

Nonetheless, additional experiments are obviously necessary in order to verify this hypothesis and to understand the particular behaviour of amikacin and butikacin; these are underway in the laboratory.

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