New Derivatives of Kanamycin B Obtained by Modifications and Substitutions in Position 6″. 2. In Vitro and Computer-Aided Toxicological Evaluation with Respect to Interactions with Phosphatidylinositol

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In a companion paper (previous paper in this issue), we report on the synthesis and microbiological evaluation of new derivatives of the aminoglycoside antibiotic kanamycin B carrying substitutions in 6″ (halogeno, or amino, amido, thioalkyl, and alkoxy groups, each series with increasingly bulkier chains). These modifications were intended to potentially modulate the interactions of kanamycin B with phospholipids since these are related to inhibition of lysosomal phospholipase activities and lysosomal phospholipidosis, an early and predictive index of the nephrotoxic potential of aminoglycosides. The new derivatives were therefore examined for inhibitory potency in vitro toward lysosomal phospholipase A_1 acting on phosphatidylcholine included in negatively charged liposomes. No simple correlation was observed between the nature or the size of the 6″-substituent and the inhibitory potencies of the corresponding derivatives, although certain groups (diethylamino, isopropylthio) caused a significant increase in inhibitory potency, whereas an *N*-acetyl-*N*-methylamino substituent had the opposite effect. 6″-Deoxy-6″-chlorokanamycin B, however, was the only derivative showing both a decrease (albeit limited) of inhibitory potency toward phospholipase A_1 associated with the maintenance of a satisfactory microbiological activity (actually equal or slightly better than that of kanamycin B). Computer-aided conformational analysis showed that this chloro substituent did not allow the molecule to insert itself very differently compared to kanamycin B or 6″-deoxykanamycin B in a monolayer of phosphatidylinositol, all three drugs adopting an orientation largely parallel to the hydrophobic–hydrophilic interface and being largely "embedded" in the bilayer at that level. In contrast, the *N*-acetyl-*N*-methylamino and isopropylthio substituents caused the corresponding derivatives to adopt an orientation largely perpendicular to the interface, because of the attraction of this substituent, and therefore of the 3″-amino sugar moiety of kanamycin B into the hydrophobic domain of the monolayer, whereas the opposite part of the drug (2′,6′-diamino sugar) protruded into the aqueous phase. No simple correlation, however, could be drawn between these changes of conformation and the relative inhibitory potencies of the derivatives.

Introduction

The search for less oto- and nephrotoxic aminoglycoside antibiotics[1,2] has been the subject of intense efforts following the recognition of their large therapeutic value in severe infections, but has met so far with only limited success.[3] In a companion paper,[4] we reported on the synthesis and microbiological evaluation of new derivatives of kanamycin B with increasingly hydrophobic substitutions in C-6″ aimed at potentially modifying the interaction of the drug with negatively charged phospholipid bilayers and thereby modulating its toxicity. The rationale of this approach stems from observations on the early cellular alterations induced by aminoglycosides in kidney.[5–12] Aminoglycosides indeed accumulate in the lysosomes of kidney proximal tubular cells and cause therein an early and conspicuous lysosomal phospholipidosis through binding to negatively charged membranes and inhibition of the activity of lysosomal phospholipases toward both acidic and neutral phospholipids, since the latter is dependent on the availability of negative charges in bilayers. The precise relationship between such alteration and the subsequent onset of tubular necrosis[11,13] and acute renal failure,[11,14] which are characteristics of aminoglycoside-induced nephrotoxicity,[1] is still undefined.[12] Recent investigations using poly-L-aspartic acid, however, have shown a direct correlation between its ability to protect against the renal toxicity induced by aminoglycosides[15] (evaluated by histological and functional changes) and its

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Table I. Effect of Kanamycin B, 6″-Deoxykanamycin B, and 6″-Deoxy-6″-Substituted Kanamycin B Derivatives on the Degradation of Labeled Phosphatidylcholine (1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) Included in Negatively Charged Liposomes by Lysosomal Extracts

drug	compd no.[a]	IC_{50},[b,c] μM±SD	no. expt[d]
kanamycin B	1	110 ± 11	10
6″-deoxykanamycin B	3	93 ± 11	2
6″-deoxy-6″-bromokanamycin B	5	123 ± 11	
6″-deoxy-6″-chlorokanamycin B	6	129 ± 16	4
6″-deoxy-6″-azidokanamycin B	7	89 ± 12	
6″-deoxy-6″-aminokanamycin B	8	83 ± 12	
6″-deoxy-6″-(methylamino)kanamycin B	9	129 ± 8	
6″-deoxy-6″-(butylamino)kanamycin B	10	99 ± 2	
6″-deoxy-6″-(dimethylamino)kanamycin B	11	96 ± 8	
6″-deoxy-6″-(diethylamino)kanamycin B	12	65 ± 2*	
6″-deoxy-6″-pyrrolidin-1-ylkanamycin B	13	81 ± 4	
6″-deoxy-6″-(methoxycarboxamido)kanamycin B	14	87 ± 13	
6″-deoxy-6″-acetamidokanamycin B	15	97 ± 10	2
6″-deoxy-6″-propionamidokanamycin B	16	99 ± 7	2
6″-deoxy-6″-butyramidokanamycin B	17	88 ± 5	2
6″-deoxy-6″-isobutyramidokanamycin B	18	125 ± 14	2
6″-deoxy-6″-pivalamidokanamycin B	19	121 ± 9	2
6″-deoxy-6″-(*N*-formyl-*N*-methylamino)kanamycin B	20	120 ± 8	
6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B	21	134 ± 7*	
6″-deoxy-6″-(*N*-propionyl-*N*-methylamino)kanamycin B	22	116 ± 5	
6″-deoxy-6″-(*N*-butyryl-*N*-methylamino)kanamycin B	23	115 ± 5	
6″-deoxy-6″-(*N*-acetyl-*N*-butylamino)kanamycin B	24	109 ± 7	
6″-deoxy-6″-(*N*-hexanoyl-*N*-butylamino)kanamycin B	25	110 ± 17	
6″-deoxy-6″-(methylthio)kanamycin B	27	96 ± 6	
6″-deoxy-6″-(ethylthio)kanamycin B	28	78 ± 6	
6″-deoxy-6″-(propylthio)kanamycin B	29	102 ± 15	
6″-deoxy-6″-(isopropylthio)kanamycin	30	60 ± 8*	3
6″-deoxy-6″-(butylthio)kanamycin B	31	77 ± 14	
6″-deoxy-6″-(methylsulfinyl)kanamycin B	32	115 ± 5	
6″-deoxy-6″-(methylsulfonyl)kanamycin B	33	118 ± 14	2
6″-*O*-methylkanamycin B	34	106 ± 8	2
6″-*O*-ethylkanamycin B	35	104 ± 10	
6″-*O*-isopropylkanamycin B	36	93 ± 15	
6″-*O*-phenylkanamycin B	37	83 ± 18	

[a] Compound number as per Scheme I of companion paper.[4] [b] Each value (±SD) represents the mean of triplicate determinations in a single experiment or the mean of all determinations of compounds tested in more than one independent experiment; *Significantly different from kanamycin B by Student *t*-test ($p < 0.05$). [c] Drug concentration causing 50% inhibition of the release of labeled lysophosphatidylcholine observed in control, measured as described in ref 18. [d] If not one.

capacity to prevent phospholipidosis.[16] In vitro, poly-L-aspartic acid prevents aminoglycoside binding to negatively charged membranes and relieves aminoglycoside-induced inhibition of lysosomal phospholipases.[17] These data suggested to us that the modulation of the binding of aminoglycosides to phospholipids, and of phospholipase inhibition, could be a lead toward the design of less toxic derivatives. Phospholipase inhibition and interactions of aminoglycosides with negatively charged lipid layers have actually been analyzed in detail by biochemical, biophysical, and computer-aided conformational analyses.[18-23] As reviewed in ref 24, modulation of these interactions can be obtained by chemical modifications compatible with the maintenance of microbiological activity such as for instance the substitution of the 1-amino group with an (*S*)-2-hydroxy-4-aminobutyryl or (*S*)-2-hydroxy-3-aminopropionyl side chain. Although the number of cationic groups carried by the molecule remains unchanged, this type of substitution leads to a change in the mode of insertion of the derivative in the negatively charged bilayer and to a decreased inhibitory potency toward lysosomal phospholipases. These compounds also show a reduced nephrotoxicity in vivo.[24] In the present work, we have investigated and report on the influence exerted by modifications of the hydrophobic/hydrophilic balance at a specific site in kanamycin B, namely around position C-6″. This position was chosen as it was hoped it would not be too critical with respect to activity. The structure, synthesis, and the microbiological evaluation of all molecules studied are given in a companion paper,[4] whereas the present report deals with their toxicological evaluation in an acellular system and a computer-aided conformational analysis of selected compounds. The structures of all other aminoglycosides mentioned in this paper, and a summary of their main properties, are given in ref 24.

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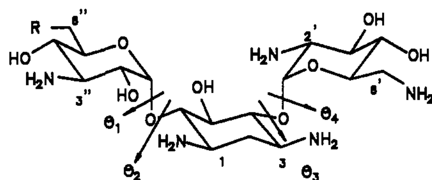

1: R=OH (kanamycin B)

3: R=H (6"-deoxykanamycin B)

6: R=Cl (6"-deoxy-6"-chlorokanamycin B)

21: R=$N(COCH_3)CH_3$ (6"-deoxy-6"-N-acetyl-N-methylaminokanamycin B)

30: R=$SCH(CH_3)_2$ (6"-deoxy-6"-isopropylthiokanamycin B)

Figure 1. Structural formulae of kanamycin B and of the 6″-substituted kanamycin B derivatives investigated by conformational analysis and definition of the torsional angles relevant to this study. The underlined figures correspond to the compound number given in Table I and in the companion paper.[4]

Results

All compounds tested (see structures in Scheme I of the companion paper)[4] inhibited lysosomal phospholipase A_1 activity toward phosphatidylcholine included in negatively charged liposomes in a dose-dependent fashion. The pattern of inhibition was similar to that described earlier for gentamicin[25] and kanamycin B.[18,26] Compounds were investigated up to a final concentration of 250 μM, at which an inhibition of at least 80% of the value of controls was obtained. This allowed calculation for each derivative of the concentration causing 50% inhibition of enzyme activity (IC_{50}; Table I). The IC_{50} of kanamycin B (1) in the present study was very close to that already reported by us earlier for this compound (112 ± 8 μM)[18] or for gentamicin (112.8 ± 19.5 μM).[19] None of the modifications made in position 6″ of kanamycin B caused a marked decrease in inhibitory potency. Increases in IC_{50} were observed for the 6″-deoxy-6″-bromo (5), 6″-deoxy-6″-chloro (6), 6″-deoxy-6″-(methylamino) (9), 6″-deoxy-6″-isobutyramido (18), 6″-deoxy-6″-pivalamido (19), 6″-deoxy-6″-(*N*-formyl-*N*-methylamino) (20), and 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino) (21) derivatives, but a statistically significant difference was obtained only for the latter. Other derivatives showed an increase in inhibitory potency compared to kanamycin B, with two of them, namely 6″-deoxy-6″-(diethylamino) (12) and 6″-deoxy-6″-(isopropylthio)kanamycin B (30), showing a most significantly ($p < 0.05$) lower IC_{50}.

Determination of the theoretical partition coefficient based on Rekker's method[27] showed large variations among the derivatives, with the lipophilic character of the drug expressed as log *P* ranging from −0.748 to 3.213. As evidenced from the data in Table I, variations of IC_{50} were by no means commensurate. Moreover, analysis of the behavior of derivatives grouped in homogeneous families failed to show any positive or negative correlation between IC_{50} values and Rekker's coefficients (data not shown).

The computer-aided conformational analysis approach was then used to compare the mode of interaction of kanamycin B with phosphatidylinositol to that of derivatives selected for the largest increase [6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (21)], or decrease [6″-deoxy-6″-(isopropylthio)kanamycin B (30)] of IC_{50} toward lysosomal phospholipase A_1. In addition, we also present data on 6″-deoxykanamycin B (3) and on 6″-deoxy-6″-chlorokanamycin B (6) since these compounds showed an antibacterial activity similar to that of kanamycin B.[4] Drugs were analyzed under their fully protonated form to mimic their behavior in lysosomes, the pH of which is estimated to lie around 5.4,[28,29] i.e. far below pK_a values of the amino groups of the aminoglycosides.[30,31] Phosphatidylinositol was considered to carry one negative charge on the phospho group at the pH (5.4) at which analysis was simulated, assuming a pK_a of ca. 2.5 for the free-acid function of phosphatidylinositol. The torsional angles of all relevant critical bonds in the fatty acid chains, in the glycerol, and of the P–O bond have been published.[19] For aminoglycosides, only the torsional angles located between the cycles (θ_1, θ_2, θ_3, θ_4 as depicted in Figure 1) were shown to play an important role in the determination of the overall conformation of the molecule. As shown in Table II, these did not significantly differ between kanamycin B (1) and 6″-deoxykanamycin B (3); angles θ_1 and θ_2 were markedly different in the 6″-deoxy-6″-chloro (6) and the 6″-deoxy-6″-isopropylthio (30) derivatives, whereas angles θ_2, θ_3, θ_4 were all three different in the 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino) (21) derivative. For each

Table II. Torsional Angles of the Isolated Aminoglycoside Molecules at a Simulated Hydrophobic–Hydrophilic Interface

drug	compd no.[a]	torsional angles, degrees θ_1	θ_2	θ_3	θ_4
kanamycin B	1	228	215	204	133
6″-deoxykanamycin B	3	244	221	218	122
6″-deoxy-6″-chlorokanamycin B	6	180	262	209	120
6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)-kanamycin B	21	249	157	101	38
6″-deoxy-6″-(isopropylthio)kanamycin B	30	171	84	217	123

[a] Compound number as per Table I and companion paper.[4]

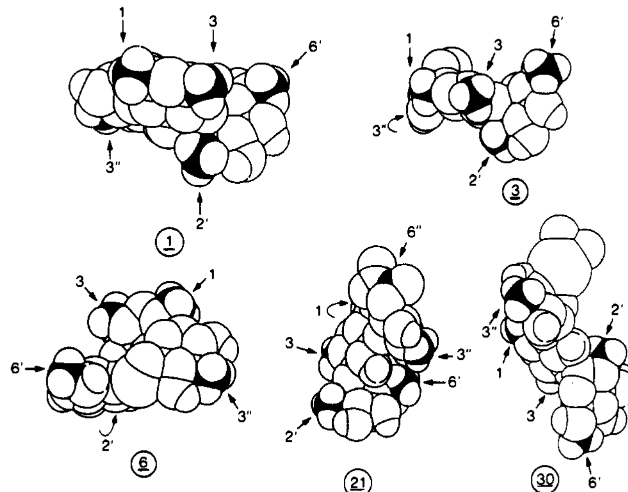

Figure 2. Most probable conformers of kanamycin B and of the 6″-kanamycin B derivatives subjected to computer-aided conformational analysis. The circled figures correspond to the compound numbers of Table I. The positions of the amino groups are indicated by the arrows pointing to the N atoms (in black) visible in the figure (see Figure 1 for numbering conventions).

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Table III. Main Characteristics of the Mixed Monolayers of Phosphatidylinositol and Kanamycin B or Kanamycin B Derivatives

drug	compd no.[a]	energy,[b] kJ/mol	relative position[c]	orientation[d]	area,[e] Å²	perturbation of monolayer
kanamycin B	1	−48.5	↓ --P--*	=	79	-
6″-deoxykanamycin B	3	−32.6	↓ --P--*	=	79	+
6″-deoxy-6″-chlorokanamycin B	6	−43.5	↓ --P--*	=	87	+
6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B	21	−24.4	↓ --P--*	⊥	92	++
6″-deoxy-6″-(isopropylthio)kanamycin B	30	−34.8	↓ --P--*	⊥	86	++

[a] As per Figure 1 and companion paper.[4] [b] Interaction energy, as defined in refs 19 and 21; value for gentamicin C1a is −44.7 kJ/mol. [c] The P refers to the phosphogroup and the * to the inositol moiety of the lipid; the arrow indicates the position of the aminoglycoside atom the most oriented toward the aqueous phase. [d] With respect to the plane of the hydrophobic–hydrophilic interface; =, parallel; ⊥, perpendicular. [e] Area of the drug molecule projected to the hydrophobic–hydrophilic interface; value for gentamicin is 52 Å².

compound, the most probable conformer at an air–water interface is shown in Figure 2. Each drug was then assembled with an increasing number of phosphatidylinositol molecules until it could not longer be accessed by additional phosphatidylinositol molecules. Kanamycin B (**1**) and 6″-deoxy-6″-(isopropylthio)kanamycin B (**30**) could be surrounded by six and 6″-deoxykanamycin B (**3**), 6″-deoxy-6″-chlorokanamycin B (**6**), and 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (**21**) could only be neighbored by five phosphatidylinositol molecules. The main characteristics of the interactions between each drug studied and a phosphatidylinositol monolayer are shown in Table III. Visualization of the assembly is given in Figure 3. In view of the mode of representation used (full atomic scale), however, the phospholipid molecules located in front of the drug are not represented in order for the latter to be seen in full, but the position of the center of the inositol moiety of the corresponding lipid is indicated. A first striking observation is that the molecules studied adopted a very different orientation. Kanamycin B (**1**), 6″-deoxykanamycin B (**3**), and 6″-deoxy-6″-chlorokanamycin B (**6**) were oriented almost parallel to the interface, and, when assembled with phosphatidylinositol, had therefore their greatest axis perpendicular to the fatty acid chains. Examination of the conformation of the isolated drug molecule (see Figure 2) largely explains this behavior. Thus, kanamycin B (**1**) adopted, at the interface, a rather "flat" configuration with three amino groups (N-1, N-3, N-6′) almost in the same plane and oriented toward the hydrophobic phase and the two other amino groups (N-3″, N-2′) facing the aqueous phase. The molecule did not adopt the crescent form previously described for gentamicin,[19,21] and did not penetrate in the bilayer further than the level of the glycerol moiety. Basically, this conformation, orientation, and degree of penetration was not modified by deoxygenation or introduction of a chloro group in C-6″, even though these changes caused the molecule to adopt a slightly more bented form. Yet, this was not sufficient to induce significant modifications of the overall orientation or of the disposition of the molecules with respect to phospholipids. It is noteworthy that the chloro group did not orient itself toward the hydrophobic phase. In sharp contrast, introduction of the *N*-acetyl-*N*-methylamino group in position 6″ (compound **21**) caused the molecule to entirely change its conformation, orientation, and mode of insertion in the bilayer. Thus, **21** was oriented perpendicularly to the hydrophobic–hydrophilic interface. When assembled with phosphatidylinositol, the two methyl groups of the 6″ substituent could interact with the fatty acid, and the 3-amino-3-deoxy-D-glucose moiety

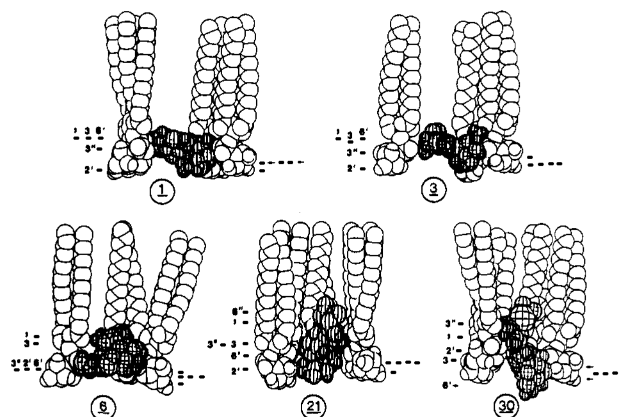

Figure 3. Space-filling configuration of mixed drug–phosphatidylinositol monolayers of each compound studied. The circled figures show the compound number, as indicated in Table I and in Figures 1 and 2. The drug atoms are represented as cross hatched. The arrows on the left of each model refer to the position of each of its amino groups (see Figure 1 for numbering conventions). The position of the center of the inositol moiety of each phospholipid surrounding the drug is indicated by the nonnumbered arrows on the right of each model; the small, complete arrows refer to the positions of the phosphatidylinositol molecules displayed on the figure; the larger arrowheads refer to those phosphatidylinositol molecules which have not been represented because falling in front of the drug and making the latter difficult to see.

(″sugar moiety) appeared much attracted toward the hydrophobic phase, whereas, thanks to the orientation of the molecule, the 2,6-diamino-2,6-dideoxy-D-glucose moiety (′sugar moiety) could somewhat protrude into the aqueous phase. A similar disposition was observed for 6″-deoxy-6″-(isopropylthio)kanamycin B (**30**). The conformation of **30** itself, however, was different. This derivative adopted a more elongated shape, so that the 6′-amino group largely protruded into the aqueous phase, whereas the thioalkyl group largely interacted with the fatty acid chains much above the glycerol. This position, including the protrusion of the ′sugar moiety, is very reminiscent of that observed for amikacin (1-*N*-[(*S*)-2-hydroxy-4-aminobutyryl]kanamycin A) or isepamicin (1-N-[(*S*)-2-hydroxy-3-aminopropionylgentamicin B).[9,24] Protrusion of **30** into the aqueous phase was much more pronounced than that of **21**. Penetration of neither of these two derivatives in the bilayer caused significant misshaping of the interface, as was reported earlier for gentamicin and amikacin.[23,25] The molecular area occupied by each compound did not markedly vary among the derivatives

studied with the exception of the 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino) derivative (**21**), which had a larger area. Compared to kanamycin B, the energy of interaction was lower for all derivatives investigated, especially for 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (**21**). 6″-Deoxy-6″-(isopropylthio)kanamycin B (**30**) and 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (**21**) caused some disorganization of the bilayer characterized by a lack of parallelism of the fatty acid chains (not illustrated in the figure, because of the removal of the phospholipids located in front of the drug molecule), which was not seen with kanamycin B, and to a lesser extent for 6″-deoxykanamycin B (**3**) or 6″-deoxy-6″-chlorokanamycin B (**6**).

Discussion

Evaluation of the potential nephrotoxicity of aminoglycosides by means of determination of the inhibitory potency toward lysosomal phospholipases measured in vitro has been proposed earlier on account of the correlations obtained between the results of these measurements and the known behavior of these drugs in vivo once pharmacokinetic parameters, including their level of tissular accumulation, have been taken into account.[18,24] Combining this biochemical approach with computer-aided molecular modeling has already provided a large bundle of valuable information and suggested a rationale to the lower nephrotoxicities of compounds such as amikacin, isepamicin, or streptomycin.[24] These compounds, compared to gentamicin, bind to a lesser extent to negatively charged liposomes, show a lower energy of interaction between the drug and negatively charged phospholipids, exhibit an orientation perpendicular to the hydrophobic–hydrophilic interface, and are more accessible to water when inserted into a phosphatidylinositol monolayer.[18,22-24] Neither streptomycin, nor amikacin or isepamicin, however, were selected or designed with these considerations in mind. The present work, therefore, represents the first systematic effort at using this approach with a homogeneous series of aminoglycoside derivatives. These were made to carry increasingly bulky hydrophobic substituents in a specific position, namely C-6″, which could potentially interact in an increasing fashion with the hydrophobic domain of the bilayer through that substituent, leaving the 2′,6′-diamino sugar moiety susceptible to interact with the hydrophilic part. Within each series, we observed distinct, albeit moderate, changes in inhibitory potency toward phospholipase A_1, with the molar concentration causing 50% inhibition falling to 54% for 6″-deoxy-6″-(isopropylthio)kanamycin B (**30**) and increasing to 122% for 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (**21**), compared to that of kanamycin B (**1**). A first conclusion of our study is therefore that variations in inhibitory potential of kanamycin B derivatives can be obtained without changes in the overall charge of the drug, which reinforces the conclusion already presented earlier[18] that the number of charges carried by the molecule is not the only determinant in enzyme inhibition, even though it plays an important role.[32] The behavior of the derivatives in the 6″-deoxy-6″-amino series moreover shows no direct correlation between the inhibitory potency and the substitutions carried by the additional 6″-amino group.

A second conclusion of the present work is that there is no systematic correlation between antibacterial activity[4] and inhibitory potency of kanamycin B derivatives toward lysosomal phospholipases, as already observed for kanamycin A derivatives[18] or a series of streptomycin derivatives.[33] Thus, 6″-deoxy-6″-chlorokanamycin B (**6**) shows a decrease in inhibitory potency (increased IC_{50} value) together with an unimpaired microbiological activity compared to kanamycin B (**1**). This decrease of inhibitory potency is, however, limited, which can be rationalized on the basis of the conformational analysis. This analysis indeed fails to reveal marked changes in the conformation or in the mode of insertion of 6″-deoxy-6″-chlorokanamycin B (**6**) in a phosphatidylinositol monolayer compared to kanamycin B (**1**). The energy of interaction was also very little modified. Kanamycin B itself does not adopt the same conformation and orientation as gentamicin C1a (or the other main components of the gentamicin complex, i.e. gentamicin C1 and C2), the latter adopting a clear-cut crescent-type conformation, with the 3- and 1-amino groups disposed on the convex side and oriented toward the aqueous phase, the N-3″ and N-6′ at each extremity of the crescent, and the N-2′ on the center of the concave side and oriented toward the hydrophobic phase.[19,21] These differences, probably, are relatively unimportant since the inhibitory potencies of gentamicin C1a and of kanamycin B are similar (IC_{50} = 100 and 112 μM, respectively). Thus, a third conclusion of our study is therefore that a major change of orientation and insertion of the drug is probably necessary to be translated into a significant change in inhibitory potency. This is indeed the case for 6″-deoxy-6″-(*N*-acetyl-*N*-methylamino)kanamycin B (**21**) and 6″-deoxy-6″-(isopropylthio)kanamycin B (**30**), which both adopted a perpendicular disposition with respect to the hydrophobic–hydrophilic interface and showed a marked decrease of their energy of interaction. Surprisingly, however, the inhibitory potencies of either compound varied in opposite direction. This is in sharp contrast with the behavior of streptomycin, isepamicin, and amikacin, and to some extent of other 1-*N*-(*S*)-2-hydroxy-4-aminobutyryl derivatives of kanamycin B and related aminoglycosides,[19,24] which all showed a decreased inhibitory potency toward lysosomal phospholipases associated to their "perpendicular" orientation. Thus, an obvious and fourth conclusion of our study is that the orientation and energy of interaction of the drug molecule with phosphatidylinositol per se are not the only parameters governing the inhibitory potency of aminoglycosides toward lysosomal phospholipases. This conclusion probably could not have been drawn from previous studies[24] because of the lack of a homogeneous series of aminoglycosides with modifications in only one position. In accordance with recent studies comparing the interaction of gentamicin and amikacin with different types of acidic phospholipids,[22,23] the present results actually suggest that a critical parameter is perhaps the accessibility of the drug to the water phase and its ability to easily exchange with this phase. Further investigations need, however, to be made to quantitatively determine accessibility and exchange rates. Finally, there is no systematic correlation between the bulkiness of the substituent carried at the 6″-position and the inhibitory potency of the corresponding derivative [compare 6″-deoxy-6″-(*N*-formyl-*N*-methylamino)- (**20**) and 6″-deoxy-6″-(*N*-hexanoyl-*N*-butylamino)kanamycin B (**25**), e.g.], suggesting that one large hydrophobic moiety is not sufficient to significantly increase the interaction of the corresponding derivative of an aminoglycoside, contrary to what is observed with mono- or diaminated cationic amphiphiles (see discussion in ref 21).

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The practical value of the approach followed appears limited at first glance, since only one compound, namely 6″-deoxy-6″-chlorokanamycin B (**6**), shows a somewhat decreased inhibitory potency toward phospholipases without loss of activity (all other compounds showing a higher IC_{50} value than kanamycin B, including **21**, have unacceptably high minimum inhibitory concentration values for critical pathogens that need to be included in the spectrum of aminoglycosides). Actually, amikacin and isepamicin already show IC_{50} ratios with gentamicin complex or kanamycin B of 1.3 and 1.9, respectively, which is superior to what we have obtained (1.2) for 6″-deoxy-6″-chlorokanamycin B. Animal and clinical studies, however, show that both amikacin and isepamicin, which in contrast to gentamicin B carry only four amino groups (see structures in ref 19 and 24), need to be administered at doses about 2.7 times higher than those of gentamicin (when expressed in moles), in account of their weaker intrinsic antibacterial activity. Yet, these drugs are definitely less toxic even at that higher dosage (see discussion and summary of animal and clinical data in ref 24). Thus, animal studies with 6″-deoxy-6″-chlorokanamycin B, or perhaps with its 3′-deoxy derivative for improved activity against *Pseudomonas aeruginosa* [such as observed for 3′-deoxykanamycin B (tobramycin)],[1] could be warranted. These studies to be meaningful, however, will need to examine not only the early lysosomal alterations (phospholipidosis) but also the overall influence of the drugs on the renal function, since the goal is to establish the lesser *nephrotoxic* character of these derivatives over sufficiently wide a dosage range to clearly establish their therapeutic advantage (see discussion in refs 1, 13, 34, and 35).

Experimental Section

Biochemical Studies. Sonicated liposomes were prepared from cholesterol, bovine brain sphingomyelin (from Sigma Chemical Co., St. Louis, MO), egg yolk phosphatidylcholine, and wheat germ phosphatidylinositol (from Lipid Products, Nr Redhill, U.K., grade 1 products) in a molar ratio of 5.5:4:4:3. Labeled phosphatidylcholine (from Amersham International plc, Amersham, U.K.; 140 mCi of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (52 mCi/mmol)/mol of phosphatidylcholine) was introduced to follow the hydrolysis of phosphatidylcholine by lysosomal phospholipases. Liposomes were prepared in acetate buffer (4 mM at pH 5.4) as described earlier[25] and briefly summarized hereunder. Lipids in organic solvents (CH_3OH–$CHCl_3$) were deposited as a thin film on the surface of a round-bottommed flask by rotary evaporation at reduced pressure. The dried film was kept under vacuum overnight and then suspended by gentle shaking in aqueous buffer. The spontaneous hydratation of lipids was performed during 1 h at 37 °C. The final lipid concentration was 10 g/L and small unilamellar vesicles were then obtained by sonication [five successive cycles of 2 min each, using a Branson B12 sonifier (Branson Sonic Power Co., Danbury, CN), equipped with 3 mm diameter titanium probe and set at 35 W at 4 °C]. Liposomes were stored under nitrogen and used within 1 week.

Inhibition of Lysosomal Phospholipases by Kanamycin B and Derivatives. Kanamycin B (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and kanamycin B derivatives, obtained as described in the companion paper,[4] were prepared as their sulfate salt and dissolved in 4 mM acetate buffer pH 5.4. The activity of lysosomal phospholipase A_1 (phosphatidate 1-acylhydrolase, EC 3.1.1.32) toward 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine included in the liposomes was determined by measuring the release of [^{14}C]-β-lysophosphatidylcholine upon exposure of the liposomes to a soluble fraction of purified rat liver lysosomes, as described previously[18,25] and briefly summarized hereunder. After 1 h of preincubation at 37 °C, the liposomes–aminoglycoside mixture (0–65 μg of aminoglycoside/μmol of phospholipid) was diluted twice by the addition of an equal volume of enzyme preparation (final protein concentration, 250 μg/mL). After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 2.5 volumes of methanol, which was evaporated under nitrogen, and the residue was dissolved in chloroform–methanol (2:1). The substrate and the reaction products were separated by thin-layer chromatography, and radioactivity was measured by scintillation counting.

Conformational Studies. Our studies were meant to examine the behavior of aminoglycosides interacting with phospholipids, i.e. at an hydrophobic–hydrophilic interface. Therefore, the method implies a two-step procedure, namely in the first stage, the calculation of the conformation and orientation of the phospholipid and of the isolated drug molecule at a lipid-water interface, and in the second stage, the calculation of the conformation of that drug molecule inserted in a lipid layer. The total energy is therefore the sum of the energy of the isolated drug molecule at the lipid–water interface and the interaction energy between this drug molecule and a monolayer. The principles of this method have been reviewed.[36] It has been used earlier to examine the interactions between aminoglycosides and acidic phospholipids.[19,21,23,33]

Isolated Molecules (Drug and Phospholipid). In the calculation procedure, the values used for the angles and bond lengths were those commonly used in conformational analysis.[37] For phospholipids, we have selected an all-trans conformation. Each molecule had n rotational angles corresponding to the relevant n torsional angles. These have been previously defined for phosphatidylinositol[19] and are shown in Figure 1 for kanamycin B. Thus, if systematic 60° changes were applied, 6^n conformations would be generated. To avoid such a large number, we have selected the structure-tree technique, where six consecutive changes of 60° each were imposed to m ($m \leq n$) torsional angles, yielding 6^m conformers in each branch of the tree. The conformational energy was calculated for each of these conformers and the most probable configurations were taken. A structure tree including the most probable of all configurations (a selection based upon the Boltzmann statistical weight of all configurations) together with their probability of existence was obtained after successive systematic analysis. At each step, the conformations for which the probability of existence was less than 5% were discarded. After systematic analysis, each conformer selected was submitted to a simplex minimization procedure by varying all torsional angles[38] with a precision of 5° in order to select the final, most probable conformer. The systematic analysis was carried out in a medium of intermediate dielectric constant representative of the membrane–water interface. A simplex minimization procedure was then performed taking into account the interface properties. At this step, the total conformational energy was calculated as the sum of four terms: the London-Van der Waals energy (E^{VdW}), the electrostatic interaction (E^{Cb}), the potential energy of rotation of torsional angles (E^{tor}), and the transfer energy (E^{tr}) of each part of the molecule from the hydrophilic to the hydrophobic phase. At each step, the molecule was oriented with the line joining the hydrophilic and hydrophobic centers perpendicular to the interface.[33,39]

Drug Molecule Inserted into the Lipid Monolayer. The method applied for monolayer formation uses the hypermatrix approach. After orientation at the lipid–water interface, the aminoglycoside position was fixed. A second molecule, i.e. the first lipid, was also oriented at the interface and was allowed to move along the x axis by steps of 0.05 nm. For each position, the lipid was rotated by steps of 30° around its long axis z' and around the first molecule. The number of positions along the x axis is l, m is the number of rotations of the second molecule around

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the first one, and n the number of rotations of the molecule itself. For each set of values of l, m, and n, the intermolecular energy of interaction was calculated as the sum of the London–Van der Waals energy of interaction (E^{VdW}), the electrostatic interaction (E^{Cb}), and the transfer energy of atoms or groups of atoms from a hydrophobic to a hydrophilic phase (E^{Tr}). Then, the second molecule was allowed to move in steps of 0.05 nm along the z' axis perpendicular to the interface, and the position of the z' axis was varied in steps of 5° with respect to the z axis to obtain the lowest interaction energy state for each set of values l, m, and n. The energy value together with the coordinates associated to each set of l, m, and n, were stored in a hypermatrix in order of decreasing value of the interaction energy. A third molecule (usually a second molecule of phospholipid) then approached the group formed by the two first molecules, and its position was defined as the first energetically favorable orientation stored in the hypermatrix, taking into account the steric and energetic constraints imposed by the presence of the first lipid molecule. For the following molecules of phospholipid, the same process was repeated in succession, but for each of them, the positions of all the molecules surrounding the drug were modified alternatively in order to find the lowest energy state. We limited this approach to the number of phospholipid molecules sufficient to surround each molecule of aminoglycoside. All calculations were performed on an IBM-compatible microcomputer equipped with a mathematical coprocessor (Intel 8087, 80287, or 80387), using the PC-TAMMO+ (theoretical analysis of molecular membrane organization) software, and the PC-MSA+ (molecular structure analysis) procedures. Graphs were drawn with the PC-MGM+ (molecular graphics manipulation) program. The procedures used have been described in previous publications on aminoglycoside–phospholipid interactions,[19,21,33] and have been the subject of extensive reviews.[36,39] The actual computer programs are available from their author (R.B.) upon request.

Determination of Partition Coefficient. This parameter was determined with the Rekker's table showing the fragmental hydrophobic constant associated to various simple chemical fragment. The lipophilic character of the drug is calculated as $\log P = \sum_{x=1}^{n} a_x f_x$ where a is the number of similar fragment present in the molecule investigated and n the number of different type of fragment present. A negative value of $\log P$ is associated with a hydrophilic molecule whereas a positive value corresponds to a more hydrophobic structure.

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