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Amphiphilic ion pairs of tobramycin with lipoamino acids

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ABSTRACT

A series of amphiphilic ion pairs of the aminoglycoside antibiotic tobramycin (TOB) with lipoamino acids (LAA) bearing an alkyl side chain of 10–14 carbon atoms are described. TOB-LAA ion pairs were obtained by reduced pressure evaporation of an aqueous-ethanol co-solution of TOB and LAAs. A different degree of substitution of TOB amine groups was obtained by using increasing the drug to LAA molar fractions (1:1 to 1:5). FTIR analysis corroborated their structure, powder X-ray diffractometry (PXRD) and differential scanning calorimetry (DSC) were used to identify the formation of new chemical species.

The prepared compounds were submitted to an *in vitro* microbiological assay against different bacterial strains, both susceptible and resistant to aminoglycosides. The presence of only one LAA moiety did not improve the *in vitro* antibacterial activity of TOB free base. Analogously, equimolar physical mixtures (PhM) of TOB with LAA failed to exert a remarkable cell growth inhibitory activity.

Noteworthy, when three or all the five amine groups of TOB were salified with LAA residues, very active compounds were produced, showing MIC values lower than the detectable limit of 0.03 μg/ml.

1. Introduction

Aminoglycoside antibiotics are bactericide agents extensively used in the clinical therapy of many infectious diseases. Among them, tobramycin (TOB; Fig. 1) is broad-spectrum antibiotic, effective against Gram negative bacteria, especially the *Pseudomonas* species. It represents a 10% component of the antibiotic complex, nebramycin, produced by *Streptomyces tenebrarius*.

TOB exerts a bactericidal activity against many bacterial strains involved in clinical infections. It is particularly indicated for the treatment of septicemia, complicated and recurrent urinary tract infections, lower respiratory infections, serious skin and soft tissue infections including burns and peritonitis, ophthalmic and central nervous system infections caused by organisms resistant to other antibiotics, including other aminoglycosides.

Bacteria can show a natural resistance to aminoglycosides because of the reduced penetration of the antibiotic inside cells, and an acquired resistance due to a low affinity of the drug for the bacterial ribosome, or drug deactivation by microbial cell enzymes [1]. A reduced cellular permeability can result in an insufficient (sub-active) concentration of the antibiotic in the target sites. Moreover, in the presence of lower drug concentrations the inactivating activity of bacterial enzymes can be more successful.

As many antibacterial drugs, TOB has been submitted to extensive investigations looking for more active derivatives, as well as to preformulation studies aimed at obtaining more stable forms and medicines with improved properties.

The sensitivity of some specific positions in the molecule of TOB to chemical modifications has been well delineated, particularly for the five amine groups. In some instances such changes resulted in strong changes of the antibacterial activity or sensitivity to inactivating enzymes [2–5].

Aminoglycoside antibiotics are generally converted to salt forms during recovery and purification procedures. For example, TOB is converted to the pentasulfate salt to obtain a water soluble compound suitable for parenteral injection. Conversion to inorganic salts can change the pharmacokinetics of the drug, but may be unable to affect their interaction with and uptake by the bacterial cells. Recently, the so-called hydrophobic ion pairing approach (HIP) has been proposed as a chemical strategy to reversibly modify the properties of drug molecules. By the HIP, in drug compounds containing ionizable groups, polar counter ions are stoichiometrically replaced with more hydrophobic ones. The resulting ion pairs, being more hydrophobic than the parent compounds, can improve drug permeability and transport through biological membranes, ensure a better systemic adsorption, and enhance the drug uptake by cells [6–8]. Furthermore, the

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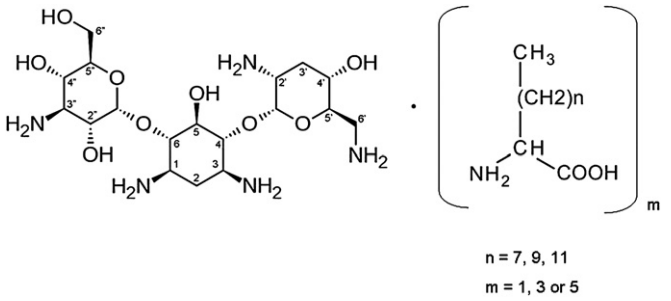

Fig. 1. General structure of TOB and lipoamino acids. Their ion pairs were obtained starting from a co-solution of the drug and LAA at a 1:1, 1:3, or 1:5 M ratio.

amphiphilic features of these complexes might be useful from a technological point of view, for instance by enhancing the drug dissolution in nonaqueous solvents and its encapsulation efficiency and retention time in various drug delivery systems [9–11]. Until very recently, some HIP studies also focused on antibiotics, to improve their absorption and biological activity, or optimize their loading in nanocarrier systems [12–15].

We have recently undertaken a series of studies concerning the reversible modification of antibiotic molecules through the formation of organic amphiphilic ion pairs with lipoamino acids (LAA). LAA have become increasingly important because of their chemical simplicity and versatility, surface activity, aggregation properties, broad biological activity and low toxicity profile [16,17]. In particular, some of us have focused the attention upon some α-amino acids bearing a saturated alkyl chain of different length in 2-position (Fig. 1). Because of the contemporary presence of a lipophilic alkyl chain and the polar amino acid head, LAA can impart amphiphilic properties to drugs, the so-called *membrane-like character* [18]. Consequently, they have been proposed as useful promoieties to modulate or enhance the interaction with and penetration through cell membranes and biological barriers of compounds which possess poor biopharmaceutical properties [19,20]. Toth and collaborators have described some conjugates of β-lactam antibiotics covalently linked to LAA [21], with the aim at enhancing their adsorption in the gastro-intestinal tract. For instance, oral administration of an ampicillin-LAA conjugate significantly improved the antibacterial activity [22]. In a more recent study, a D-glucuronic acid-LAA conjugate has been ion paired with gentamicin resulting in a better oral absorption in rodents [14].

In our lab LAA have been recently used to produce amphiphilic ion pairs of erythromycin [23]. We observed that although an electrostatic interaction between the LAA moieties and the drug occurred, as suggested by spectrophotometric analyses, the growth inhibitory activity profile of the ion pairs remained close to the parent drug against different bacterial strains, both sensitive and resistant to macrolides. However, the presence of the lipid modifier is expected to modify the *in vivo* pharmacokinetics of erythromycin and its penetration into bacterial cells.

The choice of LAA as counter ions mainly relies on their amphiphilic properties. In contrast with other markedly hydrophobic counter ions, such as fatty acids, often used for salifying drug molecules, a drug-LAA complex should retain the ability of the LAA moiety to interact with the biological membranes and facilitate the penetration of the active compound inside the target cells. We have previously proved a similar occurrence through the covalent linkage between drugs and LAA, but our aim is to verify the same possibility by a reversible ionic complexation of ionizable compounds with LAA.

The presence of multiple amino and hydroxyl groups makes TOB a hydrophilic polycation. Such a feature may affect the low activity of this antibiotic, especially against anaerobic Gram negative bacteria. Therefore, this study was designed to assess whether the amphiphilicity, induced by ion pairing TOB with the LAA moieties, will ameliorate the antibacterial activity. Moreover, from a technological point of view it is conceivable that the increased lipophilic character of TOB-LAA ion pairs would positively affect the drug encapsulation and retention in lipid-based carrier systems, such as liposomes and lipid nanoparticles; this aspect will be examined in a separate research.

LAA moieties with a different length of the side alkyl chain (10, 12, or 14 carbon atoms) (Fig. 1) were used in the present study. The

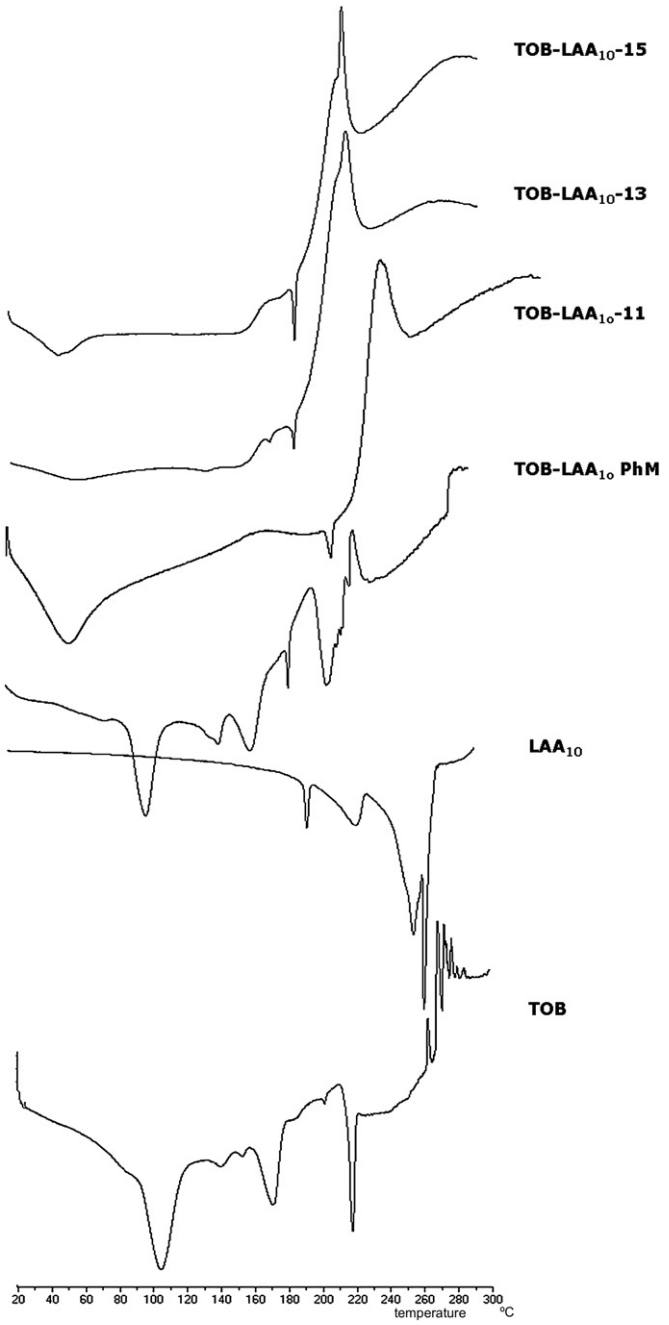

Fig. 2. DSC curves of TOB-LAA10 derivatives (endotherm down). The coevaporates were obtained at different drug-LAA molar ratios (1:1, 1:3, and 1:5); the physical mixture (PhM) was formed at a 1:1 M ratio. DSC curves of TOB (free base) of the starting lipoamino acid were included for comparison. Each sample was analyzed from 20 to 300 °C, at a heating rate of 5 °C/min and a cooling rate of 10 °C.

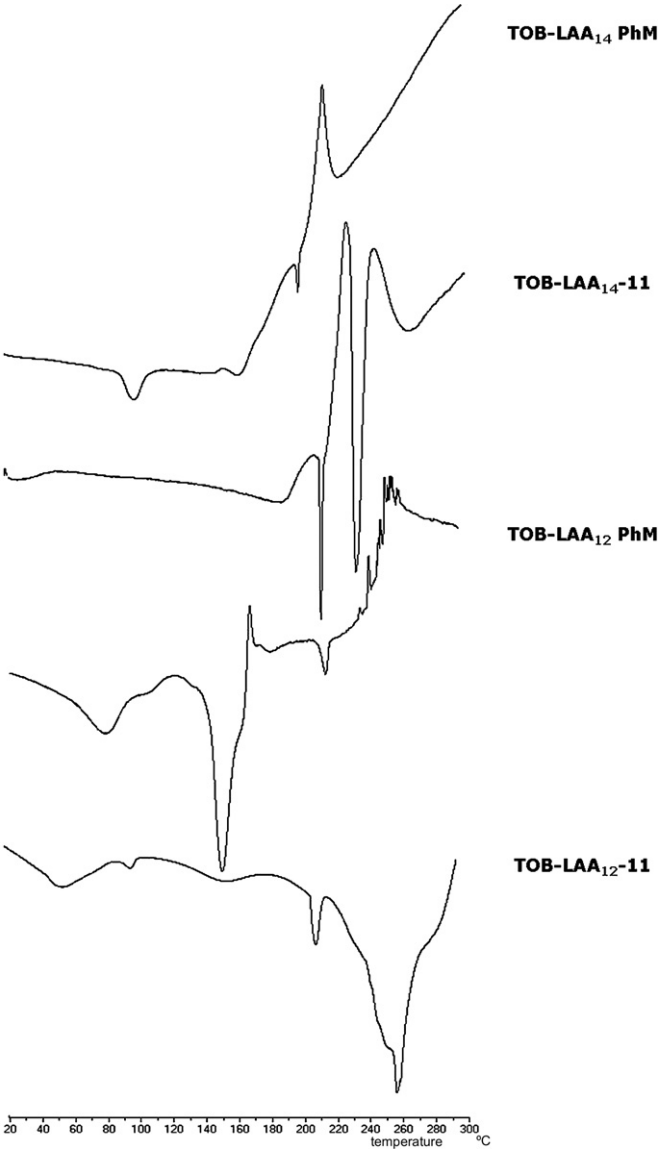

Fig. 3. DSC curves of TOB-LAA12 and LAA14 derivatives (at 1:1 M ratio). Samples were analyzed from 20 to 300 °C, at a heating rate of 5 °C/min and a cooling rate of 10 °C. Endotherm down.

ion pairs were prepared by reduced pressure evaporation of a water/ethanol co-solution of the drug (as free base) and LAA. To verify the effect of progressive ion pairing of the five amine groups present in TOB molecule, different drug to LAA molar ratios were examined (1:1, 1:3 or 1:5) labelled as TOB-LAA$_n$-11, -13 or -15, respectively.

Characterization by FTIR analysis was made to confirm the structure of the prepared ion pairs, while differential scanning calorimetry (DSC) and powder X-ray diffractometry (PXRD) were used to evaluate the formation of a new saline species in respect to the starting components. The experimental data were compared with the corresponding equimolar physical mixtures (PhM) of TOB and the different LAAs, obtained in the absence of any solvent by simple mechanical mixing of the ingredients.

In a preliminary biological assessment, TOB-LAA ion pairs and PhMs were tested *in vitro* against different bacterial strains to assess the influence of the LAA moiety upon the antibacterial activity of the drug.

2. Results and discussion

2.1. Chemistry

To investigate the interactions between TOB and LAA, their coevaporates and PhMs were analyzed in the solid state by conventional spectroscopic techniques (FTIR, PXRD, DSC).

In the DSC experiments with pure TOB (as the free base) (Fig. 2), the first observed endothermic peak can be attributed to the dehydration of the specimen, followed at 164 °C by the fusion into a metastable form. The latter then recrystallized into the stable form, as suggested by an endothermic peak at 197.5 °C, and finally melted at 217 °C [24]. Pure LAA samples showed strong endothermic peaks at the specific melting temperatures, typically around 220 and 260 °C.

The calorimetric analysis of TOB-LAA ion pairs suggested the formation of new chemical complexes. In their DSC curves the endothermic peaks of the two starting compounds were not visible and were replaced by broad signals, at variable temperature peaks, which can be assigned to the ionic domains formed by TOB amino cations and LAA carboxylic anions in the originated ion pairs (Figs. 2 and 3).

The physical mixing of TOB and the different LAA instead gave more complex DSC profiles (Fig. 2), in which the endothermic signals related to the melting of pure TOB (both the metastable and stable forms) and LAA were essentially superimposed.

Finally, the DSC curves obtained for the TOB-LAA10 ion pairs with a 1:3 and 1:5 M ratios were nearly analogous to those

Table 1
IR data (KBr) of TOB base and TOB-LAA coevaporates and physical mixtures.

Attribution[a]	TOB	TOB-LAA$_{10}$–11	PhM10	TOB-LAA$_{12}$–11	PhM12	TOB-LAA$_{12}$–13	TOB-LAA$_{12}$–15	TOB-LAA$_{14}$–11	PhM14
NH stretching (s) OH stretching (s)	3450–3200	3550–3250	3345–3240	3420–3364	3350	3348–3275	3349–3271	3489–3240	3346–3282
Aliphatic CH stretching (m)	2910	2843	2844	2923	2922	2911	2912	2851	2930
COOH stretching (w) (LAA)	–	1690	1685	1654	1685	1655	1655	1690	1685
LAA internal lactam (s)	–	–	1605	–	1606	1640, 1600 (w)	1600 (w)	–	1610
NH bending (s)	1588	1574	1574	1583	1592	1574	1574	1575	1580
CH_2 scissoring (m)	1461	1505	1463	1461	1462	1463	1463	1470	1416
In-plane OH bending	1380, 1349 (m)	1346–1336 (w)	1348–1336 (m)	1394, 1384 (w)	1394, 1387, 1349 (m)	1346, 1337 (m)	1340, 1335 (m)	1393–1335 (w)	1377–1345 (m)
CN and CO stretching (s)	1032	1011	1028	1015	1034	1019	1020	1012	1030

[a] s: strong; m: medium: w: weak intensity band.

registered for the corresponding equimolar ion pair. All showed an endothermic peak due to the melting of the stable form of TOB and a broad endothermic signal around 240–250 °C, ascribed to the melting of ion pair crystals (Fig. 2). Similar results were collected for TOB-LAA_{12}-13 and TOB-LAA_{12}-15 ion pairs, with respect to the corresponding equimolar compound (not shown). Such an information suggests that, independently on the number of amino groups that have been salified in TOB molecule, the crystalline structure and the relative thermotropic behavior of the different ion pairs were comparable, despite of the strong differences observed in the microbiological activity (*vide infra*).

The FTIR analysis of pure TOB gave strong bands around 3450-3200 cm^{-1}, at 2910 cm^{-1} and at 1588 cm^{-1}, respectively attributed to the stretching of amine and hydroxyl groups, the stretching of aliphatic CH and the bending of NH groups (Table 1) [25]. The spectra of pure LAA were mainly characterized by the presence of a complex of three sharp peaks, in the range around 1580 and 1670 cm^{-1}, due to the carboxyl group and the formation of an internal lactam with the free amine group (not shown).

Table 1 reports the FTIR data of all the tested samples; as an example, the spectra of the TOB-LAA12 derivatives are reported in Fig. 4. The IR spectra of TOB-LAA ion pairs and PhMs in a 1:1 M ratio showed few but relevant points of difference, compared to the pure ingredients. They mainly concerned the signals of the internal lactam, visible in the PhM spectra at about 1600 cm^{-1}, and the stretching of C–N and C–O bonds, that was obtained around 1230 cm^{-1} in the PhMs, similarly to pure TOB, but fell at higher fields (around 1010 cm^{-1}) for the corresponding ion pairs. Although small, such differences were observed in all the samples and confirm the formation of new chemical species between the drug and the lipophilic moieties. Noteworthy, the signal due to the lactam group was detected in the IR spectra of 1:3 and 1:5 ion pairs (Table 1). This would suggest that an aliquot of free LAA is present in these compounds and it can be explained by considering the different pka values associated with the five amine groups in TOB molecule. Actually, the basicity decrease from C-1 and C-3 amine groups (pka values roughly ranging from 6 to 7), to the C-2′ and C-3″ groups (pka between 7.8 and 8.3), to the less basic C-6′ amine group, showing a pka value of 8.9 [26]. Therefore, it is plausible that the last amine group was only partially salified by the LAAs in the ion pairs produced at a 1:5 M ratio. Elsewhere in the literature has been already observed that, in the presence of multiple ionizable groups, the process of ion pairing can reach an equilibrium prior to a complete stoichiometric substitution [27].

Additionally, the FTIR spectra of the 1:1 PhMs showed a superimposition of the signals of the starting molecules. It is noteworthy that also in these spectra a stretching band around 1580–1590 cm^{-1} was visible, confirming what observed in DSC experiments, that an ionic interaction between the carboxyl function of the LAA and the amine groups of TOB was formed, even during the mere mechanical trituration step, at room temperature and in the absence of any solvent, that was used for the production of PhMs. A critical point of the above assumption might be related to the presence of moisture in the TOB sample, as supported by the DSC analysis (Fig. 2). This

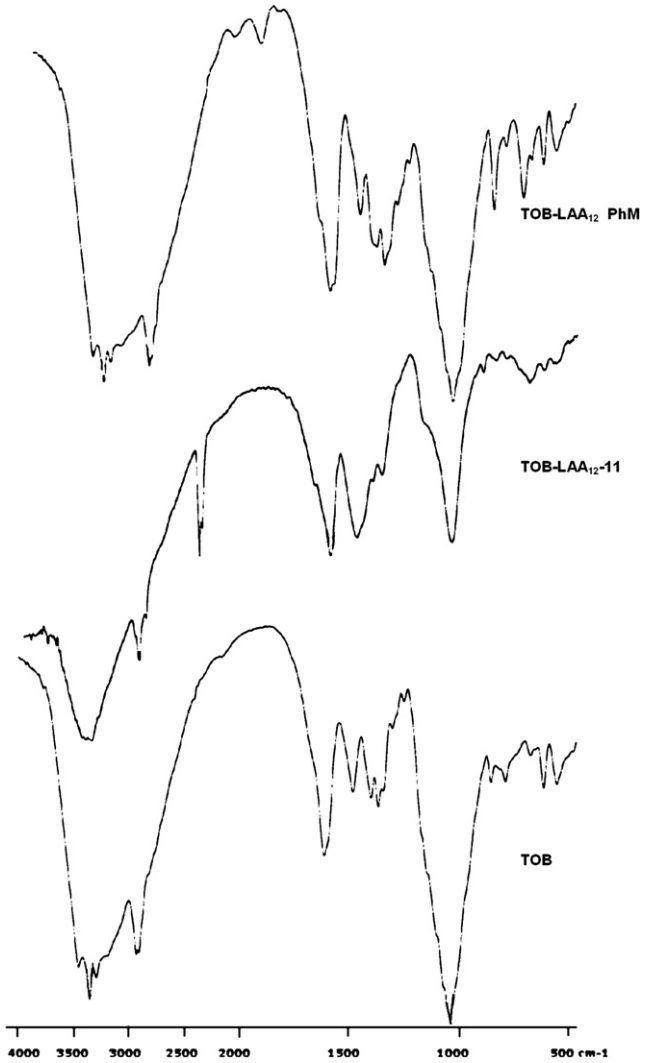

Fig. 4. FTIR spectroscopic analysis of TOB (as a free base) and TOB-LAA12 hydrophobic ion pair and PhM performed at a 1:1 M ratio.

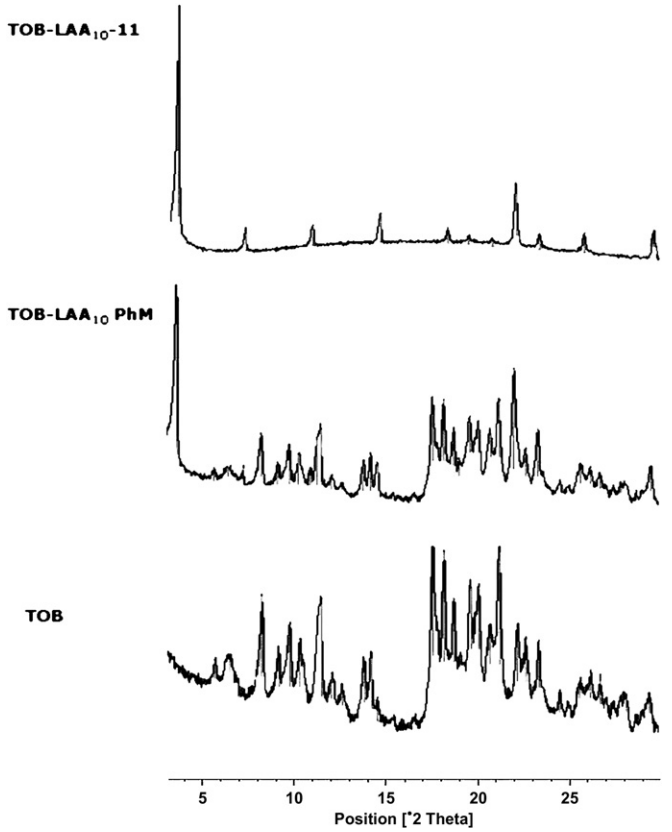

Fig. 5. PXRD profiles of TOB (free base) and TOB-LAA10 ion pair and PhM, prepared at a 1:1 M ratio.

Table 2
Solubility (mg/ml) of TOB-LAA ion pairs in different solvents, at room temperature, compared to TOB base.

Solvent	TOB base	TOB-LAA_{10}–11	TOB-LAA_{12}–11	TOB-LAA_{14}–11	TOB-LAA_{10}–13	TOB-LAA_{10}–15
Water	>50	15	15	10	10	10
pH 7.4 phosphate buffer	>50	10	15	10	7.5	7.5
Ethanol	5	>30	>30	25	>30	>30
Acetone	5	15	20	20	>30	>30
Dichloromethane	1	20	>30	>30	>30	>30

little amount of water could of course have a role in the formation of electrostatic interactions between TOB and the LAA. To verify this hypothesis, a batch of TOB/LAA10 physical mixture was prepared as described in the experimental section, but starting from a specimen of TOB free base that had been dried overnight at 70 °C under high vacuum. The resulting FTIR analysis showed that, even in the absence of residual moisture (confirmed by DSC, not shown), TOB and LAA10 were able to develop an ionic interaction, although the signal was smaller that that one observed in the previous sample (not shown). We then concluded that the ionic interaction of TOB with the counter ion, at least in part, occurred upon a simple physical mixing of the drug with the LAA.

The PXRD analysis corroborated both the DSC and FTIR results. These studies well evidenced the different nature of TOB-LAA ion pairs (coevaporates) compared to the PhMs. In Fig. 5 the behavior of LAA10 derivatives is illustrated; the other two series of compounds with the LAA12 and LAA14 gave very similar results (data not shown). All the TOB-LAA 1:1 ion pairs, independently on the LAA present, showed a diffractometric profile typical of amorphous materials, with the weak signals of the working matrix. Conversely, the corresponding PhMs gave a diffractogram nearly identical to that one of pure TOB [25].

Based on these data, it can be assumed that the simple mechanical mixing of TOB with the LAA gave a powder with the same crystalline properties of the starting drug, whereas in the coevaporates the ingredients were intimately mixed, and 'diluting' each other produced a new species with a concomitant loss of crystallinity.

2.2. TOB-LAA ion pairs solubility profile

Increasing the liposolubility is one of the main targets of the HIP approach. As a polycation molecule, TOB free base is freely soluble in water, slightly soluble in ethanol and insoluble in chloroform and ether (United States Pharmacopoeia, USP 29). In our experimental conditions, such solubility pattern was confirmed (Table 2). The formation of the coevaporates with one or more LAA moieties reduced the solubility of the drug in aqueous media, but exerted a positive influence on the solubility in the tested organic solvents (Table 2). Increase of solubility in dichloromethane and ethanol seemed also to be proportional to the number of LAA residues interacting with the drug (1:5 ≈ 1:3 > 1:1 M ratio).

This information can be important in the view of encapsulation of TOB-LAA ion pairs in polymeric or lipid-based colloidal drug carriers, such as liposomes, micelles or lipid nanoparticles.

2.3. Microbiological assays

TOB sulfate shows an inhibitory activity in various bacterial strains. Literature data indicate MIC values in the 0.25–1 μg/ml range against ATCC strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, and of 8–32 μg/ml for *Enterococcus faecalis* [28].

As the free base, in our experiments TOB had practically no activity against many Gram negative and Gram positive bacteria, with MIC values equal or higher than 4 μg/ml (Table 3). This suggests that in the base (penta-amine) form this drug is not able to penetrate into cultured bacterial cells and then inhibit their growth.

The TOB-LAA coevaporates in a 1:1 M ratio, along with the corresponding PhMs were tested in a first set of experiments. In accordance with the above analytical assessment, neither these compounds showed a notable growth inhibitory activity against the assayed strains (Table 3). The conclusion of these experiments would then be that salifying only one amino group in TOB molecule did not produce compounds able to penetrate and act into the bacterial cells.

To confirm this hypothesis, further series of coevaporates were prepared between TOB and LAA10 or LAA12, in both a 1:3 and 1:5 M ratio, so that to achieve a greater level of complexation of the basic groups in drug molecule. Interestingly, reduced MIC values were obtained (Table 3), always lower than the measurable value of 0.03 μg/ml, and anyway at least three two-fold dilutions lower than the target MICs, i.e., the expected inhibitory values for TOB sulfate against the used bacterial strains [28].

A clear experimental evidence of the present study is that salifying with LAA residues more than one amine functions in TOB molecule, up to all the five amine groups present, enhanced the growth inhibitory activity against different Gram negative bacterial strains. This is most probably due to a more efficacious and rapid

Table 3
In vitro growth inhibitory activity (MIC, μg/ml) of TOB (as the free base or penta-sulfate salt), TOB-LAA coevaporates and physical mixtures. Incubation time: 18 h.

Bacterial strain	TOB (free base)	TOB penta-sulfate[a]	TOB-LAA_{10}–11	PhM10	TOB-LAA_{10}–13	TOB-LAA_{10}–15	TOB-LAA_{12}–11	PhM12	TOB-LAA_{12}–13	TOB-LAA_{12}–15	TOB-LAA_{14}–11	PhM14
E. coli ATCC 25922	16	0.25–1	0.5	1	<0.03	<0.03	2	1	<0.03	<0.03	1	>4
E. coli ATCC 35218	16	0.25–1	0.5	0.5	<0.03	<0.03	0.5	0.5	<0.03	<0.03	1	>4
E. faecalis ATCC 29212	32	8–32	16	>4	<0.03	<0.03	32	>4	0.03	0.03	>4	>4
S. aureus ATCC 29213	16	0.25–1	2	1	<0.03	<0.03	0.5	1	<0.03	<0.03	1	>4
P. aeruginosa ATCC 27853	16	0.25–1	0.5	1	<0.03	<0.03	0.5	0.5	<0.03	<0.03	1	>4

[a] MIC values taken from Ref. [28].

penetration of the drug inside the bacterial cells, compared to the commonly used penta-sulfate TOB salt.

No evident relationship between the *in vitro* microbiological activity and the length of the side alkyl chain in the used LAA was registered. This can be due to the relatively narrow range of lipophilicity within the three used LAA residues (LAA10 to LAA14), although in many other studies on drug-LAA conjugates relevant differences have been observed using analogous substituents [29,30]. Alternatively, it could be concluded that the substitution of the free amine groups in the TOB molecule, regardless of the structure of the counterion moiety, would led to the hypothesized effect on drug uptake into bacterial cells and, hence, on their growth inhibitory effect.

3. Conclusion

The experimental findings seem to confirm the initial working hypothesis, and reinforce what has been previously observed for analogous erythromycin-LAA coevaporates: the complexation of these antibiotics with amphiphilic moieties, while maintaining or even improving the microbiological activity profile of parent drugs, can be an easy strategy to enhance the physico–chemical, pharmacokinetic and, ultimately, pharmacological properties of drugs. For instance, a specific study is in progress to assess whether the amphiphilicity of the prepared coevaporates, as demonstrated by their solubility pattern, could also positively affect the oral absorption of TOB.

Such amphiphilic character could significantly affect the penetration of TOB through biological membranes. By considering the diffuse clinical use of TOB in ocular pathologies, experiments are thus in course in our lab to assess the diffusion of TOB-LAA ion pairs through rabbit cornea, with the aim at achieving higher levels of the antibiotic in the aqueous humor and inner eye tissues [31].

4. Materials and methods

TOB free base (CAS 32986-56-4) and TOB sulfate were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). HPLC-grade water was purchased from Merck (Darmstadt, Germany); absolute ethanol was purchased from Sigma–Aldrich Chimica srl, Milan, Italy. The LAA were synthesized as described elsewhere [32].

IR spectra were registered in KBr tablets with an FTIR Perkin–Elmer 1600 spectrophotometer. PXRD data were collected on a PW3710 (Philips, Eindhoven, The Netherlands) powder diffractometer using Cu Kα radiation and a graphite monochromator, over the range $5^\circ \leq 2\partial \geq 30^\circ$ at a scanning rate of 0.005°/s. DSC experiments were performed with a Mettler DSC12E calorimeter, connected to a Lauda Ecoline RE 207 thermocryostat. A sample of pure indium was used to calibrate the instrument. The detection consisted of a Mettler Pt 100 sensor, with a thermometric sensitivity of 56 μV/°C, a calorimetric sensitivity of about 3 μV/mW and a noise less than 60 nV (<1 mV). Each DSC scan showed an accuracy of ±0.4 °C and reproducibility and resolution of 0.1 °C. Samples (5–10 mg) were sealed in a 40-μl aluminum pan, using an empty pan as reference. Each sample was analyzed from 20 to 300 °C, at a heating rate of 5 °C/min and a cooling rate of 10 °C.

4.1. TOB-LAA ion pair preparation

1:1 Molar Ratio. TOB-LAA_{10}-11, TOB-LAA_{12}-11, and TOB-LAA_{14}-11, equimolar ion pairs between TOB and the LAA bearing a side alkyl chain of 7, 9, or 11 carbon atoms, respectively (Fig. 1) were prepared by co-evaporation of a co-solution of the two components. TOB base (0.3 mmol) was dissolved in water, while the appropriate LAA (0.3 mmol) was dissolved under magnetic stirring in absolute ethanol. The two solutions were mixed for about 4 h at 40 °C and then at overnight room temperature. Ethanol and part of the water were removed under high vacuum at an external temperature of 40 °C. Residual water was finally removed by freeze-drying (Edward Modulyo). The resulting fluffy, white powders were stored in tight closed glass vials at 4 ± 1 °C until use.

1:3 Molar Ratio. TOB-LAA_{10}-13 and TOB-LAA_{12}-13 were obtained in a similar manner, starting from 0.3 mmol TOB and 0.9 mmol of the chosen LAA.

1:5 Molar Ratio. TOB-LAA_{10}-15 and TOB-LAA_{12}-15 were obtained in a similar manner, starting from 0.2 mmol TOB and 1 mmol of the chosen LAA.

4.2. Preparation of the physical mixtures

TOB-LAA equimolar PhMs (PhM10, PhM12, and PhM14) were prepared by mixing the two components in a porcelain mortar for 30 min. The products were stored in a refrigerator in closed glass vials until use.

4.3. Solubility determination

The solubility profile of coevaporates in a range of pharmaceutically related solvents (water; 0.13 M phosphate buffer solution, pH 7.4; ethanol; acetone; and dichloromethane) was measured at room temperature. To a known volume of each solvent (about 2 ml) in capped glass tubes, small amounts of TOB or TOB-LAA ion pairs were progressively added. The mixture was vortex-mixed and submitted to turbidimetry analysis (Shimadzu UV-1601; Shimadzu Italia, Milan, Italy). The early measurement of an absorbance at 650 nm was considered as the solubility threshold. Experimental data are reported in Table 2.

4.4. Microbiology

4.4.1. Strains

E. coli ATCC 25922, *E. coli* ATCC 35218, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853, were investigated in this study.

4.4.2. Susceptibility test procedure

The antimicrobial activity of TOB-LAA coevaporates and PhMs was determined in comparison with that of the free drug, with MICs determined by using the standard broth microdilution assay [33]. The sample suspension was added so that to obtain an equivalent drug concentration with respect to that of the free drug solution, thus allowing to evaluate the effectiveness of the counter ion moiety by a direct comparison of the results. Cation-adjusted Muller-Hinton broth (CAMHB) was used except for streptococci, where CAMHB added of 2.5% lysed horse blood was used.

A stock solution of TOB base (320 μg/ml) was prepared in water; the stock solutions of tested samples were obtained by dissolving them in dimethylsulfoxide and then diluting to 10 ml with the culture broth. The further dilutions were obtained as proposed by the CLSI [33]. A total of 10 concentrations of each sample were prepared. A suspension of microorganisms was added to each well to have a bacterial concentration of 10^3 CFU/ml in each well.

A positive control (growth) consisting of organisms in broth, a negative control (sterility) consisting of uninoculated broth, and a drug control consisting of broth containing the highest concentrations of TOB, TOB-LAA ion pairs or PhMs (concentrations 1, 10, and 100 times higher than those used in the experiments) were included for each tested bacterial strain. Plates were sealed with transparent acetate film and incubated at 37 °C under atmospheric conditions for up to 18 h. Each dilution was assayed six times and

six additional times on a different day with all the formulations to ensure reproducibility of results; at least four out of the six values for each assay were considered for MIC calculation.

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