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Research Article

Amphiphilic Erythromycin-Lipoamino Acid Ion Pairs: Characterization and *In Vitro* Microbiological Evaluation

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Abstract. A series of amphiphilic ion pairs of erythromycin (ERY) with lipoamino acids (LAAs) were produced. The ion pairs were prepared by evaporation of a water/ethanol co-solution of the drug and LAA bearing an alkyl side chain of 10–16 carbon atoms. For the sake of comparison, equimolar physical mixtures were prepared by triturating ERY and the LAA in the absence of any solvent. FTIR spectroscopy confirmed the structure of ion pairs, while differential scanning calorimetry and powder X-ray diffractometry were used to assess the formation of new saline species. The solubility pattern of the coevaporates in different aqueous and organic solvents confirmed their amphiphilic properties. ERY–LAA ion pairs were submitted to an *in vitro* microbiological assay against different bacterial strains, both susceptible and resistant to macrolides. The presence of the LAA moiety was shown not altering the antibacterial spectrum of activity of the drug. These results can be the basis for a further evaluation of ERY–LAA ion pairs as a mean to improve the penetration of the drug inside bacterial cells and to optimize the loading of ERY in lipid-based nanocarriers.

KEY WORDS: amphiphilicity; antibacterial activity; coevaporates; ion pairs; lipoamino acids; physical mixtures.

INTRODUCTION

Erythromycin (ERY; Fig. 1) is the head of the macrolide class of antibiotics. It is clinically used since 1953, and it is active against Gram-positive cocci, such as *Staphylococcus aureus*, pneumocoscci, streptococci, and Gram-negative cocci. It is also active against *Bordetella perutussis*, *Brucella*, *Mycoplasma pneumoniae,* and also intracellular microorganisms like *Rickettsia*.

Resistance to ERY shown by many bacterial strains is due to different mechanisms, among which an impaired permeability of the bacterial cells, resulting in a reduction of the drug concentration in the cytoplasm to insufficient (sub-active) levels.

In particular, most Gram-negative bacteria possess an intrinsic resistance to many antibiotics, including ERY, due the presence of an outer membrane (OM) rich in lipopolysaccharides and proteins, which protects the internal peptidoglycan wall (1). Antibacterial drugs can cross OM by two main pathways: hydrophobic compounds enter by a passive route, whereas hydrophilic antibiotics diffuse through porins, water-filled channels existing in the OM (1–3).

Many commercial medicines contain prodrugs or derivatives of ERY. Instability at the gastric pH, unpleasant taste, and limited solubility in water are the main factors that have prompted the research for alternative derivatives and salts of ERY (4–6). For instance, erythromycin-A 2′-ethylsuccinate is used as an oral suspension in children to reduce the unpleasant taste of the drug. Topical therapy of acute skin infections in patients with atopic eczema, with both ERY acistrate and ERY stearate, was able to eradicate the infection due to *S. aureus* in more than 60% of cases (7).

Among the described salts of ERY are the nalidixate (8), taurate (9), maltobionate (10), melibionate (11), penicillanate (12), and fumarate (13), all obtained by reaction of ERY free base with the respective acids. Other derivatives, such as ethylsuccinate, estolate, gluceptate, stearate, and lactobionate are already on the market as medicinal products (14). All these compounds share the advantage of being highly soluble in water and in many organic solvents (9).

Lipoamino acids (LAA; Fig. 1) are α-amino acids bearing an alkyl side chain, whose length, and structure can be modified to modulate their physicochemical properties. Because of the presence of both the alkyl chain and the polar amino acid head, LAA can impart amphiphilic properties (membrane-like character) to the drugs to which they are associated (15). LAA have been therefore proposed as useful promoieties to modulate or enhance the interaction with and penetration through cell membranes and biological barriers of many drug compounds that show poor biopharmaceutical properties (16–19).

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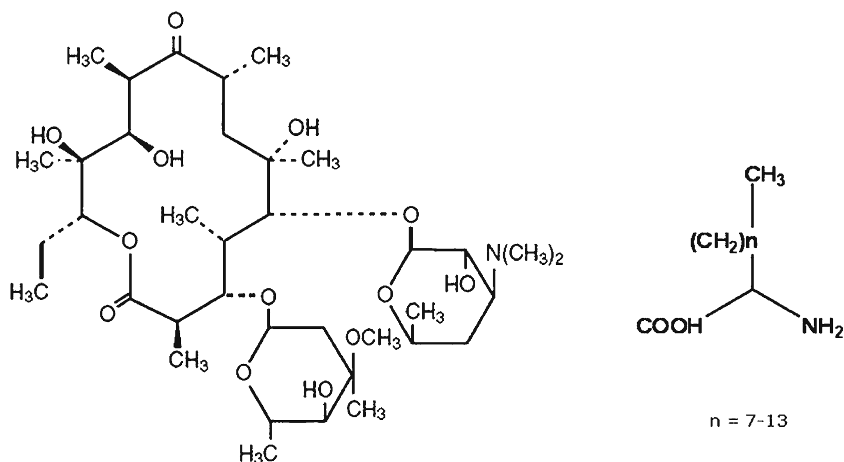


Fig. 1. Chemical structure of erythromycin and lipoamino acids

Toth and co-workers described some conjugates of β-lactam antibiotics with LAA and tested their activity against different bacterial strains (20). Although these antibiotics are active orally, they are not completely adsorbed in the gastrointestinal tract; thereby, their conjugation to LAA might increase oral bioavailability. In the case of ampicillin, for instance, conjugation to LAAs through a reversible ester linkage positively affected the oral drug adsorption and improved the drug activity (21). Similarly, the production of amphiphilic ion pairs between a LAA-sugar derivative and gentamicin has shown to enhance the absorption of the antibiotic in the gastrointestinal tract of rats (22).

We have recently started a series of studies concerning the production of new saline species of antibiotics using different LAA as counter-ions. The peculiar physicochemical properties of these derivatives should affect their biological behavior; particularly, an improvement of the oral absorption of the drug is expected, as well as an increase of drug penetration inside bacterial cells. Moreover, the amphiphilic features of drug-LAA ion pairs may be of great benefit to allow a higher loading and a better retention of the antibiotics in lipid-based delivery systems, such liposomes and lipid nanospheres.

In this work, the production and characterization of a series of amphiphilic ion pairs of ERY with LAAs bearing an alkyl side chain of 10–16 carbon atoms was reported. The ion pairs were prepared by evaporation of a water/ethanol co-solution of ERY and the chosen LAA. Compounds were characterized by FTIR analysis, to confirm their structure, and by differential scanning calorimetry (DSC) and powder X-ray diffractometry (PXRD), to evaluate the formation of a new saline species with respect to the starting ingredients. The experimental data were compared with equimolar physical mixtures (PhM) of ERY with the various LAAs.

Both the coevaporates and PhMs were submitted to an *in vitro* microbiological assay against different bacterial strains, both sensitive and resistant to macrolides, to verify the influence of the LAA counter-ion on the antibacterial activity profile of ERY.

MATERIALS AND METHODS

ERY free base and absolute ethanol were purchased from Sigma-Aldrich Srl (Milan, Italy). HPLC-grade water was purchased from Merck (Darmstadt, Germany) was used in the study. 2-Amino-D,L-decanoic acid (LAA10), 2-amino-D,L-dodecanoic acid (LAA12), 2-amino-D,L-tetradecanoic acid (LAA14), and 2-amino-D,L-hexadecanoic acid (LAA16) were synthesized from diethyl acetamido malonate and the appropriate alkyl bromide using published procedures and the spectral data for these compounds matched the literature data (23).

Elemental analysis was performed using a Carlo Erba 1106 instrument. Samples were kept overnight at 40°C under vacuum before the analysis; found values were within ±0.4% of the theoretical ones. FTIR spectra were registered in KBr tablets with a Perkin-Elmer 1600 spectrophotometer. PXRD data were collected on a PW3710 (Philips, Eindhoven, The Netherlands) power diffractometer using Cu Kα radiation and a graphite monochromator, over the range $5° \leq 2\partial \geq 30°$ at a scanning rate of 0.005°/s.

DSC experiments were performed with a Mettler DSC12E calorimeter, connected to a Haake D8-G thermocryostat (Haake Mes-Technik, Karlsruhe, Germany). 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 30 to 280°C, at a heating rate of 5°C/min.

Chemistry

ERY–LAA Ion Pairs Preparation

ERY–LAA10, ERY–LAA12, ERY–LAA14, and ERY–LAA16, the respective ion pairs of ERY with the LAA having an alkyl side chain of 7, 9, 11, or 13 carbon atoms (see Fig. 1) were prepared by evaporation at reduced pressure of a co-solution of the two components. ERY base (0.3 mmol) was dissolved in water, while the LAA (0.3 mmol) was slowly dissolved under magnetic stirring in absolute ethanol. The obtained solutions were mixed for about 4 h at 40°C and then at room temperature overnight. Ethanol and part of the water were removed off under high vacuum at an external temperature of 40°C. To remove the residual water, the sample was frozen in liquid nitrogen and lyophilized overnight (Modulyo

Table I. Solubility (milligrams per milliliter) of ERY–LAA Coevaporates in Different Solvents at Room Temperature, Compared to ERY Base

Solvent	ERY	ERY–LAA10	ERY–LAA12	ERY–LAA14	ERY–LAA16
Water	2.1	7.5	7.5	7.5	7.5
pH 7.4 phosphate buffer	1.8	5	7.5	15	15
Ethanol	>20	10	15	50	50
Acetone	>20	2.5	7.5	>20	20
Ethyl acetate	>20	1	2.5	2.5	2.5
Dichloromethane	>20	5	15	>20	20

freeze-dryer system; Edwards, Trezzano sul Naviglio, Milan, Italy). The resulting fluffy white powders were stored in tight closed glass vials at 4±1°C until use.

Physical Mixtures

ERY–LAA PhMs were prepared by mixing the two ingredients in a 1:1 molar ratio in a porcelain mortar for 30–40 min. Also these mixtures (PhM10, PhM12, PhM14, and PhM16) were stored in the refrigerator in tight closed glass vials.

Solubility Determination

The solubility profile of the prepared coevaporates in a range of pharmaceutically related solvents (water; 0.13 M phosphate buffer solution, pH 7.4; ethanol; acetone; ethyl acetate; dichloromethane) was measured at room temperature. To a known volume of each solvent (about 2 ml) small amounts of ERY or ERY–LAA ion pairs were progressively added. The mixture was vortex-mixed and submitted to turbidimetry analysis (Shimadzu UV-1601). The first measurement of an absorbance at 650 nm was considered as the evidence of having reached the solubility limit. Solubility data are gathered in Table I.

Microbiology

Strains

Escherichia coli ATCC 25922, *E. coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, were investigated for this study.

Susceptibility Test Procedure

The antimicrobial spectrum of activity of ERY–LAA ion pairs was investigated in comparison with that of the free drug, using the MIC values calculated by the standard broth microdilution assay (24). Each ERY–LAA ion-pair suspension was added in order to obtain an equivalent drug concentration with respect to that of the free drug solution. Mueller-Hinton broth was replaced by Iso-Sensitest broth (Oxoid, Basingstoke, UK) as previously described (25). Briefly, a stock solution of each ERY–LAA ion-pair (2,560 mg/ml in Iso-Senstitest broth) and its further dilutions (512 mg/ml in Iso-Sensitest broth) were obtained from an original stock in water at 5,120 mg/ml. The further dilutions were obtained as proposed by the National Committee for Clinical Laboratory Standards (24). A total of 11 concentrations of each sample were prepared. A suspension of organisms (1 μl of a suspension containing 10^7 CFU/ml) was added to each well. A positive control (growth) consisting of organisms in broth, a negative control (sterility) consisting of uninoculated broth, drug control consisting of broth containing the highest concentrations of drug, and drug-free conjugated (concentrations 1, 10, and 100 times higher than those used throughout the experiments) were included for each bacterial strain tested. Plates were sealed with transparent acetate and incubated at 37° C under atmospheric conditions for up to 18 h. Each assay was repeated six times for each double-dilution of every sample and six additional times on a different day with all formulations, to

Table II. *In Vitro* Antibacterial Activity of ERY–LAA Coevaporates and PhMs

Bacterial strain	ERY	ERY–LAA10	ERY–LAA12	ERY–LAA14	ERY–LAA16	PhM10	PhM12	PhM14	PhM16	LAA12
E. faecalis ATCC 29212	0.25	1	1	0.5	2	2	0.5	1	1	>128
S. aureus ATCC 29213	0.5	0.25	0.5	0.25	0.25	1	0.5	0.5	0.5	>128
E. coli ATCC 25922	64	64	32	64	64	128	32	64	64	>128
E. coli ATCC 35218	64	64	64	64	64	128	32	64	64	>128
P. aeruginosa ATCC 27853	>512	>128	128	>128	>128	128	64	128	>128	128

The activity of pure LAA12 and ERY free base was reported for comparison. MIC values (micrograms per milliliter) were calculated basing on the actual ERY concentration in each sample. Incubation time: 18 h

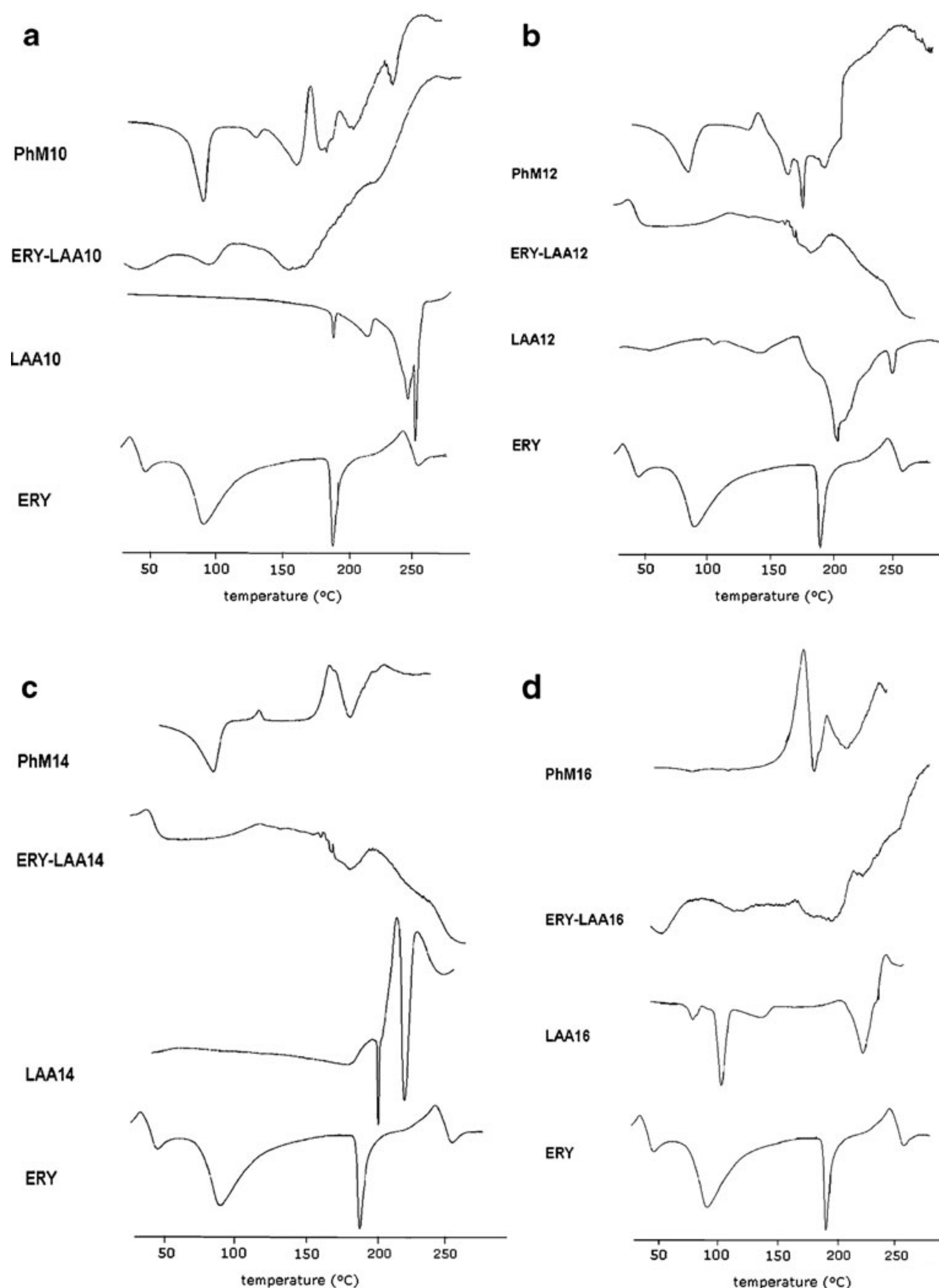


Fig. 2. DSC curves of ERY derivatives with: **a** LAA10, **b** LAA12, **c** LAA14, and **d** LAA16. Exotherm up

ensure the reproducibility of results. The reported MIC (Table II) correspond to at least four identical values out of six measurements.

RESULTS AND DISCUSSION

Chemistry

ERY base, having a pK_a around 8.9, is instable at pH values below 4 and is absorbed in the superior part of the gastrointestinal tract, where it is largely in an ionized form. It then shows an irregular oral absorption, a limited stability in the gastric environment, and a low solubility on water (2 mg/ml). To overcome such limits, different derivatives have been proposed, like salts, esters, and prodrugs, with the common aim at increasing the overall bioavailability and potency of the antibiotic, along with a better toxicological profile (10).

In this paper, we prepared some new derivatives of the drug, by ion-pairing ERY with LAA. According to previous studies, the conjugation of LAA promoieties can improve the amphiphilic character of antibiotics and other drugs (18,19,21,22). We hypothesized that an analogous effect can be achieved by using LAAs as counter-ions of an amine drug, such as ERY, instead of covalently conjugating an LAA residue to the drug.

Consequently, ERY–LAA ion pairs are expected to be able to improve some features of this drug, for instance in terms of absorption through biological membranes and passive penetration into bacterial cells. Both the ERY–LAA coevaporates, obtained by ion-pairing ERY free base with LAAs, as well as the PhM formed by the mere trituration of dry ERY and LAAs showed interesting physicochemical properties in the solid state.

The ion-pairing approach often has a main result, an increase of the solubility of drugs in apolar environments. Thereby, we evaluated the changes in solubility of ERY due to its ion-pairing with the various LAAs in polar and apolar solvents of pharmaceutical interest (water, pH 7.4 0.13 M phosphate buffer solution, ethanol, acetone, dichloromethane, ethyl acetate; Table I). All the coevaporates were more soluble than ERY in water and in the phosphate buffer solution. In the organic solvents, the lower homologues ERY–LAA10 and ERY–LAA12 were slightly less soluble than ERY, whereas the longer-chain homologues ERY–LAA14 and ERY–LAA16 showed the same or even higher solubility than the parent drug, especially in ethanol. Only ethyl acetate showed to have limited solvent properties for these ion pairs.

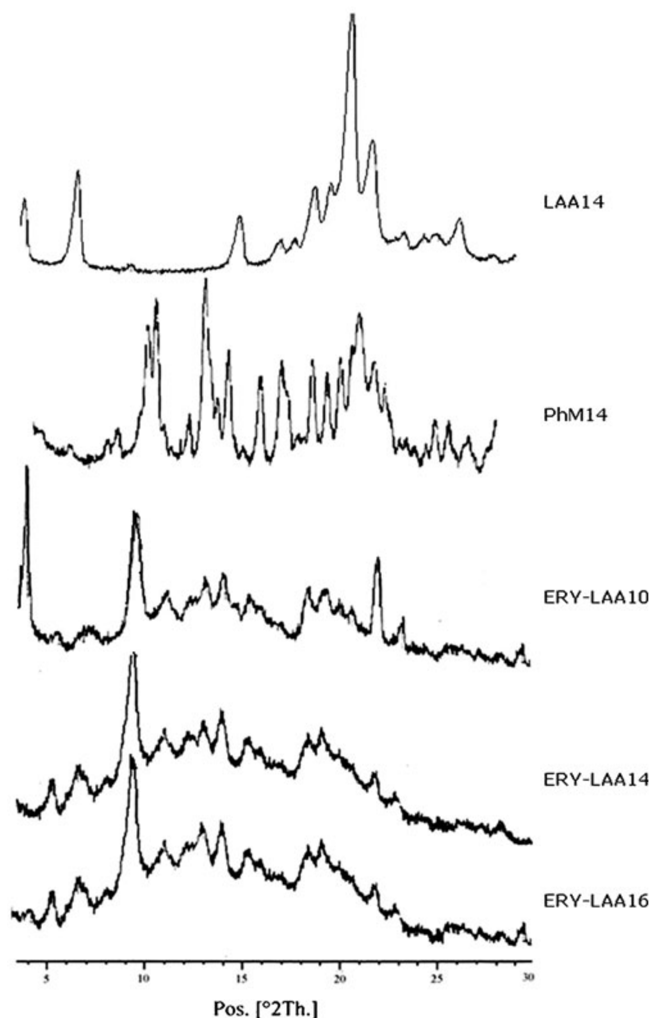


Fig. 3. PXRD profiles of ERY ion pairs and one physical mixture with the LAAs

The experimental solubility data suggest that LAA ion pairs of ERY possess an amphiphilic character, which can become helpful both for the formulation of controlled delivery carriers of this antibiotic, and for modifying the absorption profile of the drug after systemic administration. For instance, ERY salts and prodrugs have been shown to have different oral absorption levels and times than the free antibiotic (6,26,27), as well as to give different local bioavailability than ERY in specific sites (28–30).

The physical state of ERY–LAA coevaporates and PhMs was investigated by different conventional techniques. DSC curves of pure ERY showed a strong endothermic peak corresponding to its melting point, around 193–195°C, along with a broad signal between 70°C and 130°C, attributed to the loss of crystallization water, and a smaller, broad signal around 50°C, due to moisture evaporation (Fig. 2a). Pure LAAs instead showed strong endothermic melting peaks in the range between 200°C and 260°C (Fig. 2a–d).

Calorimetric analysis of ERY–LAA ion pairs (Fig. 2a–d) suggested the formation of a new chemical entity, in whose DSC curves the endothermic peaks of the two starting ingredients were not more visible, and a new, large endothermic peak appeared between 150°C and 190°C, probably due to the gradual melting of the formed ion-pair.

The ERY–LAA PhMs instead showed a more complex DSC profile. Both pure ERY and LAA endothermic signals were still present, along with the broad peak corresponding to the melting of the ERY–LAA ion-pair. This latter finding is of great significance, since it suggests that by a mere mechanical trituration, in the absence of any solvent apart

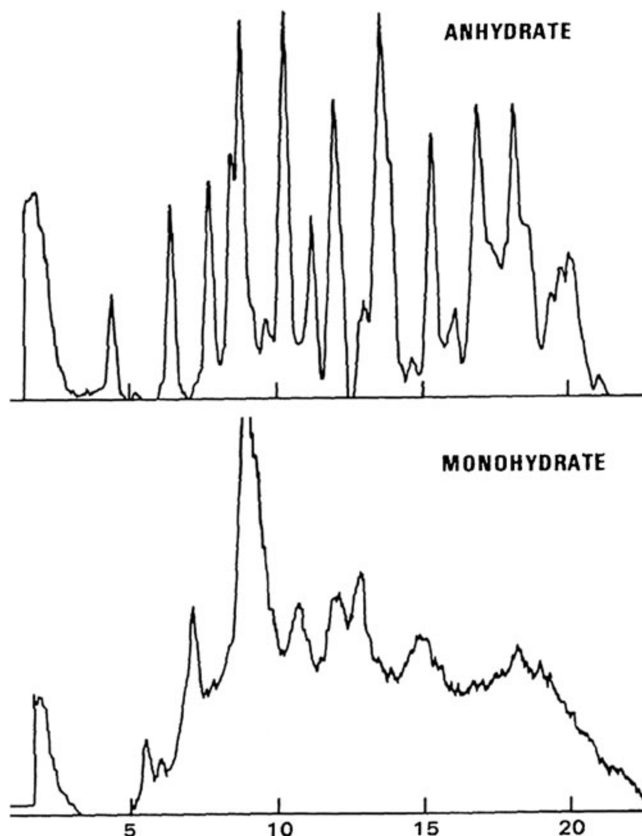


Fig. 4. PXRD profile of anhydrous and monohydrate ERY *cf.* ref. (31)

from the water present in the crystals of the commercial ERY sample, the LAAs tended to form stable ionic bonds with the basic groups present in the drug molecule.

However, the different nature of the ERY–LAA ion pairs (coevaporates), with respect to the corresponding PhMs, was clearly substantiated by the PXRD studies (Fig. 3). In fact, all the assayed ion pairs, despite the kind of LAA present, showed an identical diffractometric pattern, largely superimposable to that one registered for the crystals of monohydrate erythromycin (Fig. 4) (31) and in which the

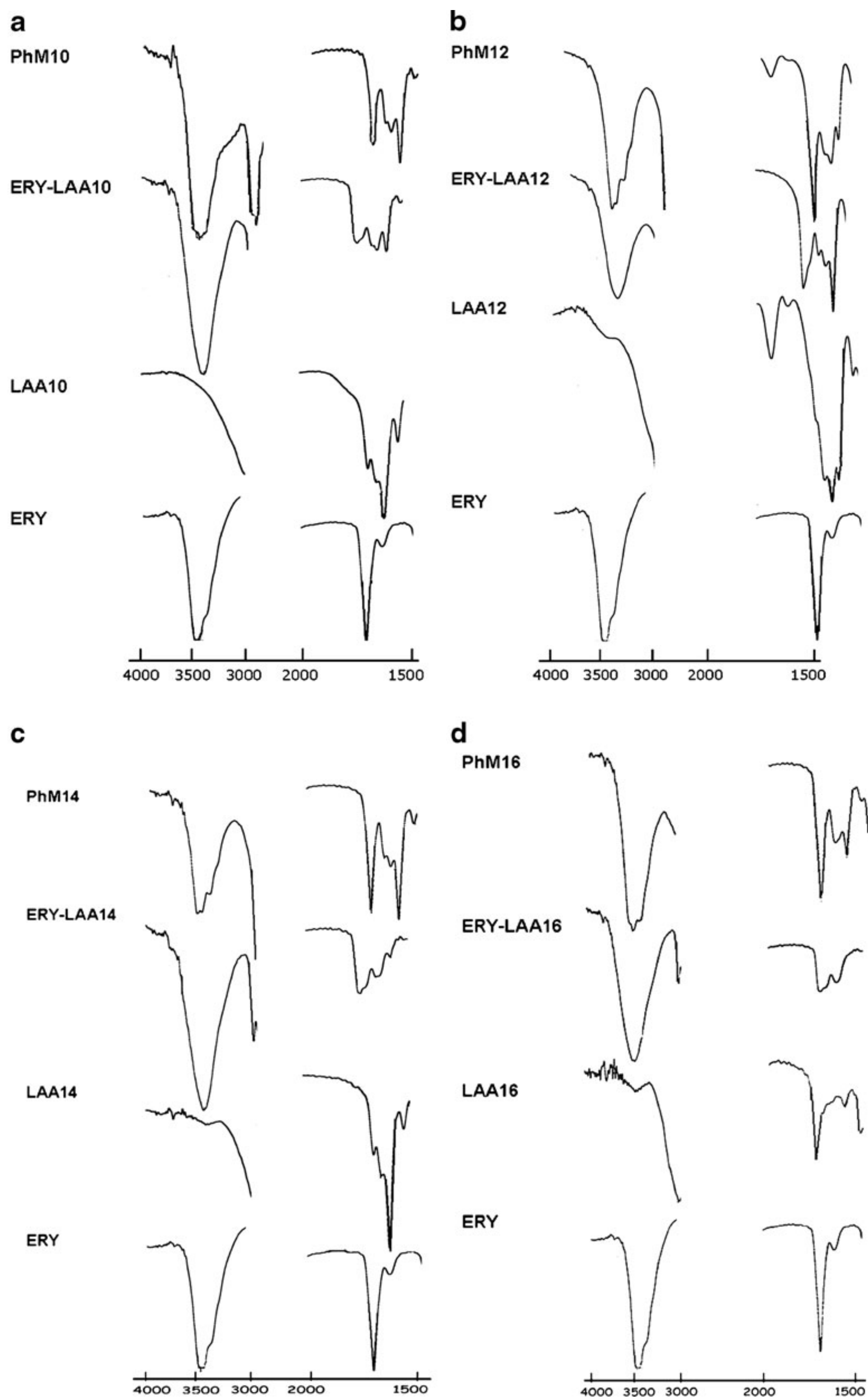
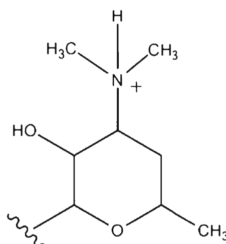


Fig. 5. FTIR spectra of ERY derivatives with: **a** LAA10, **b** LAA12, **c** LAA14, and **d** LAA16

proper signals of pure LAA were not clearly found. Conversely, the spectrum of PhMs was rich in signals, a half of whom corresponding to the profile of drug crystals. This confirmed its nature of a “mixed” powder between two distinct ingredients, ERY and LAA, among which no strong chemical bond was formed, and thus no novel crystalline entity originated.

The results of FTIR analysis are resumed in Fig. 5. Pure ERY shows some strong bands around 3,470 cm^{-1} and at 1,712–1,640 cm^{-1}, respectively due to the OH groups (that form hydrogen bonds), and to the ketone and lactone carbonyl groups (9,32). IR spectra of the starting LAAs are all characterized mainly by the presence of a strong stretching signal, between 1,650 and 1,750 cm^{-1}, due to the presence of the carboxy group and the formation of internal lactam with the free amine group.

In the ion pairs formed with the LAAs, the signal around 3,400 cm^{-1} was broadened and slightly shifted to higher fields, while the bands relative to the carbonyl groups, both deriving from the drug and the LAA residue, appeared, of course, attenuated because of the reciprocal dilution effect between the two components. These variations, along with two other changes in the FTIR spectra, confirm the formation of a new compound (9): the shift of the carbonyl groups signal towards lower fields (to about 1,725 cm^{-1}) and the appearance of a new signal around 1,580 cm^{-1}, that suggests the formation of a quaternary ammonium salt of ERY, with the possible structure below shown (13):



The FTIR spectra of the corresponding PhMs showed an evident superimposition of the own signals of the two starting components. However, it is noteworthy that also in these spectra, a stretching band is evident around 1,580–1,590 cm^{-1}, confirming what was observed in the DSC analysis, *i.e.*, the development of an ionic interaction between the LAA carboxyl function and the drug amine groups, even after the simple dry mixing at room temperature.

Microbiology

The *in vitro* antibacterial spectrum of activity of the synthesized coevaporates was compared with that one of the parent drug by the MIC method (Table II). Different ERY-susceptible strains were tested, along with some Gram-negative strains (*E. coli* and *P. aeruginosa*), against which the aminoglycoside antibiotics are less effective. As a further comparison, one of the starting LAA (LAA12, that bears a nine-carbon long alkyl side chain; *cf.* Fig. 1) and the various physical mixtures of ERY with LAAs were included in the assay.

Pure ERY (as the free base) showed the known activity profile, with MIC values of 0.25 and 0.5 µg/ml against *E. faecalis* and *S. aureus*, respectively, and lack of activity against *E. coli* and *P. aeruginosa*. As expected, the mere lipoamino acid counterpart (LAA12) was devoid of any antibacterial activity against all the tested strains.

The antibacterial activity of ERY–LAA ion pairs with the different LAA moieties was close to that of the parent drug, and even higher against the *S. aureus* strain. Finally, ERY–LAA PhMs gave a growth inhibitory activity slightly lower than that of the corresponding coevaporates.

As concerns the less susceptible Gram-negative strains, although most derivatives showed the same activity profile of ERY, both the ion pairs and PhM with LAA12 showed a better activity than the parent drug, with MIC values one order lower in some instances.

In general, these results confirmed that forming ERY ion pairs with the LAAs did not distress the drug *in vitro* antibacterial effects. In view of the aims of the present research, that is, using LAA derivatives as a mean to efficiently carry the antibiotic in lipid nanocarriers, or also to improve the penetration of ERY inside bacterial cells, the collected experimental microbiological data can be considered as positive results and will prompt towards further technological and biological studies.

In particular, the relative increase of activity against Gram-negative bacteria with respect to the parent drug, shown by some ion pairs, would deserve a separate investigation on the involved mechanisms, possibly as a strategy to overcome the permeation-based bacterial resistance to ERY.

CONCLUSIONS

Within a research focused on the preparation of amphiphilic ion pairs between antibiotics and LAA (33), a series of ERY ion pairs with different LAA moieties have been synthesized and characterized. An *in vitro* microbiological assay confirmed that these compounds keep the antibacterial activity profile of the free drug. Based on of other literature evidences (9,27), it can be hypothesized that ERY–LAA ion pairs will show a similar antimicrobial activity profile also *in vivo*, where the presence of the LAA moiety can affect the pharmacokinetics and biodistribution of the drug.

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