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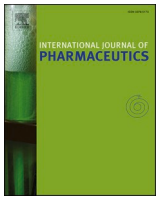

Development of nanovehicles for co-delivery of colistin and ArnT inhibitors

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ABSTRACT

Antimicrobial resistance (AMR) represents a critical global health challenge, with increasing prevalence among high-priority pathogens such as *Pseudomonas aeruginosa*. Colistin, a last-resort antibiotic, faces limitations in efficacy due to toxicity and bacterial resistance, primarily driven by lipid A modifications that impair colistin binding. In *P. aeruginosa*, resistance to colistin is mainly due to activation of the *arn* operon whose last enzyme is ArnT. This study explores a liposomal nanocarrier approach to co-deliver colistin with an ArnT inhibitor, isostevic acid (ISA), aiming to restore colistin's efficacy against resistant *P. aeruginosa* strain. We designed liposomes incorporating colistin in the aqueous core and ISA within the lipid bilayer, optimizing formulations to achieve stable, high-efficiency encapsulation by varying the cholesterol/egg phosphatidylcholine ratios. These co-loaded liposomes demonstrated enhanced antimicrobial activity, significantly lowering the minimum inhibitory concentration (MIC) of colistin against resistant strain. The dual-drug liposomes also achieved bactericidal effects at lower colistin concentrations compared to the free drug, attributed to the synergistic action of ISA as an adjuvant that locks colistin resistance mechanisms. The results suggest that liposome-mediated co-delivery of colistin and ISA offers a promising strategy to counteract colistin-resistant infections. This approach could improve the clinical management of multidrug-resistant *P. aeruginosa* and highlights the potential for liposomal systems to modulate drug release and target bacterial resistance mechanisms.

1. Introduction

Antimicrobial resistance (AMR) has become a compelling issue of global public health. AMR was predicted to rapidly increase the frequency of life-threatening infections and failure of treatments (in some cases leading to death), which has been recently estimated to reduce life expectancy by 1.8 years in the next decade (AMR leaders, 2024). This scenario seems unlikely to be reversed in the immediate future, due to the insufficient investments in research and development (R&D) of new antimicrobials, as well as the absence of effective strategies to regulate the use of antibiotics, particularly excessive and improper use. Antimicrobials R&D is negatively impacted by the expected low incomes discouraging private investments, leaving this task to public and philanthropic associations.

In 2017 the World Health Organization (WHO) published a bacteria priority pathogen list which, in 2024, has been updated based on changing trends in resistance, distribution of bacterial infections and emergence of new resistance mechanisms; here, *Pseudomonas aeruginosa* is a high priority pathogen (WHO 2024). The current antibacterial pipeline contains 97 drugs, about half of which are traditional

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antimicrobial agents, with only 4 active against high priority pathogens, two of them against carbapenem-resistant *P. aeruginosa* (WHO 2023b), which makes this list insufficient to face up this crisis demanding an urgent need to develop innovative antimicrobials.

AMR can also be constrained by regulating the use of existing antibiotics (WHO, 2023a). This is very important as the consequence of antimicrobial resistance is expected to be particularly severe when pathogens are resistant to antimicrobials critically important for human health. Polymyxins, in particular colistin, are classified as "Highest Priority Critically Important Antimicrobials" due to the increased use of colistin to treat infections and the spread of colistin resistance (WHO 2023b).

Colistin is a cationic polypeptide, more precisely a cyclic eptapeptide with a tripeptide side chain acylated at the N-terminus by a fatty acid tail, which accounts for its antimicrobial activity but also toxicity (Poirel et al., 2017). Colistin targets the outer membrane of Gram-negative bacteria by electrostatic interaction between the positive charge of its diaminobutyric acid (Dab) and the negatively charged phosphate groups of the lipid A component of lipopolysaccharide (LPS). This interaction displaces divalent cations (Ca^{2+}, Mg^{2+}) from the outer membrane leading to LPS destabilization with consequent inner membrane permeabilization, leakage of cytoplasmic materials and ultimately cell death (Poirel et al., 2017; Sabnis et al., 2021).

In Gram-negative bacteria, the primary mechanism of colistin resistance is lipid A modification. This may occur by covalent addition of 4-amino-4-deoxy-L-arabinose (Ara4N) or phosphoethanolamine (PEtN) which, by reducing the net positive charge of LPS, inhibits colistin binding to LPS (Poirel et al., 2017; Moffatt et al., 2019). In *P. aeruginosa,* lipid-A aminoarabinosylation has been reported as the main colistin resistance mechanism. Accordingly, mutations that activate the *arn* operon, thus increasing production of Ara4N, have been identified in colistin resistant isolates (Barrow et al., 2009; Miller et al., 2011; Lo Sciuto & Imperi, 2018;); plasmid-mediated expression of the *mcr-1* gene, which is responsible of PEtN transfer to lipid A, was not associated with colistin resistance in *P. aeruginosa* and *Achromobacter* spp in Cystic Fibrosis (CF) isolates (Pedersen et al., 2018, Liu et al., 2024); mutants defective in the lipid-A aminoarabinosylation are incapable to develop colistin resistance (Petrou *et al.*, 2016, Lo Sciuto & Imperi, 2018). Based on these findings, we and others, have recently identified compounds that, potentially acting as ArnT inhibitors, behave as adjuvant of polymyxins to mitigate polymyxin resistance (Ghirga et al., 2020, Quaglio et al., 2020, Zimmerman et al., 2020). While Zimmerman and colleagues used a large-scale robotic screen to identify compounds inhibiting the growth of colistin resistant *Escherichia coli*, our group adopted a structure-guided virtual screening to identify inhibitors of colistin resistance in an *in house* library of natural products. The target was ArnT, whose crystal structure was available (Petrou *et al.*, 2016). This approach led to the discovery of the ent-beyerane diterpenes, as a scaffold for development of ArnT inhibitors, that selectively potentiate colistin activity against *P. aeruginosa* resistant strains (Quaglio et al., 2020). By a structure – activity-relationship (SAR) study around the initial diterpene, novel colistin-resistance breakers have been identified (Quaglio et al., 2020), among them the isostevic acid (ISA) showed colistin adjuvant activity against colistin resistant *P. aeruginosa* (Quaglio et al., 2020).

The ent-beyerane diterpenes are characterized by poor aqueous solubility, which is expected to negatively affect their activity in aqueous environments. This limitation can be overcome by using an adequate delivery system to improve their biopharmaceutical poor features. Liposome-mediated drug delivery provides several benefits, including the possibility to deliver both hydrophobic and aqueous soluble drugs, presenting diverse formulative and composition options and versatile properties besides being biocompatible (Abbasi et al., 2023). This vehicle also opens the possibility of the co-loading of hydrophobic drugs together with amphipathic compounds, such as colistin, for developing a combined therapy. We therefore selected liposomes for the concomitant delivery of colistin and colistin adjuvants.

ISA was selected as prototype of diterpenoid ArnT inhibitors for the development of nanovehicles for the co-delivery of both colistin and its adjuvant, i.e. ArnT inhibitor. To design a high compatible and versatile liposomal formulation, egg phosphatidylcholine (EPC) and cholesterol were selected as liposome components. The presence of cholesterol in the liposome formulation has been demonstrated to be crucial for nanocarrier biopharmaceutical features including drug loading and release, and stability. In this study, the co-encapsulation of ISA and colistin (COL) was explored by first looking at the effect of cholesterol/EPC molar ratio of liposome composition. Subsequently, different co-loaded liposomes were produced by varying COL:ISA dose ratios and their antimicrobial activity analyzed against colistin resistant *P. aeruginosa.*

2. Material and methods

2.1. Bacterial strains, culture media and chemicals

P. aeruginosa strains: PA14 col^R5, KK1 col^R3 and TR1 col^R1, *in vitro*-evolved colistin-resistant mutants (Lo Sciuto & Imperi, 2018). Mueller Hinton broth (MH) was utilized for all bacterial assays. Unless otherwise specified, all chemicals were procured from Merck (Milan, Italy), including colistin (cod. C4461). Isostevic acid was synthesized as previously reported (Quaglio et al., 2020). Egg phosphatidyl choline (EPC) was kindly gifted by Lipoid (Ludwingshafen, Germany). Acetonitrile (ACN), chloroform ($CHCl_3$), methanol (CH_3OH), trifluoroacetic acid (TFA) were provided by Honeywell Riedel-de-Haën TM (Morris Plains, NJ, USA). Ethanol (EtOH) was provided by VWR (Radnor, Pennsylvania, USA).

2.2. Drug quantification

The quantification of COL was performed as previously reported by RP-HPLC. The system was equipped with a Kromasil 100–5-C18 column (5 μm, 100 Å, 4.0x125 mm, AkzoNobel), eluted at 1 mL/min in a gradient mode of water and acetonitrile both added of 0.05 % v/v TFA. Acetonitrile was increased from 20 to 65 v/v% in 15 min and then to 95 v/v% in 5 min. The UV detector was set at 214 nm. COL retention time was found to be around 10.3 min. The AUC of COL eluted peaks resulting from the sum of colistin A and colistin B peak areas was used to derive COL concentration referring to a calibration curve in the 0.01–1 mg/mL concentration range. Quantification of ISA was performed by LC- MS, employing Xevo G2-S QTof with Acquity H Class UPLC equipped with a Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm column run at 0.3 mL/min in a gradient mode of water and acetonitrile as mobile phase. Acetonitrile was increased from 10 to 90 v/v% in 8 min. Detection was collected in negative ion mode. ISA concentration was assessed from the AUC by referring to a calibration curve of isostevic acid in the 0.25–2.5 μg/mL concentration range. Podocarpic acid was used as internal standard at 1 μg/mL due to structure similarity. For quality control, calibration curves were routinely generated before sample analyses.

2.3. Liposome formulation

Liposomes were formulated by thin layer hydration method using egg phosphatidylcholine (EPC) and cholesterol. EPC and cholesterol stock solutions (10 mg/mL) were prepared in ethanol, COL stock (10 mg/mL) was prepared in 10 mM HEPES at pH 7.4 and ISA (10 mg/mL) was stocked in methanol. The lipid film was prepared by mixing 30 mg of 5 mol% cholesterol/lipids and 0.3–15 mol% feed ratios of ISA with respect to total lipids in a round bottom flask. The organic solvent was removed under reduced pressure using a rotatory evaporator at 40 °C for 30 min to generate the lipid film that was then stored overnight under vacuum. The lipid film was hydrated with 1 mL of 10 mM HEPES, pH 7.4 or 1 mL of 2.5 mg/mL COL solution in 10 mM HEPES, pH 7.4. The

formulation was then subjected to freeze and thaw treatments and 11 times extrusion through 200 nm polycarbonate membrane filters (Whatman – Clifton, NJ, USA) using a mini-extruder (Avanti Polar Lipid, Alabaster, AL, USA). Preliminary studies were performed by preparing: i, liposomes with cholesterol/lipids ratio in the 0–50 mol% range formulated with 5 mol% ISA feed; ii, liposomes with cholesterol/lipids ratio in the 0–50 mol% range formulated by lipid film hydration with 2.5 mg/mL COL solution. When needed, fluorescently labelled liposomes were also prepared by including 0.3 % mol/mol 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-Cyanine 5.5 in the ethanolic lipid mixture. Non-encapsulated drugs were removed by a validated procedure. Briefly, freshly prepared liposomes, diluted 4-fold in 10 mM HEPES pH 7.4, and were ultracentrifuged at 40.000 rpm at 4 °C under vacuum for 45 min. The supernatant was then removed, the pellet recovered, and the initial volume restored using the same buffer. ISA and COL were assessed according to the analytical procedures reported above. Phospholipid recovery was quantified by Stewart assay (Stewart, 1980).

2.4. Size, zeta potential analysis and stability tests

The mean hydrodynamic size, the polydispersity index (PDI), and the zeta-potential of freshly prepared liposomes were measured by Dynamic Light Scattering (DLS) using Zetasizer Ultra from Malvern Panalytical Ltd (Malvern, UK). Before analysis, liposomes were diluted to 100 μg/mL in MilliQ water. Measurements were carried out in triplicate and the size was reported as Intensity.

Stability tests. 2.5 mg/mL liposomes in 10 mM HEPES pH 7.4 were incubated at 37 °C and underwent size and PDI analysis at different time points by DLS upon dilution to 100 μg/mL in MilliQ water. Measurements were carried out in triplicate for three independent formulations and the size was reported as Intensity.

2.5. Release study

1.3 mL of liposomes diluted to 2.5 mg/mL in 10 mM HEPES pH 7.4 were transferred into a 50 KDa cut-off Float-A-Lyzer (Spectrum Labs, Inc –Rancho Dominguez, CA, USA) and dialyzed against 5 L of 10 mM HEPES at pH 7.4. The study was carried out at 37 °C, and the medium was replaced twice per day. At scheduled time points aliquots (30 μL) of each liposomal sample were withdrawn and analyzed to assess residual drug concentration as reported above. The dialysis procedure was validated before the release study to ensure both COL and ISA were diffusing through the 50 KDa cut-off Float-A-Lyzer. Complete diffusion of drugs was confirmed after 6 h.

2.6. Antimicrobial susceptibility assays

Antimicrobial susceptibility was assessed by the minimal inhibitory concentration (MIC) assay as previously reported (Ghirga et al., 2020), The reference strain PA14 col^{R}5 was used as a COL resistant *P. aeruginosa* reference strain. Briefly, bacterial cultures in Mueller-Hinton broth (MH) grown to mid-exponential phase, were diluted to 5x10^{5} CFU/mL in MH in 96-well microtiter plates (150 μL volume per well) with increasing concentration of COL solution (15.000 IU/mg), 0–64 μg/mL range, or 2-fold increasing concentration of liposomal formulations or blank liposomes. Growth was measured (OD_{600}) after 24 h of incubation at 37 °C under static conditions in a microplate reader (CLARIOstar Plus); before reading the plates were incubated in a microplate shaker at 250 rpm for 10 min. Growth inhibition was expressed as the percentage growth reduction with respect to non-treated controls (corresponding to 100 %). Additionally, half maximum inhibitory concentration (IC_{50}) was determined by using GraphPad Prism and 90 % inhibition concentration (IC_{90}) was calculated from the IC_{50} data as it follows according to Eq. (1) where H represents the Hill slope.

$$IC_{90} = [90/(100-90)]^{1/H} \bullet IC_{50} \quad (1)$$

2.7. Cell viability assay of the formulations

Cytotoxicity was assessed using a modified version of a previously described method (Sardo et al., 2019). Briefly, Calu-3 (ATCC HTB-55) cells were seeded at a density of 10^{4} cells per well in 96-well microtiter plates in 200 μL of complete RPMI medium. 96 h after seeding, fresh medium containing serial two-fold dilutions of the various treatments, or equivalent amount of blank liposome, was added to each well. Liposomal formulations were tested in a range of concentration of 0 to 500 μg/mL COL or equivalent lipid concentration for ISA loaded or blank liposomes. After incubation for 3 or 18 h at 37 °C, the medium was removed, cells were washed thrice with PBS and the culture medium was replaced with 200 μL of MTT, 0.5 g/L. The plates were incubated for an additional 3 h at 37 °C. The culture supernatant was then discarded and 200 μL of DMSO was added to each well to solubilize the formazan crystals. OD_{570} was measured using a microplate reader. Cell viability was calculated as a percentage relative to the control wells containing non-treated cells, which were set to 100 %.

3. Results and discussion

3.1. Development of liposomal formations for the delivery of COL and ISA

When considering neutral liposomes and the physicochemical properties of COL and ISA (Fig. 1), co-loaded liposomes are expected to encapsulate COL, mostly in the aqueous core, by weak interactions with the lipid bilayer, while ISA, should be incorporated mostly in the liposome bilayer (Wang et al., 2018; Wallace et al., 2012; Bearer and Friend, 1980). Thus, this rationale design was followed to produce dual drug liposomes for the simultaneous delivery to bacterial cells of colistin and its adjuvant, ISA (Fig. 1).

Liposomes were formulated with cholesterol and egg phosphatidyl choline (EPC), which are typical components of pharmaceutical formulations for delivery of both hydrophilic and hydrophobic drugs (Fukuda et al., 2017; Song et al., 2022). EPC has been widely used to formulate hydrophobic drugs (Lee, 2020). Indeed, ISA is a good candidate for loading into liposomes according to its lipophilicity, as predicted by its high logP (5.61) and it is completely insoluble in water.

Since cholesterol/lipids ratios in liposome composition is known to affect the biopharmaceutical features of liposomes (Song et al., 2022; Briuglia et al., 2015; Magarkar et al., 2014), liposomes, either blank or

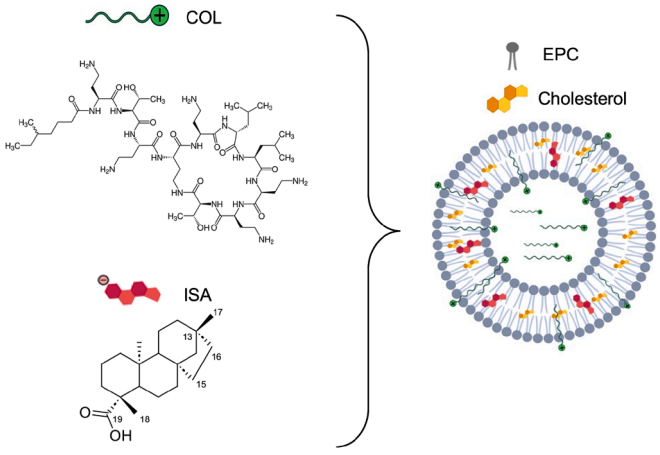

Fig. 1. Rational design of dual drug liposome with COL and ISA. Chemical structure of COL and ISA and representative scheme of the co-loaded liposomes with the two drugs.

loaded with COL or ISA, were produced with a range of 0–50 % cholesterol/lipids molar ratios (Wang et al., 2018; Mukherjee et al., 2019; Briuglia et al., 2015). The resulting liposomes were similar in size (170–200 nm) and had a similar polydispersity index (PDI), lower than 0.2, suggesting that cholesterol/EPC ratio did not significantly affect these features (Fig. S1). Additionally, these dimensional features, being below the average lung mucus mesh size (100–200 nm), are considered suitable for pulmonary administration (Suk et al., 2009).

All cholesterol/EPC compositions formulated, yielded a similar COL encapsulation efficiency (EE ~ 20 mol%) and loading capacity (LC ~ 1 mol%) (Fig. 2A-B), which were in fair agreement with similar formulations reported in the literature (Wallace et al., 2012; Bearer and Friend, 1980). COL has a negative Log P (https://pubchem.ncbi.nlm.nih.gov/compound/Colistin,https://doi.org/10.1101/2022.08.17.504228), which should favour its localisation into the aqueous liposome core. Nevertheless, since COL contain an alkyl chain in its structure, interaction with lipid bilayer could take place as reported in the literature (Wallace et al., 2012). This interaction could allow for partial COL integration into the lipid bilayer which seems in agreement with the slight increase of zeta potential upon liposome loading with COL (Fig. 2C). Differently, the encapsulation efficiency of ISA reached almost 100 % in formulations with a cholesterol/lipids ratio up to 10 mol%, characterized by a constant loading capacity; both parameters progressively decreased as the cholesterol/lipids ratio increased above 10 mol% ratio (Fig. 2D-E). Similarly, the zeta potential increased at cholesterol/lipids ratio above 10 mol% ratio (Fig. 2F). Collectively, these results are consistent with a higher amount of ISA associated with the lipid bilayer in formulations with a low cholesterol/lipids ratio.

Therefore, we selected liposomes with 5 or 10 mol% cholesterol/lipids ratio for further characterization. The liposome size and PDI of the single drug liposomes, either loaded with COL or ISA, did not change over time up to 150 h, after which a progressive increase was observed (Fig. S2). *In vitro* drug release study showed an initial burst of ~ 40 % followed by slow and complete COL release in about 130 h without significant differences between the two formulations (Fig. S3A). Differently, ISA release was faster from liposomes with the lower cholesterol/EPC ratio (Fig. S3B).

3.2. Antimicrobial activity of single-drug loaded liposomes

It has been suggested that high levels of cholesterol in liposomal formulations would improve liposome-bacteria interaction which in turn is expected to increase the antimicrobial activity of the loaded drugs (Briuglia et al., 2015; Xie et al., 2022). Based on this, the antimicrobial activity of COL encapsulated in liposomes with low (5 mol%) or high (30 mol%), cholesterol/lipids ratio was evaluated using the COL resistant *P. aeruginosa* strain PA14 col^{R}5 (Lo Sciuto & Imperi, 2018). Liposomal formulations, both low or high cholesterol, showed higher antimicrobial activity when compared to free COL, as indicated by the two-fold reduction, from 64 to 32 μg/mL, of the MIC value (Table 1). Accordingly, at 32 μg/mL growth inhibition was complete in samples treated with the liposomal formulations (5 % mol or 30 %mol), whereas free COL reduced bacterial growth by 83 % (Fig. 3).

As expected, control cultures treated with blank liposomes, at the same concentrations as those loaded with COL, showed no significant effect on bacterial growth (Fig. S4). Thus, based on the physicochemical characteristics and the antimicrobial activity, liposomes with 5 mol% cholesterol/lipids were selected for the development of dual drug loaded liposomes.

3.3. Co-loading of ISA and COL

COL and ISA co-loaded liposomes (COL:ISA-L) were prepared using 5 mol% cholesterol/lipids, fed with a constant amount of COL (2.5 mg/mL), and increasing ISA, from 0.3 to 15 mol%, with respect to total

Table 1
COL, COL-L(5%) and COL-L(30%) MIC (μg/mL).

	COL	COL-L(5 %)	COL-L(30 %)
PA14 col^{R}5	64	32	32
KK1 col^{R}3	32	32	ND
TR1 col^{R}1	128	128	ND

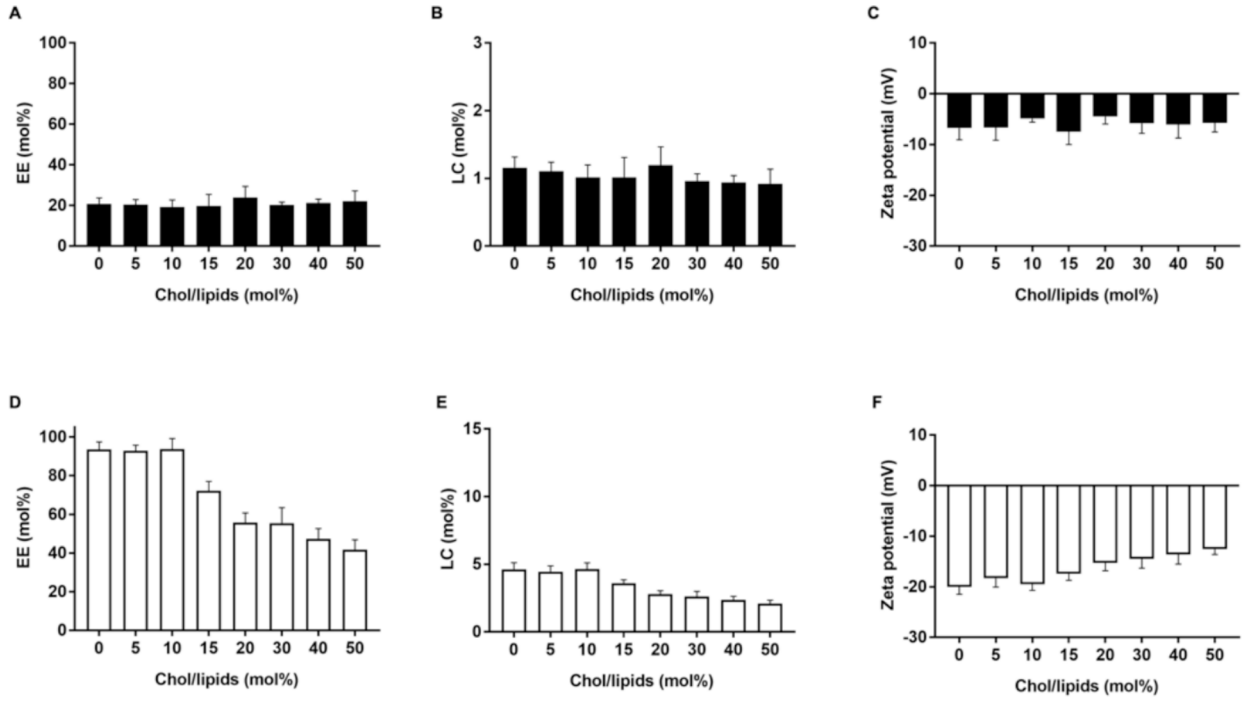

Fig. 2. Single drug encapsulation profiles of COL (A-C) and ISA (D-F) in liposomes formulated with 0–50 mol% cholesterol/lipids ratio. EE, encapsulation efficiency (A, D); LC, loading capacity (B, E); zeta potential (D, F).

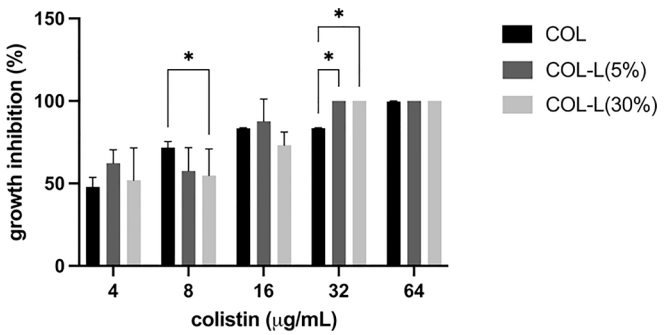

Fig. 3. Antimicrobial activity of single drug loaded liposomes. PA14 col^{R}5 growth inhibition (%) by COL loaded liposomes formulated with 5, COL-L (5 %), or 30, COL-L (30 %), cholesterol/lipids ratio, and used at the indicated concentration of COL. COL, control cultures treated with COL solution. Growth inhibition (%) was determined respect to untreated controls. Data are mean ± SD of three independent experiments. Statistics, Two-way ANOVA; *p < 0.05.

lipids. The resulting liposomes showed a constant COL loading, as assessed by encapsulation efficiency, and loading capacity (Fig. 4A, Fig. S5A). At variance, the encapsulation of ISA was higher at low loading feeding (EE > 92 % up to 5 mol% ISA feeding), with a progressive decrease down to 40 % at 15 mol% feeding (Fig. 4A). Additionally, the co-loaded liposomes showed similar size and PDI, irrespective of the ISA/lipids ratio in the formulations (Fig. 4B). The zeta potential of the co-loaded formulation, reported in Fig. 4C, was less negative with respect to single drug liposomes loaded with ISA (Fig. 2F), which is expected due to electrostatic interaction between ISA, embedded into the lipid bilayer, and COL.

Similarly, to single drug loaded liposomes, COL and ISA were released from the co-loaded particles with biphasic profiles characterized by a burst release in the first 3 h, followed by a slower release, nearly complete in about 130 h (Fig. 5A-B). In particular, COL release was not significantly affected by increasing amount of ISA and nicely overlapped that of single drug loaded liposomes, while ISA release was slower from the co-loaded liposomes respect to that from the single drug loaded liposomes, irrespective of the amount of ISA feed (0.3 to 2.5 % mol) suggesting that COL may affect the permeability of the liposome membrane (Fig. 5B).

Stability profile studies, showed that the co-loaded formulations, similarly to the single drug liposomes, were stable at 37 °C for about 150 h, suggesting that the co-loading of the two drugs did not alter the colloidal stability of the liposomes (Fig. S5B).

3.4. Antimicrobial activity of the co-loaded liposomes

We have previously observed that ISA, when combined with COL at sub-MIC concentration (8 μg/mL), inhibits the growth of PA14 col^{R}5 to about 60 % at concentration ranging from 16 to 64 μM, supporting its putative ArnT inhibitor activity (Quaglio et al., 2020). This result is further corroborated by preliminary *in vitro* binding study showing that ISA binds *P. aeruginosa* ArnT with a K_d of 31.76 +/- 5.6 mM (Fig. S6). Thus, taking into consideration the physicochemical properties of the dual-drug liposomes, the formulations with increasing amounts of ISA, from 0.3 to 2.5 mol%, were selected for the antimicrobial tests. These formulation are characterized by the following dose ratio of COL and ISA (COL:ISA μg/mL: μM): 4:1, 2:1, 1:1 and 1:2. Each formulation, used at two-fold serial dilutions, is expected to expose microorganism to COL and ISA in the following range of concentration: COL, 4–64 μg/mL; ISA 1–128 μM.

The antimicrobial activity of the dual drug liposomes was compared to that of single drug liposomes or free COL (Fig. 6). Inhibition of the bacterial growth by COL:ISA-L was equal or lower to that induced by COL-L, or free COL up to 16 μg/mL of COL. Indeed, in the 4–16 μg/mL range, the antimicrobial activity of COL was higher respect to single and dual drug liposomes, irrespective of the ratio of COL and ISA in the liposomes. Differently, at 32 μg/mL COL, liposomes, either dual or single drug, appeared more active than free COL. At the highest concentration of COL, 64 μg/mL, no bacterial growth was observed in cultures treated with the different liposomal formulations or free colistin, which agrees with the MIC (64 μg/mL) of the tester strain. Next, the IC_{50} and IC_{90} was assessed for the different formulations tested. IC_{50} was not very informative as very similar for all the liposomal formulations, whereas one of the co-loaded formulations [COL:ISA-L(2:1)] showed the lowest IC_{90}. In particular, this formulation showed an IC_{90} equal to 19.04 (μg/mL), which is 2.7- and 1.7-fold lower than COL-L and free COL, respectively (Table 2). As expected, control cultures treated with ISA-L did not show any bacterial growth inhibition, which confirmed the adjuvant activity of isostevic acid (Fig. S7).

To further support this result, the bacterial cells viability of the treated cultures was determined by plating small aliquots on non-selective plates and monitoring bacterial growth (Fig. 7). Bactericidal activity was observed in samples treated with COL:ISA-L(2:1) and COL:ISA-L(1:1) at 64 μg/mL COL and ISA 32 μM or 64 μM, respectively. Differently, the formulations COL:ISA-L(4:1) or COL:ISA-L(1:2) did not show bactericidal activity. It may be speculated that these formulations did not release a sufficient amount of ISA, which may be the case for the COL:ISA-L(4:1), used at the maximal concentration of 16 μM ISA. *In vitro* drug release studies showed that the release of ISA is negatively affected by COL, suggesting that the different activity of the dual drug liposomes may be due to a sub-optimal drug release in the context of a bacterial culture. Free COL, while inhibiting the growth completely at 64 μg/mL, did not show bactericidal activity. Overall, COL:ISA(2:1)-L liposomes exhibited higher bactericidal activity PA14 col^{R}5 than other formulations and free COL.

As a preliminary study on the applicability of dual-drug liposomes, and to rule out the possibility that the activity of the dual drug formulations is strain-dependent, *P. aeruginosa* strains with different genetic backgrounds were tested. Two *in vitro*-evolved colistin-resistant clinical strains, isolated from Cystic fibrosis patients, KK1 col^{R}3 and TR1 col^{R}1 were selected for this study (Lo Sciuto & Imperi, 2018). Both strains

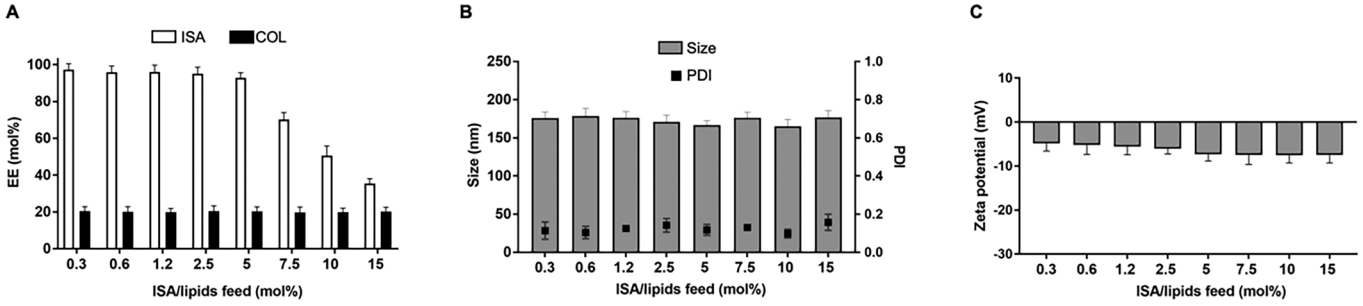

Fig. 4. Biopharmaceutical features of dual drug loaded liposomes. Encapsulation efficiency (A), size and PDI (B), zeta potential (C) of COL:ISA-L at increasing ISA/lipids feed ratio.

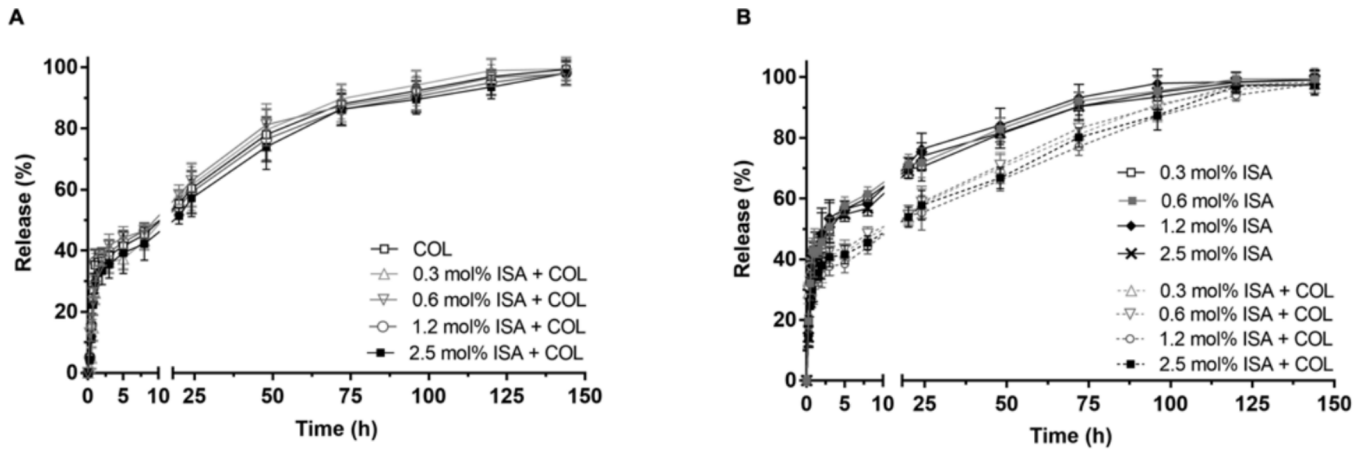

Fig. 5. Release profiles from 5 mol% cholesterol/lipids liposomes loaded with single drugs or co-loaded with different ISA feed and constant COL feed. (A) COL release from liposomes loaded with only COL at 2.5 mg/mL feed, and from liposomes coloaded with 0.3–2.5 mol% ISA feed and 2.5 mg/mL COL feed. (B) ISA release from liposomes loaded with ISA at 0.3–2.5 mol% feed, or coloaded with 0.3–2.5 mol% ISA and 2.5 mg/mL COL.

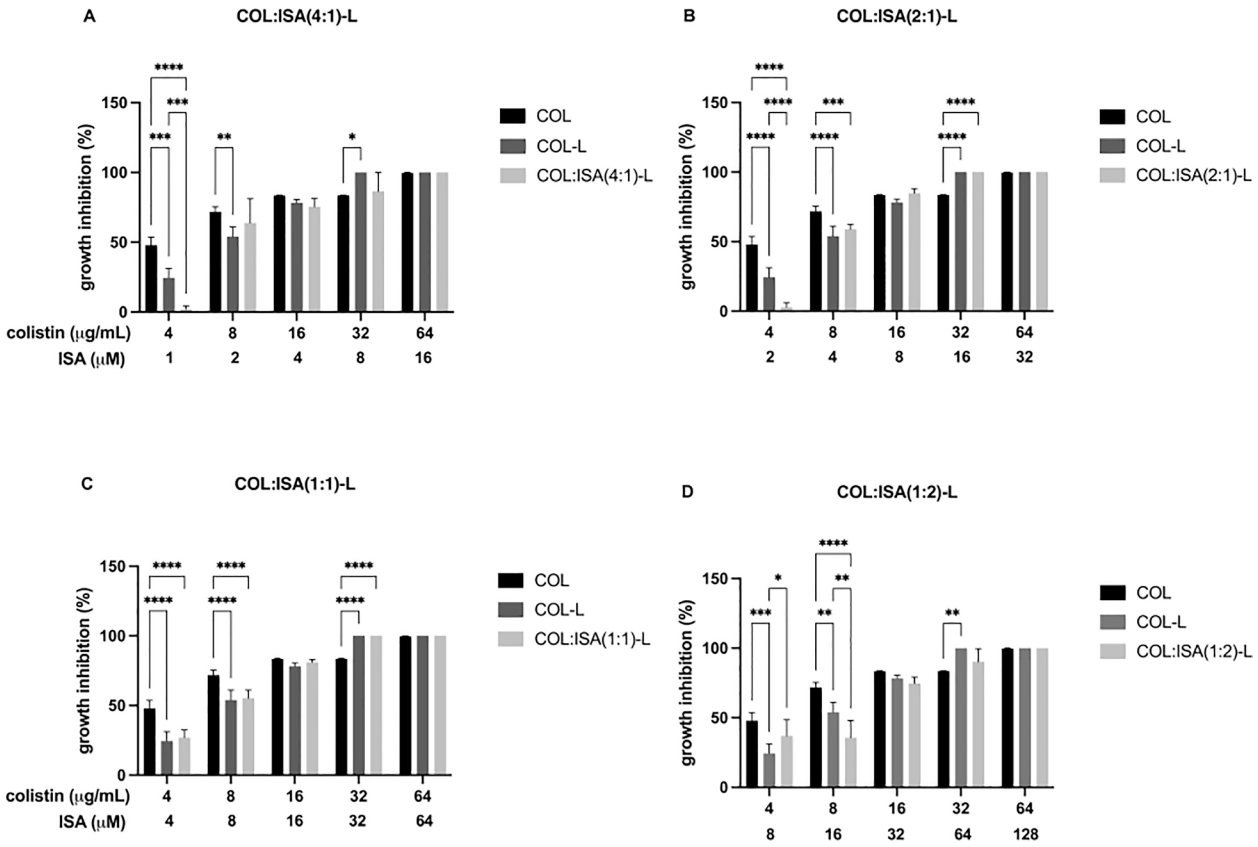

Fig. 6. Dose-dependent effect of the co-loaded liposomes on PA14 col^{R}5 growth. (A) COL:ISA-L, 4:1 dose ratio (B) COL:ISA-L, 2:1 dose ratio; (C) COL:ISA-L, 1:1 dose ratio; (D) COL:ISA-L, 1:2 dose ratio. Data are mean ± SD of three independent experiments. Statistics, Two-way ANOVA; ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

acquired *arn* operon-dependent COL resistance with a COL MIC of 32 μg/mL (KK1 col^{R}3) and 128 μg/mL (TR1 col^{R}1), respectively (Table 1). The most active formulations on the reference strain PA14 col^{R}5, COL:ISA(2:1)-L and COL:ISA(1:1)-L, reduced the COL IC_{90} in both strains, although more efficiently in the case of TR1 col^{R}1 (Table 2). In particular, the IC_{90} of COL-L (172.52 μg/mL) was reduced to 76.37 μg/mL by COL:ISA(2:1)-L and to 88.13 μg/mL by COL:ISA(1:1)-L (Table 2). In the case of the KK1 col^{R}3 strain, COL:ISA(2:1)-L caused a lower reduction of COL IC_{90} with respect to COL-L, from 14.43 to 10.85 μg/mL. In both strains, the single drug liposomes (COL-L) showed higher IC90 than free COL, possibly due to the time of drug release from the liposomes. Similar results were obtained with the growth inhibition assay showing that the COL:ISA(2:1)-L formulation was more active against TR1 col^{R}1 compared to KK1 col^{R}3 (Fig. S8). Interestingly, bactericidal activity was observed in TR1 col^{R}1 treated with both dual drug formulations at 64 μg/mL COL (Fig. S8C-D). Differently, these formulations did not show bactericidal activity on KK1 col^{R}3 (Fig. S8A-B). Although, a more extensive analysis is required on a greater number of clinical strains, these preliminary results suggest that the efficacy of the dual drug formulations, in particular COL:ISA-L(2:1), may be extended to clinical

Table 2
Colistin half maximum (IC_{50})[a] and 90% (IC_{90})[a] inhibitory concentrations of COL and liposomal formulations for different *P. aeruginosa* strains.

				COL:ISA-L			
		COL	COL-L	4:1	2:1	1:1	1:2
PA14 col^{R}5	IC_{50}	4.00	7.38	7.96	7.59	7.01	8.30
	IC_{90}	32.54	34.56	29.53	19.04	32.92	52.79
KK1 col^{R}3	IC_{50}	3.17	6.48	ND	5.40	5.66	ND
	IC_{90}	7.59	14.43	ND	10.85	11.63	ND
TR1 col^{R}1	IC_{50}	17.24	21.41	ND	15.45	18.51	ND
	IC_{90}	121.34	172.52	ND	76.37	88.13	ND

[a] μg/mL.

isolates with high resistance to COL.

3.5. Biocompatibility and mobility tests

To evaluate the applicability of these formulations, in particular for the treatment of respiratory bacterial infections by *P. aeruginosa*, the cytocompatibility of the liposomal formulations was tested with a human lung cell line, the Calu-3 cell. For this, cell monolayers were incubated with increasing concentration of liposomes, covering a high range of COL concentration, up to 500 μg/mL, which is about 8 times more than the MIC of PA14 col^{R}5, and within the MIC distribution for COL, from 0.06 to 128 μg/mL, reported by Eucast (MIC EUCAST 2024). COL-L, ISA-L and COL:ISA-L incubated for a short (Fig. 8A) or long (Fig. 8B) time period with Calu-3 cells, did not affect cell viability suggesting that all the liposomal formulations possessed high biocompatibility. Only prolonged exposure to high COL-L concentrations showed a limited, and non-significative, reduction of cell viability (~10 %) (Fig. 8B).Fig. 9..

Additionally, considering the lung as a possible target site, the mobility of the liposomes in the mucus was analysed. Fluorescence recovery after photobleaching (FRAP) assay was carried out by using Cy5.5 labelled liposomes dispersed in mucin containing medium as model to investigate the liposome mobility in the mucus.

The FRAP images and the fluorescence profiles show a faster ISA-L fluorescence recovery compared to COL-L and COL:ISA-L, indicating

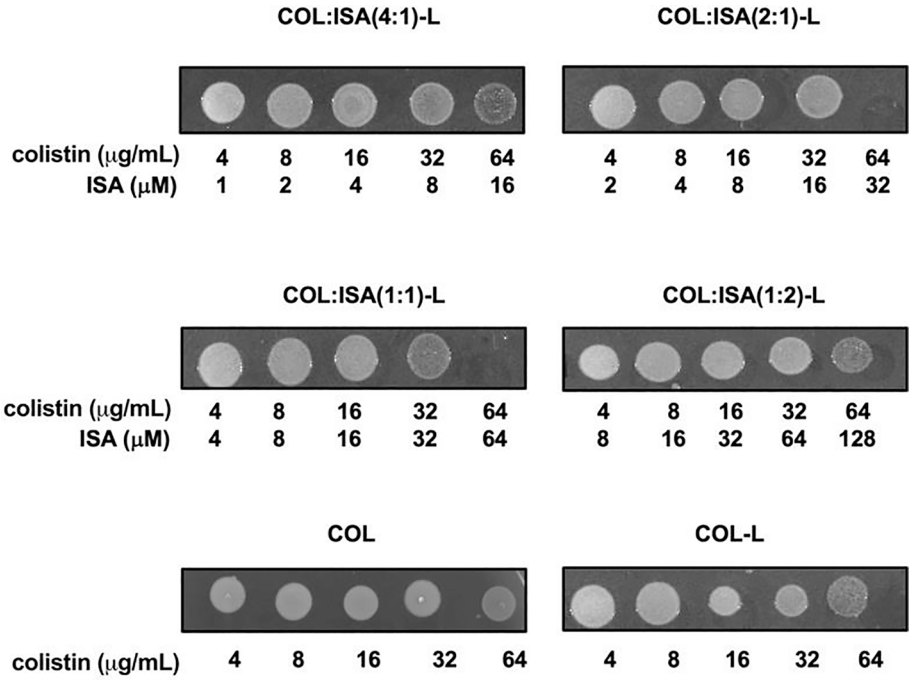

Fig. 7. Effect of liposomal formulations on PA14 colR5 cell viability. Spots represent the growth resulting from a 5-μL aliquot of cultures treated with the indicated formulations at the concentration reported below the images. Representative images of three independent experiments are shown.

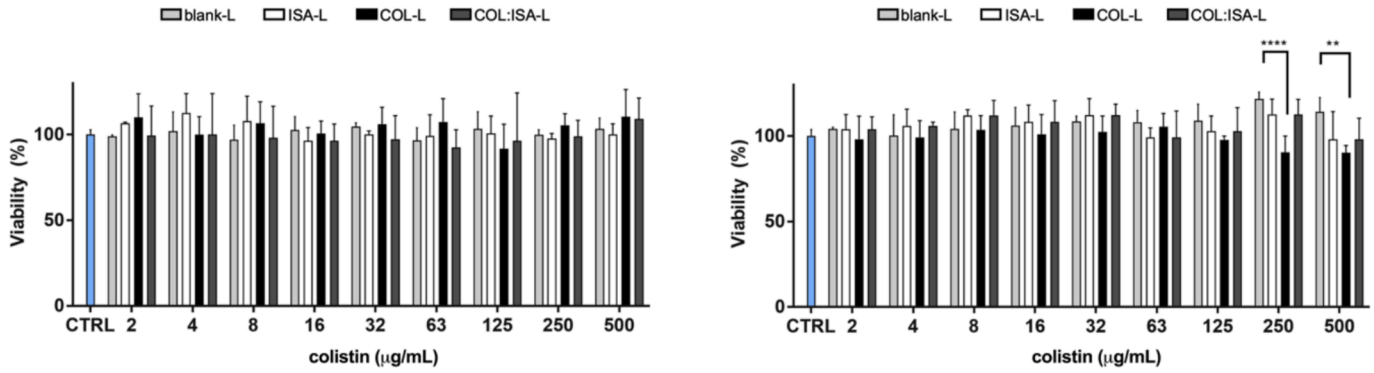

Fig. 8. Calu-3 cell viability (%) after treatment for 3 (left) and 18 h (right) with drug-free liposomes (blank-L) or liposomes loaded with ISA (ISA-L), COL (COL-L) and COL and ISA co-loaded (COL:ISA-L). Cells were exposed to liposomes at two-fold increasing concentration of colistin, in the range of 2–500 μg/mL. Formulations non containing colistin were tested at the lipid concentrations corresponding to COL loaded liposomes.

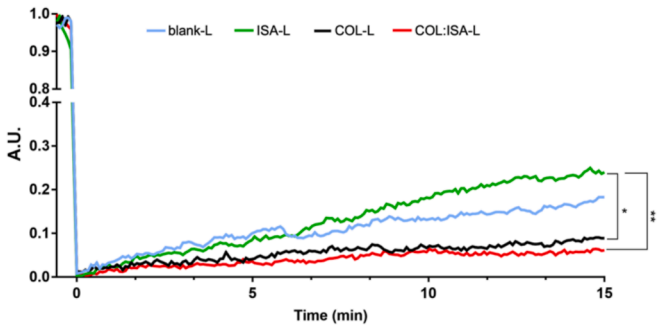

Fig. 9. Fluorescence recovery after photobleaching (FRAP) of drug-free (blank-L), ISA loaded (ISA-L), COL loaded (COL-L) and COL and ISA and co-loaded (COL:ISA-L) liposomes. Fluorescence recovery profile of liposomes after photobleaching. Data are normalized between 0.0 and 1.0 A.U., representing respectively fluorescence before and after photobleaching. Liposomes were generated with 5 mol% cholesterol/lipids; those containing ISA were produced with 5 mol% ISA feed; those containing COL were produced by hydrating the lipid film with 2.5 mg/mL COL solution. $^{**}p < 0.01$, $^{*}p < 0.05$.

higher mobility within the mucin medium (Fig. 9 and S9). The different mobilities well correlate with the zeta potential of liposomes. Indeed, the literature reports that the diffusion of lipid-based particles in mucus inversely correlates with the zeta potential (Zöller et al., 2024). Thus, liposomes with higher zeta potential (COL-L and COL:ISA-L) may have more interactions with mucin and lower mobility with respect to those with lower zeta potential (ISA-L).

4. Conclusion

In this study, we explored the potential of liposomes as delivery vehicles for the simultaneous administration of COL and COL-adjuvants. ISA, an ent-beyerane-based ArnT inhibitor with COL adjuvant activity (Quaglio et al., 2020), was co-encapsulated with COL, in liposomes. This is made possible by the assembly of liposomes encapsulating COL, which is amphiphilic, in the aqueous core and lipid bilayer and isostevic acid in the lipid bilayer. Liposomal formulations with biopharmaceutical features suitable for the treatment of COL resistant *P. aeruginosa* strains, possibly in the context of lung infections, were produced and demonstrated to allow COL activity on different *P. aeruginosa* strains. As expected by the adjuvant activity of ISA, the dual drug formulations were more active than the single drug liposomes, particularly on strains with high COL resistance. This enhanced efficacy could be attributed to several factors, including prolonged retention time and better interaction with the bacterial cell surface, which is the primary target of COL. Additional advantages of liposomal formulations include: the ability to fuse with bacterial membranes and/or biofilm structures (Scheeder et al., 2023; Rukavina *et al.*, 2016); the controlled, and possible targeted, drug release, which contribute to reduce the well-known toxicity of COL (Wadia and Tran, 2014; Lim et al., 2010; Dai et al., 2022). Overall, our study demonstrates that liposome-mediated delivery of COL and ent-beyerane-based compounds represents a promising strategy to overcome some of the major limitations associated with the use of COL, such as toxicity and resistance due to LPS-modifications, particularly in the context of lung infection by *P. aeruginosa*.

Although promising results have been obtained, additional work is required to further optimize the liposomal formulations and to extend their applications to other COL resistant strains, both clinical isolates and other Gram-negative bacteria, such as *Klebsiella pneumoniae* (Ghirga et al., 2020). Studies have been undertaken to produce a lyophilized product, which allows for long term stability and storage of the formulation. Preliminary results showed that sucrose can be properly used as lypoprotectant as it can allow the reconstitution of the formulation with same colloidal features of the starting product. Additionally, *in vivo* studies are required to evaluate the pharmacokinetics, biodistribution, and therapeutic efficacy of these liposomal formulations in relevant infection models. If successful, such strategies could provide a significant breakthrough in the fight against multidrug-resistant bacterial infections, offering a more effective and safer approach to treating these challenging pathogens.

CRediT authorship contribution statement

Valentina Pastore: Writing – original draft, Methodology, Investigation, Formal analysis. **Jessica Frison:** Methodology, Investigation. **Cristiano Pesce:** Investigation, Formal analysis, Data curation. **Mariya Ryzhuk:** Methodology, Investigation. **Mariangela Garofalo:** Investigation, Formal analysis, Data curation. **Martina Cristoferi:** Investigation, Formal analysis. **Silvia Cammarone:** Methodology, Investigation, Formal analysis, Data curation. **Giorgia Fabrizio:** Investigation. **Maria Carmela Bonaccorsi Di Patti:** Formal analysis, Investigation. **Deborah Quaglio:** Methodology, Formal analysis, Data curation. **Francesca Ghirga:** Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francesco Imperi:** Formal analysis, Conceptualization. **Mattia Mori:** Formal analysis, Conceptualization. **Paolo Caliceti:** Funding acquisition, Conceptualization. **Bruno Botta:** Funding acquisition, Conceptualization. **Fiorentina Ascenzioni:** Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Stefano Salmaso:** Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2025.125515.

Data availability

Data will be made available on request.

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