BMC Chemistry

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Catechol-substituted siderophore colistin exhibits superior antimicrobial activity than its unmodified polypeptide counterpart

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Abstract

Catechol moieties have been covalently coupled to the last-resort polypeptide antibiotic colistin via esterification and amidation reactions, inspired by the superior antimicrobial action of cefiderocol, i.e., a catechol-substituted siderophore cephalosporin. Among the tested strategies, the incorporation of the catechol motif by amidation reduces by 50% the minimum concentration to inhibit the growth of a clinical strain of uropathogenic *Escherichia coli* (*E. coli*) in its planktonic form. Its minimum bactericidal concentration is reduced by 25% after chemical modification. The tested modified antibiotic did not show cytotoxicity against human fibroblasts and keratinocytes at bactericidal doses. Additionally, due to the potential nephrotoxicity of colistin, the cytotoxicity of this catechol-substituted siderophore colistin was evaluated in a 3D model of human renal organoids showing no cytotoxicity at the doses tested. The chemical incorporation of catechol groups to existing antibiotics can reduce the doses to exert a fast antimicrobial action reducing the chances to develop antibiotic resistance.

Keywords Colistin, Antibiotic, Catechol, Siderophore, Polymyxin E, Esterification, Amidation

Introduction

Cefiderocol is a last resort cephalosporin used in the treatment of infections caused by multidrug resistant (MDR) gram-negative aerobic bacteria including strains having carbapenem resistance genes. This antibiotic incorporates a chlorocatechol group at the end of the side chain of a cephalosporin acting as a siderophore. This chlorocatechol chelates extracellular iron (an essential element for the synthesis of several metabolic and regulatory proteins of bacteria) and drives the antibiotic-iron inside bacteria using the ferric iron transporter system in the outer membrane of gram-negative bacteria [1]. In the periplasmatic space the chelated iron is released and the antibiotic prevents peptidoglycan cell wall synthesis by its attachment to penicillin-binding proteins [2].

In the competition with other species, several natural antibiotics of bacterial origin known as sideromycins, such as albomycins and salmycins (Fig. 1), have evolutionary used the same antimicrobial mechanism to introduce intracellularly an antibiotic covalently coupled to an iron-chelating agent [3]. This nature-inspired antibiotic delivery strategy reduces the minimum concentrations required to exert inhibitory and bactericidal action. For

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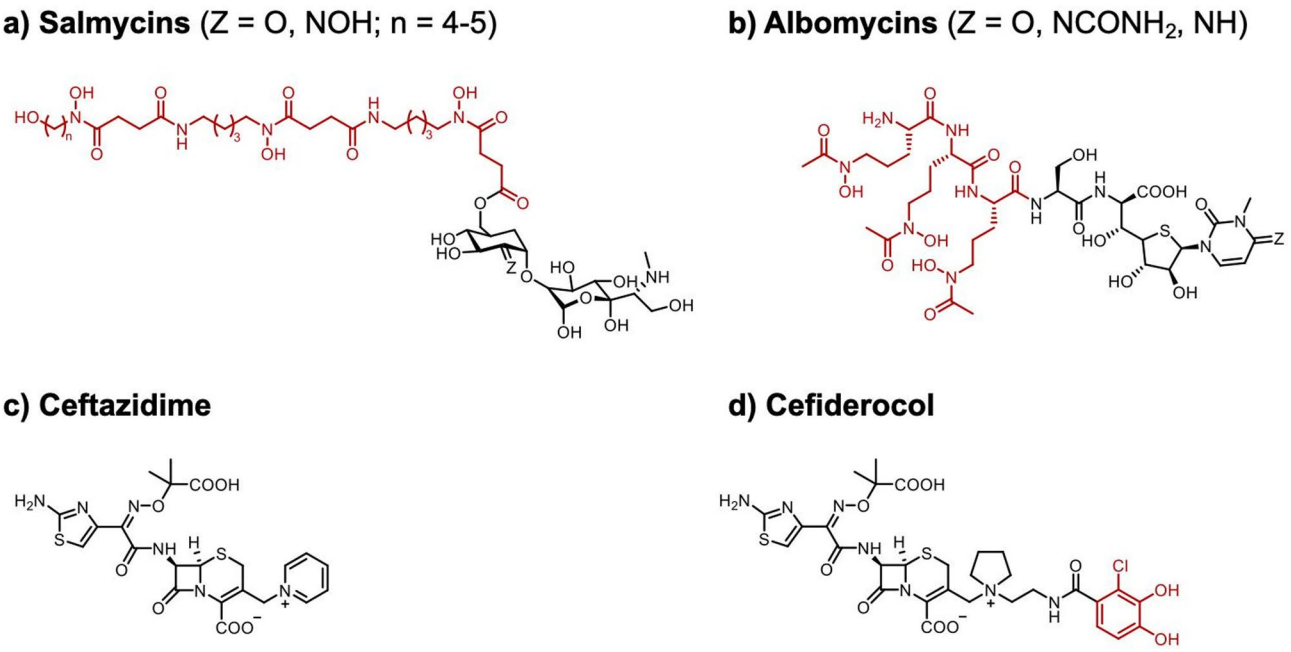

Fig. 1 Molecular structure of natural sideromycins of bacterial origin, (**a**) salmycins, (**b**) albomycins. Structure of a synthetic cephalosporin (**c**) ceftazidime and (**d**) siderophore- cephalosporin conjugates (cefiderocol). Labelled in red the iron chelating moiety

instance, compared to ceftazidime, a third-generation cephalosporin, the Minimum Inhibitory Concentration (MIC) reported was 4 times lower when using cefiderocol in iron-containing medium against *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 and up to 16 times lower when using an iron-depleted medium, corroborating its siderophore activity [1]. During infection, to counteract this antimicrobial action, bacteria upregulate their siderophore production to compete with those siderophore-antibiotic conjugates [4]. On the other hand, the infected host immune cells produce neutrophil gelatinase-associated lipocalin, as part of the innate immune response in order to scavenge iron-containing bacterial siderophores [5]. Nevertheless, cefiderocol, being included in the model list of essential medicines, has been granted approval by the for the treatment of adults with complicated urinary tract infections, including pyelonephritis, and hospital-acquired bacterial pneumonia, and ventilator-associated bacterial pneumonia. The has approved it for the treatment of infections caused by gram-negative aerobic organisms in adults with reduced treatment options [6]. The iron chelating ability of catechols has inspired the development of siderophore-antibiotic conjugates since the 1980s. The pioneering work of Ohi et al. [7] demonstrated that different penicillins having catechol groups show a preferential *P. aeruginosa* uptake and enhanced antimicrobial action (i.e., from 30 to 60-times greater than that of the parent ureidopenicillin lacking catechol groups used as control). They also demonstrated that the number and position of the hydroxyl groups on the benzene ring play an important role in the antimicrobial action observed being the isomers having two adjacent groups superior to any of the other conformations tested. The enhanced antimicrobial action of several catecholates (e.g., enterobactin, bacillibactin, etc.) and also tris-catechol enterobactin mimics conjugated to antibiotics have been extensively reviewed elsewhere [8].

Not only cefiderocol but also other novel antibiotics in different stages of development present an iron-scavenging catechol bound to β-lactam antibiotics through periplasmatic cleavable linkers which release the antimicrobial in the outer membrane of gram-negative bacteria [9]. Other authors have developed linkers which cleave releasing the antibiotic in the cytoplasmic space by covalently coupling bidentate catechol analogues to antibiotics (e.g., trimethoprim) [10]. Independently of the periplasmatic or cytoplasmic delivery, catechol groups stand out as the most common iron-complexing agents used to conjugate different antibiotics due to the flexibility of the organic synthesis, where the attachment position and the length and arrangement of the linker play a pivotal role [9].

Inspired by cefiderocol, herein, we hypothesized that the inclusion of a catechol group in another last resort essential antibiotic, colistin, might enhance its antimicrobial action. Colistin (polymyxin E) is a polypeptide antibiotic whose positively charged peptide ring binds to the electronegative phosphate moieties on the lipid A subunits of the outer membrane lipopolysaccharides of gram-negative bacteria producing inner membrane

permeabilization and intracellular leakage [11]. Due to its neuro- and nephrotoxicity it is used on resistant gram-negative bacteria as last resort antibiotic only [12]. Several other surface-acting membrane-disrupting peptides are used in antimicrobial and oncolytic therapies [13].

Plasmid-mediated mobilized colistin resistance (mcr) genes have been identified globally in animals, humans, the environment, and in animal-derived food products [14]. Several combination regimens of some antibiotics, antimicrobial peptides and metals have demonstrated to overcome colistin resistance and reduce the antimicrobial concentrations required to inhibit bacterial growth. For instance, MacNair et al. [15] demonstrated that the combination of colistin with rifampins and macrolides is able to reduce the MICs in murine models of mcr-1 positive *Klebsiella pneumoniae* (*K. pneumoniae*). The use of this hydrophobic antibiotics, conventionally active against gram-positive bacteria, disrupts the outer-membrane of mcr-1 strains enhancing the effect of colistin. Antimicrobial peptides (e.g., β-defensin-2 and MSI-78) have also been used in combination with colistin to circumvent the resistance of bacterial strains of *E. coli*, *K. pneumoniae*, *Acinetobacter baumannii* (*A. baumannii*) and *P. aeruginosa* harbouring the mcr-1 and −2 genes taking advantage of the pore-forming ability of those antimicrobial peptides [16]. Repurposed non-antibiotic drugs have also demonstrated the ability to reduce inhibitory and bactericidal concentrations. For instance, Domalaon et al. [17] showed that, compared to monotherapy, the combination of anthelmintic salicylanilides and colistin reduced the MIC values for colistin-resistant *E. coli* 94,474 clinical isolates (i.e., harbouring the colistin-resistant mcr-1 gene plasmid) re-sensitizing the bacteria to the antibiotic due to the genetic and phenotypic responses induced by the salicylanilides. MCR-1 is a phosphoethanolamine (pEtN) transferase that modifies the pEtN binding group in lipid A subunits of the outer membrane lipopolysaccharides preventing colistin from binding. This transferase includes a necessary Zn(II) cofactor in its active site, which can be irreversibly substituted by silver ions restoring the efficacy of colistin, as shown by Zhang et al. [18] in murine models of peritonitis and in wound infections with mcr-1 positive *K. pneumoniae* 9607.

Besides the use of those combination therapies, the use of siderophores can also reduce the concentration needed to inhibit the growth of clinical isolates. In early studies, the colistin antimicrobial effect was potentiated by the addition of catechol-type flavonoids as adjuvants that dysregulate iron homeostasis and promote ROS (reactive oxygen species) generation due to an excessive accumulation of ferrous iron in four representative MDR isolates of gram-negative bacteria (including *Salmonella typhimurium*, *E. coli* and two strains of *K. pneumoniae*) [19]. Phenolic acids (i.e., caffeic acid, gallic acid and protocatechuic acid) bearing catechol groups also demonstrated to enhance the antimicrobial action of colistin due to the increased generation of superoxide anions on *A. baumannii* AB5075 [20]. Other adjuvants are also used to block resistance mechanisms on pathogenic resistant bacteria. For example, Minrovic et al. [21] developed 2-aminoimidazole-based adjuvants which downregulate the PmrAB system, responsible for the phosphoethanolamine modification of the lipid A anchor of lipopolysaccharides, which confers *A. baumannii* resistance to colistin. Other strategies explore the use of endogenous iron to mediate the conjugation of chlorocatechol-containing siderophores to antibiotics bearing pyridine groups such as pyridomycin [22] improving the antimicrobial action of the antibiotic alone.

In those previous examples colistin and catechol adjuvants were simultaneously added. In this work, for the first time, we have covalently bound catechol units to commercial colistin using two synthetic routes and study the antimicrobial effect of the released modified colistin in the bacterial periplasmatic space. We have followed two synthetic routes using 3,4-dihydroxybenzoic acid as reagent to link catechol units. On the one hand, we have used an esterification reaction where the catechol group was bound to colistin through an ester bond with the hydroxyl groups of threonine (Thr) residues. On the other hand, we have used an amidation reaction to statistically connect catechol units to colistin through an amide bond to the amino groups in colistin (2,4-diaminobutyric acid, Dab residues) (Fig. 2). The efficacy of both novel siderophore-colistin molecules has been benchmarked against non-modified colistin using a clinical isolate of uropathogenic *E. coli* in its planktonic and sessile forms.

Results and discussion

Synthesis and characterization of catechol-substituted colistin

Three catechol-substituted colistin samples were synthesized following two different pathways. The carboxylic acid group of the commercially available 3,4-dihydroxybenzoic acid, or protocatechuic acid, was used to covalently attach colistin through either esterification or amidation reactions (Fig. 3a and b, respectively).

Regarding the esterification pathway, *para*-methoxybenzyl (PMB) was used as protecting group of 3,4-dihydroxybenzoic acid obtaining product **1** under mild basic conditions. Selective deprotection of PMB ester gave PMB-protected benzoic acid **2**, following a previously reported protocol (relevant spectroscopic data are collected in Figures S1 to S4) [23]. Since the amine groups of the Dab residues of colistin can be chemically active, they were also protected by *tert*-butyloxycarbonyl (Boc) groups [24], giving product **3** with a highly improved

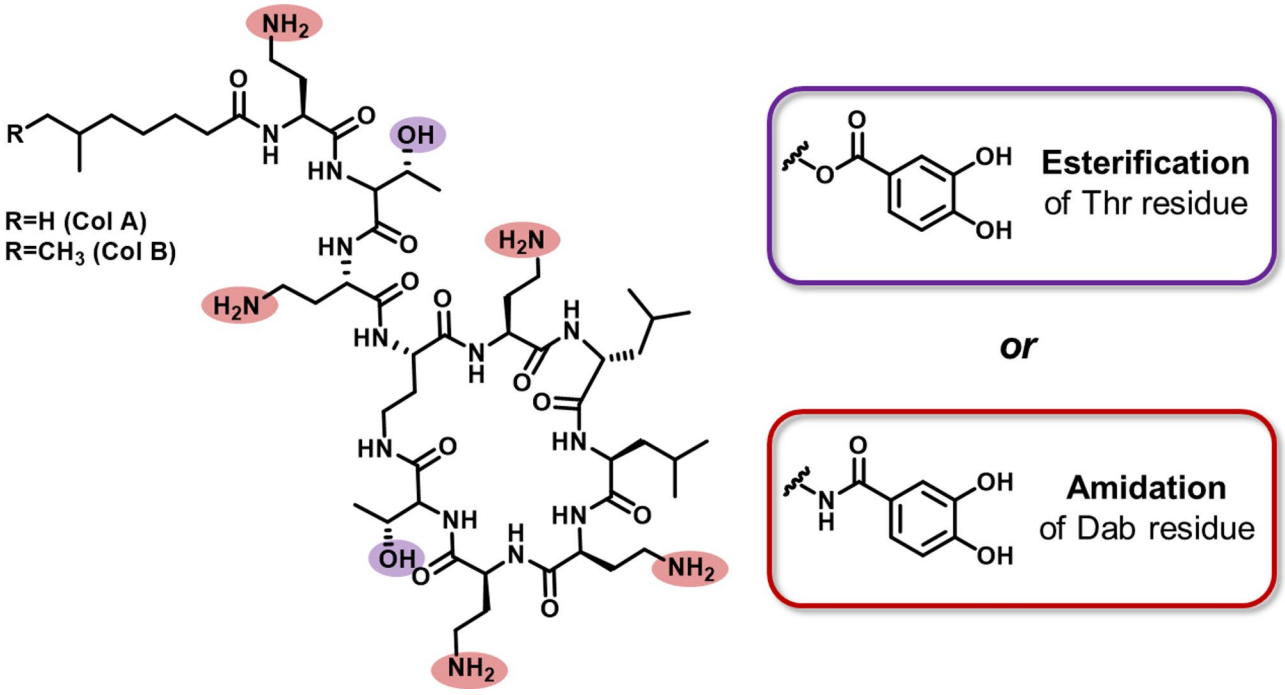

Fig. 2 Design of target reactive sites in the colistin molecule to introduce catechol units by esterification or amidation reactions

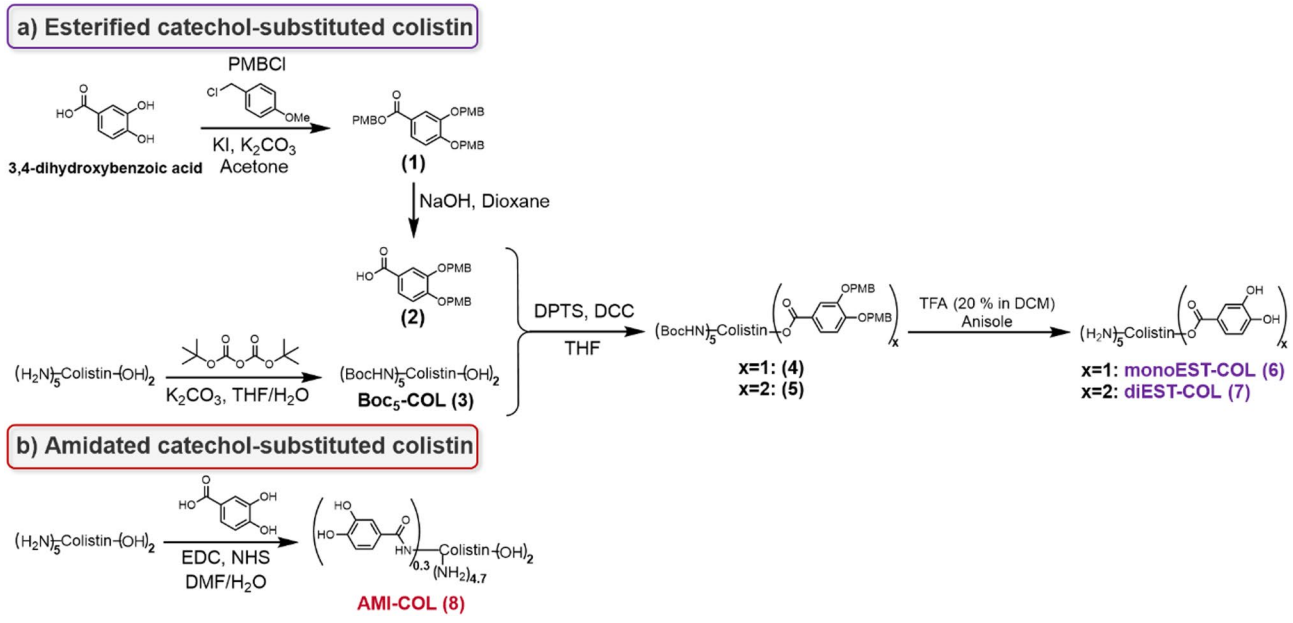

Fig. 3 Synthetic scheme for catechol-substituted colistin by (**a**) esterification or (**b**) amidation reactions

solubility in organic solvents. The correct development of the reaction was validated by ^{1}H NMR spectroscopy by the apparition of the methyl Boc signal at 1.37 ppm (Figures S6 and S7), as well as by the observed modification in the solubility of the product, turning to completely insoluble in water after Boc-protection. Then, esterification was carried out by the Steglich reaction, coupling **2** onto either one [4] or the two [5] colistin Thr residues. Both products were obtained from the same reaction pot and separated by flash column chromatography on silica gel. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra confirmed the apparition of catechol aromatic signals from δ 7.64 to 6.85 ppm, overlapped with -N*H*-Boc signals, and of benzylic protons (ArCH_2O) at 5.22–5.15 ppm (Figures S8 and S9 for **4** and S10 for **5**). Integration of the OH protons at 4.99–4.88 ppm also

decreased from 2 H in product **3** to 1H in **4**, or they completely disappeared in **5**. Due to the presence of two esterification sites in **3**, functionalization at either the extracyclic or intracyclic Thr residues yielded **4** as a mixture of isomers. Target **monoEST-COL (6)** or **diEST-COL (7)** were obtained by simultaneous deprotection of both PMB and Boc-protecting groups under acidic conditions. They were purified by size exclusion chromatography (SEC) using Sephadex® G-10 as stationary phase and ammonium bicarbonate volatile buffer (0.1 M, pH 7.8) as mobile phase. Although the complete deprotection was confirmed by the disappearance of Boc and PMB signals in both ^{1}H NMR spectra (Figure S11 and S12 for **monoEST-COL** and **diEST-COL**, respectively), the limited solubility of **diEST-COL** in water, dimethyl sulfoxide (DMSO) or mixtures of both, explains the poor resolution in the spectrum. Still, **monoEST-COL** was found to be moderately soluble in water.

In order to synthetize the amidated catechol-substituted colistin, a single-step procedure was first attempted using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) in a mixture of water and *N*, *N*-dimethylformamide (DMF), to ensure the solubility of all the reactants at room temperature. Purification was performed by precipitation in cold acetone. However, despite various attempts to optimize the reaction conditions, including different acid-to-colistin ratios, solvents, activating system, pH of the solution, temperature and protecting the hydroxyl groups of the catechol molecule, a complete amidation was not reached in any case (see Tables S1-S4, Supporting Information). Therefore, the isolated **AMI-COL** corresponds to a mixture with an average of non-substituted colistin (70%) and catechol-amidated colistin (30%), according to the integration of the aromatic ring signals shown in the ^{1}H NMR spectrum (Fig. 4a). This mixture composition was found to be reproducible in different independent batches using the same conditions. Amidation is also assumed to occur in any of the 5 possible amine groups or, although less likely, in more than one of the same molecule, obtaining a mixture of positional isomers. Diffusion-ordered spectroscopy (DOSY) experiments confirmed the attachment of the catechol motif, since it reported one unique diffusion coefficient for ^{1}H NMR resonances of catechol and colistin protons (Fig. 4b).

Biological evaluation

The microbiological activity of the three catechol-substituted colistin derivatives (as well as that of unmodified colistin) was tested by the broth microdilution method against a clinical uropathogenic *E. coli* strain (UTI *E. coli*). As previously reported, experiments were conducted under iron-depleted conditions in order to induce the production of iron transporters mimicking the conditions of human tissues and fluids infected by bacteria [25–28]. Iron-depleted broth (ID-CAMHB) was prepared as Hackel et al. [25] reported. Briefly, Mueller Hinton Broth 2 was stirred with Chelex 100 resin (100 g of resin per liter of broth) for 2 h and filtered through a 0.2-micron filter to remove the resin. Then, pH was adjusted to 7.3 using 0.1 M HCl solution and the broth was supplemented with calcium chloride (22.5 mg/L), magnesium chloride (11.25 mg/L) and zinc sulphate (0.56 mg/L). Finally, ID-CAMHB was filtered again with a 0.2-micron filter and autoclaved.

For unmodified colistin used as reference, the determined MIC (concentration that reduce bacteria growth, at least, 2 log respect to an untreated control) and Minimum Bactericidal Concentration (MBC, concentration which completely eradicates bacteria) values were 600 and 1000 ppb, respectively (Table 1). In the case of the **monoEST-COL**, no improvement (i.e., reduction in the MIC values) was observed, as the determined MIC value was 750 ppb, while the MBC value could not be reached

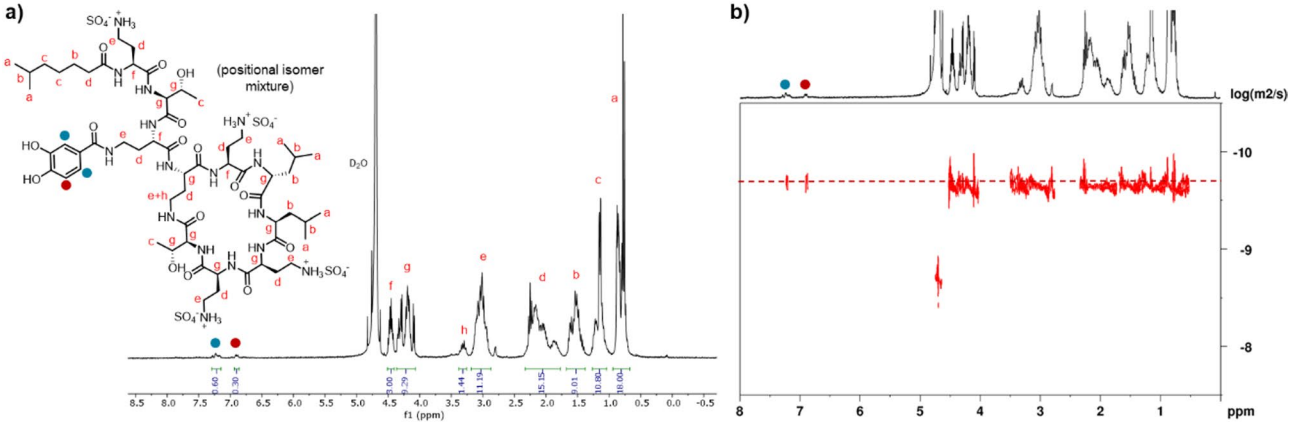

Fig. 4 (**a**) ^{1}H NMR and (**b**) DOSY spectra of **AMI-COL** (D_2O, 400 MHz). For the sake of clarification, protons located in the same area of the spectrum are labelled with the same letter

Table 1 Microbiological activity of colistin and colistin-derivative samples against UTI *E. coli* strain

Sample	MIC (ppb)	MBC (ppb)
Colistin	600	1000
monoEST-COL	750	> 2000
diEST-COL	2500–5000	> 10,000
AMI-COL	300	750

within the concentration range tested, being above 2000 ppb (Table 1). On the other hand, for the **diEST-COL**, the determined MIC and MBC values were higher than those for **monoEST-COL** (in the range of 2500–5000 ppb for MIC and above 10000 ppb for MBC, Table 1). In both cases, the MIC and MBC values determined for the synthesized compounds via esterification reactions were higher than those obtained for unmodified colistin, indicating that the incorporation of catechol units into the hydroxyl groups of Thr residues, had reduced its bactericidal action. Best reported mechanism of action of colistin is based on the interaction between the positively charged groups of the molecule (such as amino groups) and the negatively charged phosphate groups of lipid A, a component of the lipopolysaccharides in the outer membrane of Gram-negative bacilli [29]. Since the inclusion of catechol moiety in **monoEST-** and **diEST-COL** does not affect the amino groups in colistin molecule, the reduction in the bactericidal activity must be attributed to other factors. As previously mentioned, **monoEST-COL** was moderately soluble in water, while **diEST-COL** was poorly soluble in water or water/DMSO mixtures. Given that the culture medium is primarily aqueous, this reduced solubility (i.e., reduced bioavailability) compared to that of the colistin sulphate used as reference could explain the reduction in the bactericidal activity of the samples synthetized through esterification reactions compared to that observed for the colistin sulphate.

Alternatively, **AMI-COL**, which resulted from the statistically amidation of the colistin and showing a higher water solubility than the ester derivatives, was studied. The results obtained for this compound, compared with those of unmodified colistin, are shown in Fig. 5a. Compared to unmodified colistin, MIC and MBC values were reduced to 300 and 750 ppb, respectively (Table 1). Compared to the non-modified colistin, this reduced antibiotic MBC, would eradicate bacteria completely and quickly leaving less time for bacteria to mutate and develop resistance, and, at the same time, unwanted side effects would also be minimized.

Regarding the mechanism of action of colistin, once the interaction between the amino groups of the colistin and lipid A occurs, the molecule enters the bacterium. Inside the bacterium, the hydrophilic groups (free amino groups) of the fatty acid chains of colistin interact with the inner membrane, causing to lose its integrity and leading to cell lysis [29]. In the case of **AMI-COL**, the inclusion of the catechol moiety in the colistin molecule results in the statistical loss of one amino group in 30% of colistin molecules. Based on this, it should reduce the interaction between the compound and the bacterial outer membrane, thereby decreasing its bactericidal activity. However, the results suggest an effective entry of the compound into the bacteria due to the presence of catechol acting as siderophore, as its bactericidal activity is greater than that of the unmodified colistin.

To verify its siderophore character, the bactericidal activity assay was repeated at the MIC concentration for both samples (unmodified reference colistin and **AMI-COL**) in a culture medium where iron was not depleted (Mueller-Hinton Broth 2, CAMHB). As can be seen (Fig. 5b), the effect of unmodified colistin was similar in both culture media, reducing the bacterial growth by, at least, 2 logs. However, in the case of the **AMI-COL**, when using the culture medium with iron, bacterial growth was not reduced at the same level at which bacterial growth inhibition was observed in the medium without iron. Therefore, we can conclude that **AMI-COL** acts as a siderophore.

In previous studies, reported by Masakatsu et al. [30], it was found necessary to use iron-free culture media to obtain reproducible and comparable results with in-vitro models when determining the MIC of cefiderocol. As a result, the Clinical and Laboratory Standards Institute Subcommittee on Antimicrobial Susceptibility Testing approved the microdilution assay using iron-free culture medium in 2016 to reliably assess the bactericidal activity of siderophore-based antimicrobial compounds [31]. Taking these recommendations into account, the results obtained could indicate that, while colistin does not have a mechanism of action dependent on iron transporters to enter the bacterium and similar results were obtained in medium with and without iron, the mechanism of action for **AMI-COL** would indeed depend on these iron transporters, because a reduced antimicrobial action was observed in an iron containing medium. This suggests that colistin has been herein modified to have properties similar to those of other siderophore antibiotics such as cefiderocol.

With this bacterial entry strategy, disruption of the bacterial inner membrane would not be possible, as **AMI-COL** would be located in the cytoplasm rather than embedded in the membrane. However, another secondary mechanism of action has been reported for colistin, the inhibition of the NADH-quinone oxidoreductase, an essential enzyme in the cellular respiration of Gram-negative bacteria [32, 33].

Beyond its antibiotic activity against Gram-negative bacteria, colistin has also inhibitory effects on biofilm formation [34, 35]. The antibiofilm activity of **AMI-COL**

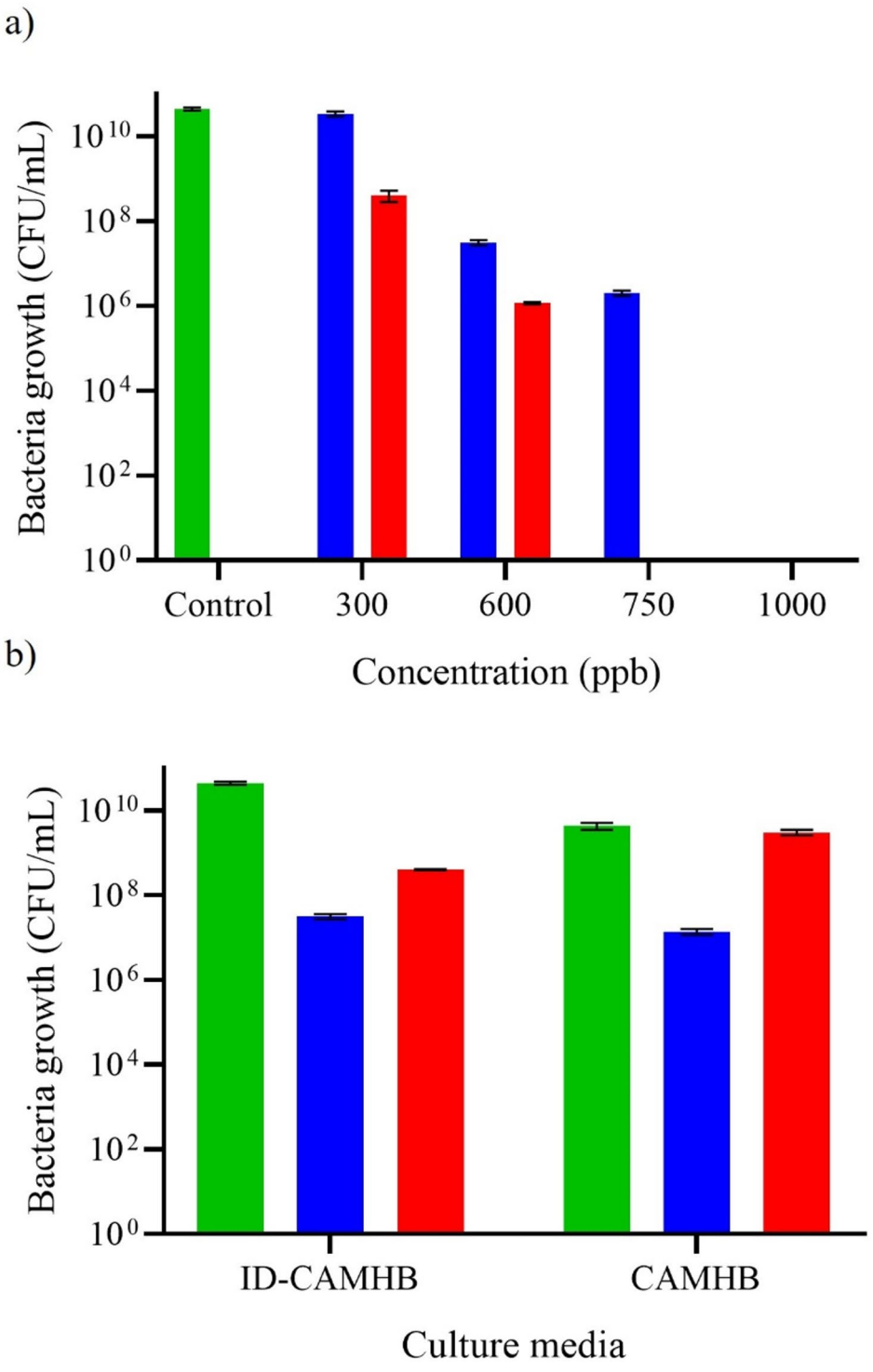

Fig. 5 (**a**) Bactericidal activity of commercial colistin (blue) and **AMI-COL** (red) against UTI *E. coli*. Control sample (untreated bacteria) is shown in green. (**b**) bacteria growth at MIC concentration for control (untreated samples, green), colistin (600 ppb, blue,) and **AMI-COL** (300 ppb, red). Values are shown as Colony Forming Units per mL (CFU/mL). CAMHB stands for Mueller-Hinton Broth 2, and ID-CAMHB stands for iron depleted Mueller-Hinton Broth 2

was tested to investigate whether the inclusion of the catechol group in the colistin structure affects biofilm formation or erradication. The ability of both unmodified reference colistin and **AMI-COL** to inhibit biofilm formation from an UTI *E. coli* culture, as well as their ability to disrupt a preformed mature biofilm of this bacterial strain, were evaluated.

The percentage of biofilm obtained (normalized respect to a control without any bactericidal treatment) for the biofilm inhibition assay is presented in Fig. 6a). Both reference colistin and **AMI-COL** samples were able to inhibit biofilm formation (showing dose-dependence), at a concentration of 300 ppb and higher. However, the inclusion of the catechol group in **AMI-COL** did not enhance its activity as biofilm inhibitor for the studied strain of *E. coli*. A higher percentage of biofilm formed was found in samples treated with **AMI-COL** compared to those treated with colistin (at concentrations of 300 and 600 ppb). At higher concentrations, where the percentage of biofilm formed was less than 50%, there were no significant differences in the treatment with both compounds.

The concentration of viable bacteria present in the samples was also studied to determine whether the inhibition of biofilm formation was caused by a lower number of bacteria present or because the biofilm production mechanisms were blocked (Fig. 6c). For the reference colistin, it was observed that, while the concentration of the compound increased, the bacterial counts and the percentage of biofilm present both decreased. However, for the lower concentrations of **AMI-COL**, it was observed that although the percentage of biofilm decreased (up to 54% for 600 ppb of **AMI-COL**), the bacterial concentration was not altered (no statistically significant differences were found between the treated sample and the control sample).

On the contrary, neither of the two studied samples, reference colistin and **AMI-COL**, were able to eradicate mature biofilms of UTI *E. coli*. At all the concentrations tested, the percentage of biofilm present in the samples varied between 77% and 115%, indicating that, once the biofilm was formed, neither of these antimicrobials could disrupt it (at the tested concentrations). This was confirmed by the analysis of viable bacteria within the

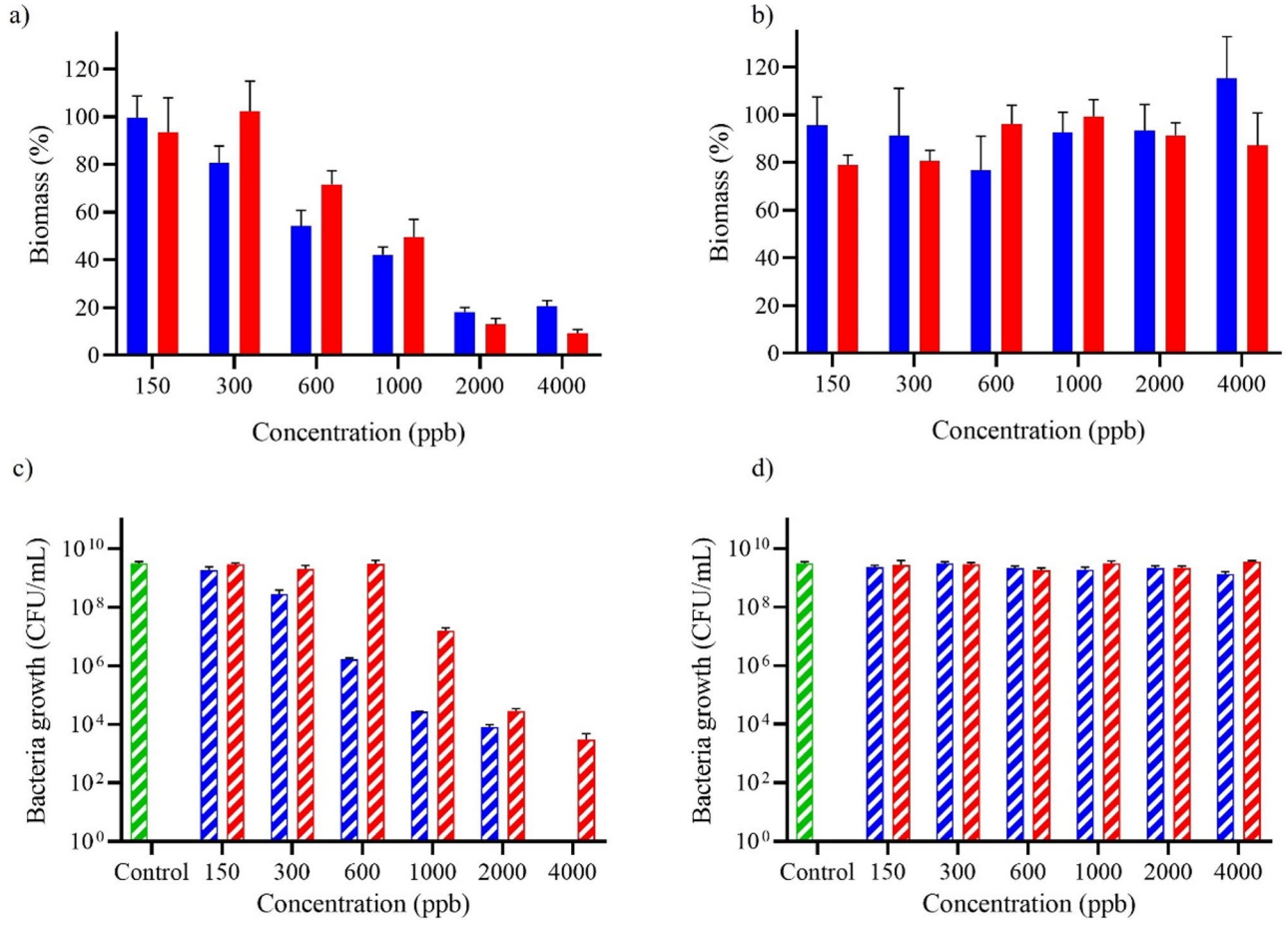

Fig. 6 Anti-biofilm activity of colistin (blue) and **AMI-COL** (red). (**a**) and **c**), inhibition of biofilm growth. (**b**) and **d**), disruption of mature biofilms

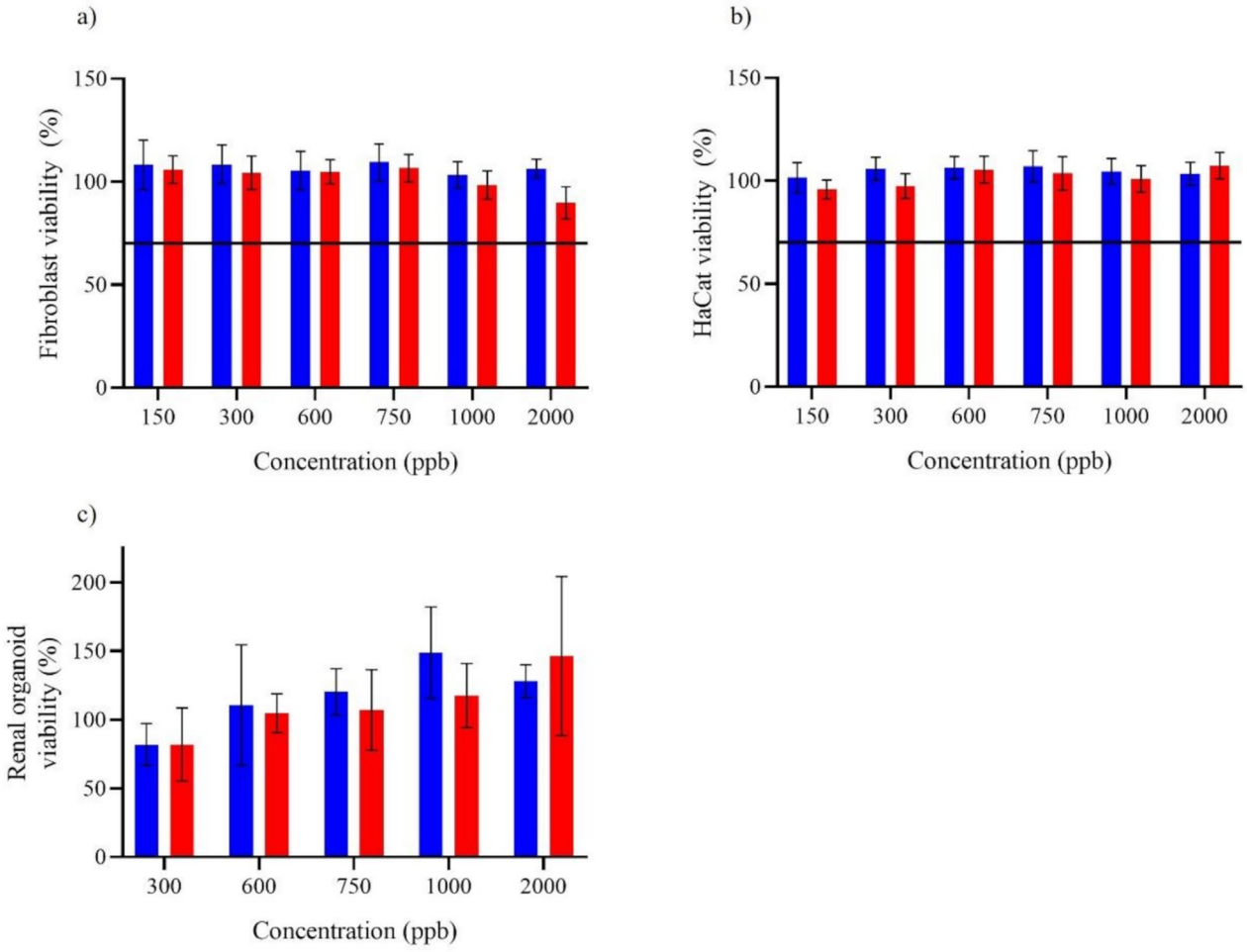

Fig. 7 Cytotoxicity studies for colistin (blue) and **AMI-COL** (red)

biofilm (Fig. 6d). In this experiment, bacterial load was not reduced in any case, with bacterial concentrations reaching levels similar to the untreated control samples (~10^9 Colony Forming Units per mL, CFU/mL).

Finally, the cytotoxicity of tested compounds against eukaryotic cells was also evaluated. Two 2D cell culture models, one of human fibroblasts and one of keratinocytes (HaCat), as well as a 3D renal organoid model, were used. A renal organoid was selected due to the reported nephrotoxicity of colistin. In all cases, the ISO 10993-5:2009 norm was followed, which sets the cell viability limit at 70% to consider a medical device as safe. As shown in Fig. 7, neither of the two samples (reference colistin nor **AMI-COL**) reduced the viability of any cell line below 70%, at the studied concentrations. Since the modification introduced in the colistin molecule did not result in increased cytotoxicity, **AMI-COL** can still be considered as an antibiotic, as it did not exhibit greater cytotoxicity than reference colistin at the concentrations and cellular models studied and it is selective against bacteria.

Conclusions

Here, we report the synthesis of three new colistin-derivatives, including 3,4-dihydroxybenzoyl unit as catechol siderophore moiety in order to enhance their bactericidal activity. Samples synthetized by esterification reaction (**monoEST**- and **diEST-COL**) were functionalized with one or two catechol moieties per molecule, respectively. However, those samples did not present better bactericidal activity than unmodified commercial colistin despite of the presence of the siderophore unit. **AMI-COL** was obtained by an amidation reaction to obtain the statistical amidation of one amino group in 30% of colistin molecules showing better bactericidal activity than the reference colistin. For **AMI-COL**, a decrease of MIC value from 600 ppb to 300 ppb and the MBC from 1000 to 750 ppb were observed. The different antimicrobial behaviour of the **AMI-COL** in media with iron or having iron depleted suggests that the catechol group acts as a siderophore, sequestering extracellular iron, driving its bound colistin to the bacterial interior and improving its bactericidal activity. Cytotoxicity of **AMI-COL**

was also evaluated, presenting no cytotoxicity against human fibroblasts and HaCat 2D cellular models as well against 3D renal organoid models, at bactericidal concentrations. The chemical incorporation of catechol groups to existing antibiotics can reduce the doses to exert their antimicrobial action potentially reducing the chances of developing antibiotic resistance.

Experimental section

Reagents

Colistin sulphate, used as reference in biological evaluation and reagent for esterification and amidation reactions, was purchased from Biosynth. 4-methoxybenzyl chloride was purchased from TCI Europe. Blue Cell Viability Assay kit, Dulbecco's Modified Eagle Medium (DMEM), hydrochloric acid (HCl, 37%), L-glutamine, a mix of antibiotics and antimycotics (penicillin-streptomycin-amphotericin B, PSA) and trypticase in soy agar plates (TSA plates) were purchased from VWR. EDC, 3,4-dihydroxybenzoic acid, acetic acid (≥ 99%), anisole, calcium chloride solution ($CaCl_2$, 1 M), crystal violet, di-tert-butyl decarbonate, K_2CO_3, KI, magnesium chloride hexahydrate ($MgCl_2$), Mueller Hinton Broth 2, *N*-acetylcysteine, NHS, *N*, *N*'-dicyclohexylcarbodiimide (DCC), Sephadex® G-10 gel filtration resin, trifluoroacetic acid and zinc sulphate monohydrate ($ZnSO_4$) were obtained from Sigma-Aldrich. Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Labclinics and Advanced F-12 medium and B-27 supplement were purchased from Gibco. R-spondin3 conditioned medium was obtained from Immunoprecise, EGF from Peprotech, Rho-kinase inhibitor Y-27,632 from Abmole and A83-01 from Tocris Bioscience.

Chemical characterization

^{1}H NMR, carbon-13 Nuclear Magnetic Resonance (^{13}C NMR), ^{1}H ^{1}H homonuclear correlation spectroscopy (COSY) and DOSY spectra were recorded on a Bruker 400 MHz NMR spectrometer. The experiments were performed at room temperature in different deuterated solvents ($CDCl_3$, DMSO-d_6 or D_2O).

Catechol-substituted colistin compounds synthesis protocol

*Synthesis of 4-methoxybenzyl 3,4-bis((4-methoxybenzyl) oxy)benzoate**(1)***. 3,4-Dihydroxybenzoic acid (2 g, 12.98 mmol), KI (6.9 g, 41.53 mmol) and K_2CO_3 (12.6 g, 90.86 mmol) were mixed in acetone (85 mL) for 30 min at 40 ºC, getting a white slurry. 4-Methoxybenzyl chloride (6.6 g, 41.83 mmol) was subsequently added dropwise and the reaction was stirred at reflux temperature for 3 days. The mixture was cooled to room temperature and the solvent evaporated to dryness. The remaining solid was dissolved in water (80 mL) and extracted with dichloromethane (3 × 80 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and the solvent was removed under vacuum. The product was purified by flash column chromatography on silica gel, initially eluted with hexane/ethyl acetate (9:1), gradually increasing the polarity to finish with a (6:4) mixture. The target compound was obtained as a pale-yellow solid. Yield: 60%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.63–7.61 (2 H, m), 7.37–7.32 (6 H, m), 6.92–6.86 (7 H, m), 5.24 (2 H, s), 5.12 (2 H, s), 5.09 (2 H, s), 3.82 (3 H, s), 3.80 (6 H, s). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.2, 159.2, 159.1, 159.0, 152.6, 147.7, 129.9 (2 × CH), 129.5 (2 × CH), 129.4 (2 × CH), 127.7, 127.4, 127.2, 123.4, 122.0, 114.6, 113.9 (2 x CH), 113.8 (2 × CH), 113.7 (2 × CH), 113.2, 69.9, 69.7, 65.7, 55.1, 55.1, 55.0.

*Synthesis of 3,4-bis((4-methoxybenzyl)oxy)benzoic acid**(2)***. Compound **1** (3.5 g, 7.73 mmol) was dissolved in 1,4-dioxane (40 mL). NaOH aqueous solution (2 M, 15 mL) was subsequently added and the reaction was stirred at 80 ºC for 5 h. After cooling the reaction to room temperature, the solvent was evaporated to dryness. The remaining residue was stirred in water and acidified to pH 2 by dropwise addition of 1 M HCl aqueous solution, until a white precipitate appeared. The solid was filtered and washed with cold hexane. Yield = 64%.^{1}H NMR (DMSO-d_6, 400 MHz, δ (ppm)): 12.67 (1H, bs), 7.54–7.51 (2 H, m), 7.39–7.33 (4 H, m), 7.14 (1H, d, J = 9.0 Hz), 6.96–6.90 (4 H, m), 5.11 (2 H, s), 5.06 (2 H, s), 3.75 (3 H, s), 3.74 (3 H, s). ^{13}C NMR (DMSO-d_6, 100 MHz, δ (ppm)): 167.5, 159.5, 159.4, 152.7, 148.1, 129.9 (2 × CH), 129.7 (2 × CH), 129.3, 129.0, 123.9, 123.6, 115.2, 114.3 (2 × CH), 114.2 (2 × CH), 113.6, 70.3, 70.2, 55.6, 55.5.

*Synthesis of Boc-protected colistin**(3)***. Synthesis of **3** was performed following a previously reported protocol [24]. A solution di-*tert*-butyl dicarbonate (8.8 g, 39.45 mmol) in THF (100 mL) was added to a solution of colistin sulphate (2 g, 1.58 mmol) in distilled water (80 mL). The reaction mixture was stirred at room temperature for 5 min. A solution of K_2CO_3 (5.4 g, 39.45 mmol) in distilled water (20 mL) was added dropwise to the reaction mixture, which was then stirred overnight at room temperature. The mixture was then transferred to a separating funnel, and saturated NaCl solution (120 mL) and tetrahydrofuran (THF) (120 mL) were added. After 30 min, when two phases were clearly differentiated, the organic phase was collected and the aqueous one extracted again with THF (2 × 60 mL). The combined organic extracts were dried with $MgSO_4$, filtrated and the solvent eliminated by rotary evaporation. The residue was purified by flash column chromatography on silica gel using first dichloromethane as eluent and increasing progressively polarity to dichloromethane/methanol 92/8. The desired product was obtained as a pale-yellow powder. Yield = 75%. ^{1}H NMR (DMSO-d_6, 400 MHz, δ

(ppm)): 8.80–7.61 ppm (8 H, m), 7.50–6.15 ppm (8 H, m), 4.91 (2 H, m), 4.43–3.76 ppm (12 H, m), 3.20–2.87 ppm (12 H, m), 2.15 (2 H, m), 1.98–1.48 ppm (20 H, m), 1.37 ppm (45 H, s), 1.27–0.99 (11 H, m), 0.94 – 0.76 ppm (18 H, m).

Synthesis of ***monoEST-BocCOL(4)*** *and* ***diEST-BocCOL(5)***. Compounds **2** (178.7 mg, 0.45 mmol) and **3** (500 mg, 0.3 mmol) were dissolved in dry THF under Ar. 1,4-Dimethylpyridinium *p*-toluenesulfonate (DPTS, 44 mg, 0.15 mmol) and DCC (186 mg, 0.9 mmol) were subsequently added while cooling the mixture into an ice bath. The reaction mixture was left to warm to room temperature while stirred for 3 days. After that, the solvent was eliminated by rotary evaporation. Both compounds were separated by flash column chromatography on silica gel, initially eluted with dichloromethane/methanol 97/3, gradually increasing the polarity to 94/6. Compounds **4** and **5** were obtained in approx. 6:4 ratio and were used for deprotection without further purification. Overall yield = 55%.

^{1}H NMR of **4** (DMSO-d_6, 400 MHz, δ (ppm)): 8.69–8.39 (2 H, m), 8.29–7.91 (6 H, m), 7.64–7.51 ppm (2 H, m), 7.39–7.32 ppm (4 H, m), 7.20–7.02 (3 H, m,, 6.96–6.85 ppm (6 H, m), 6.73–6.53 (4 H, m), 5.22–5.15 ppm (1H, m), 5.13–5.04 ppm (4 H, m), 4.99–4.88 ppm (1H, m), 4.73–4.61 ppm (1H, m), 4.43–3.76 ppm (10 H, m), 3.73 ppm (6 H, s), 3.16–2.84 ppm (12 H, m), 2.10–1.42 ppm (25 H, m), 1.37 ppm (45 H, s), 1.25–1.02 (12 H, m), 0.94 – 0.76 ppm (18 H, m).

^{1}H NMR of **5** (DMSO-d_6, 400 MHz, δ (ppm)): 8.52 (2 H, s), 8.33–7.94 (6 H, m), 7-62-7.45 ppm (4 H, m), 7.45–7.38 ppm (8 H, m), 7.23–7.08 (3 H, m), 7.06–6.83 ppm (10 H, m), 6.83–6.54 (5 H, m), 5.22–4.95 ppm (9 H, m), 4.75–4.59 ppm (2 H, m), 4.56–3.91 ppm (10 H, m), 3.74 ppm (12 H, s), 3.24–2.80 ppm (12 H, m), 2.05–1.45 ppm (35 H, m), 1.37 ppm (45 H, s), 1.25–0.95 (29 H, m), 0.94 – 0.76 ppm (18 H, m).

Synthesis of ***monoEST-COL(6)*** *and* ***diEST-COL(7)***. The deprotection of the Boc and PMB groups was carried out simultaneously and following previously reported methods [23, 24]. As a general procedure, anisole (5 mmol) was added to a solution of **4** or **5** (0.008 mmol) in half of the total volume of dichloromethane (0.015 M relative to **4** or **5**). Trifluoroacetic acid (1 mmol, 20% vol over dichloromethane) was diluted with the remaining half of the solvent and added dropwise to the reaction mixture at 0 ºC. The reaction was stirred at room temperature for 3 and 4 h for **6** and **7**, respectively. The resulting transparent oil was decanted and the remaining solvent was removed under vacuum at room temperature. The residue was then dispersed in ammonium bicarbonate buffer (1 mL, 0.1 M, pH 7.8) and purified by SEC using Sephadex® G-10 and the previously mentioned buffer. After lyophilization, both compounds were obtained as white solids. Yields = 54% [6] and 48% [7].

^{1}H NMR of **6** (D_2O, 400 MHz, δ (ppm)): 7.41 ppm (2 H, m), 6.85 ppm (1H, d, J_1 = 7.9 Hz), 5.40 ppm (1H, m), 4.50–3.95 ppm (12 H, m), 3.32–2.82 ppm (12 H, m), 2.43 1.10 (33 H, m), 1.03–0.75 ppm (18 H, m).

^{1}H NMR of **7** (D_2O, 400 MHz, δ (ppm)): 7.41–7.20 ppm (4 H, m), 6.72–6.62 ppm (2 H, m), 4.50–3.95 ppm (12 H, m), 3.06–2.75 ppm (12 H, m), 2.22–1.78 (14 H, m), 1.64–1.35 ppm (9 H, m), 1.33–1.04 ppm (10 H, m), 0.95 – 0.54 ppm (18 H, m).

Synthesis of ***AMI-COL(8)***. Synthesis of **AMI-COL** was attempted using various methods, which are described in the Supporting Information (Tables S1-S4). The following explanation corresponds to one of the methods tested that resulted in the highest conversion of **AMI-COL**.

3,4-Dihydroxybenzoic acid (29.6 mg, 0.19 mmol) was dissolved in H_2O/DMF (1/1, 0.5 mL). EDC (44.1 mg, 0.23 mmol) and NHS (27.1 mg, 0.23 mmol) were dissolved in H_2O/DMF (1/1, 0.5 mL) and added dropwise to the reaction mixture. The pH of the solution was adjusted to approx. pH 5 with HCl aqueous solution (1 M), and then it was allowed to stir for 1 h at room temperature until no unreacted acid was detected by thin layer chromatography. Afterwards, colistin sulfate (200 mg, 0.16 mmol) was solubilized in water (2 mL) and dropwise added to the reacting mixture. The pH was then adjusted to approx. pH 9 with NaOH aqueous solution (1 M, maximum basic pH before colistin precipitation) and the reaction was kept stirring at room temperature overnight. The residue was evaporated to dryness and re-dissolved in distilled water (0.5 mL). Then, it was precipitated in cold acetone (2.5 mL) three times, isolating the product by centrifugation. Final compound was dried under vacuum overnight, obtaining a white powder. The isolated product corresponds to a mixture of colistin (70%) and catecholamidated colistin (30%), according to ^{1}H NMR spectrum. Sulphate groups are assumed to act as counterions. Yield = 32%. ^{1}H NMR (D_2O, 400 MHz) δ (ppm): 7.29–7.15 ppm (0.6 H, m), 6.94–6.86 ppm (0.3 H, m), 459 – 448 ppm (3 H, m), 4.43–4.15 ppm (9 H, m), 3.44–3.31 ppm (1H, m), 3.24–2.94 ppm (11 H, m), 2.41–1.84 ppm (14 H, m), 1.76–1.43 ppm (9 H, m), 1.33–1.10 (10 H, m), 1.03–0.75 ppm (18 H, m).

Biological evaluation

MIC and MBC determination

MIC and MBC were determined against a clinical UTI *E. coli* (kindly donated by Dr. Alicia Lacoma, Germans Trias i Pujol Research Institute, Badalona, Spain) strain following the standard microdilution method [36]. Stock solutions of tested compound were prepared in DMSO (2 mg/mL for **monoEST-** and **diEST-COL**) or sterile water (1 mg/mL for **colistin** and **AMI-COL**). Briefly,

stock solutions of the compounds were diluted in the culture medium (until reaching tested concentration) and mixed with the bacteria inoculum, at a final bacteria concentration of 10^5 CFU/mL. Samples were incubated at 37 °C under shaking (150 rpm) and, after 24 h, were diluted in DPBS and spot-plated in TSA plates. Colonies present in plates were counted after 16 h of incubation at 37 °C. Positive control (untreated bacteria) samples were also tested.

Antibiofilm activity

The ability of the tested compounds to inhibit the formation of biofilm and to disrupt mature biofilms produced by an UTI *E. coli* strain were evaluated. For the biofilm inhibition, bacteria concentration was adjusted to 10^7 CFU/mL in ID-CAMHB and different concentrations of tested compounds were added to each sample. Solutions were cultured in 96-well microplates for 24 h at 37 °C without shaking. Microplates were kept in a box with a constant level of humidity (to avoid water evaporation) and, after this time, ID-CAMHB was discarded and samples were washed twice with DPBS.

For mature biofilm disruption, bacteria concentration was adjusted to 10^7 CFU/mL in ID-CAMHB and cultured for 24 h in a 96-well microplate (without shaking at 37 °C). Then, ID-CAMHB was discarded and samples were washed twice with DBPS, to remove planktonic bacteria. Different concentrations of tested compounds in ID-CAMHB were added to samples and then incubated for 24 h more (37 °C without shaking). During the experiment, microplates were kept in a box with a constant level of humidity. After this time, ID-CAMHB was removed and samples were washed twice with DPBS.

To quantify the biomass present in the samples, 100 µL of crystal violet solution (1 mg/mL in sterile water) was added to washed biofilms and incubated for 10 min at room temperature. Then, the solution was discarded and samples were washed 4 times with DPBS. After the last wash, samples were treated with 100 µL of acetic acid (30% v/v in sterile water) and absorbance was read at 570 nm in a Varioskan LUX microplate reader (Thermo Fisher Scientific, USA). Biofilm growth was calculated by data interpolation, assigning 100% biofilm growth to untreated samples (positive control samples).

To quantify the bacteria, present in the samples, the microdilution method was employed. 100 µL of DPBS were added to each washed sample and samples were sonicated for 1 min in an ultrasonic bath to disrupt the biofilm and release bacteria present within it. Then, bacteria were diluted and seeded as previously described for the MIC and MBC calculation, in order to quantify the bacterial load present in each sample.

Cell toxicity evaluation

2D cytotoxicity assays The toxicities of colistin and synthetized compounds were evaluated in a 2D model with two cellular lines, human dermal fibroblasts (fibroblasts, Lonza) and HaCat (kindly donated by Dr. Pilar Martin-Duque). Both lines were cultured in DMEM (supplemented with L-glutamine, 2 mM; PSA, 1% v/v and foetal bovine serum, 10% v/v) at 37 °C in a 5% CO_2 atmosphere.

Cells were seeded at a density of 6000 cells/well for fibroblasts and 8000 cells/well for HaCaT in a 96-well microplate and cultured for 24 h. Then, culture medium was replaced by fresh medium having different concentrations of the tested compounds. After 24 h, cytotoxicity was evaluated by measuring cell metabolism with the Blue Cell Viability assay. Culture medium was substituted for the Blue Cell Viability solution (10% v/v in DMEM), samples were cultured at 37 °C for 4 h and then fluorescence ($\lambda_{ex}/\lambda_{em}$ 530/590 nm) was read in a Varioskan LUX microplate reader. Viability was determined by interpolation, assigning 100% of cellular viability to untreated control samples.

3D cytotoxicity assays Kidney organoids used in this study were provided in frozen aliquots by the Biobank of the Aragon Health System (see Supplementary information for detailed information).

Once thawed, the organoid pellets were resuspended in Cultrex growth factor reduced BME-2 (Biotechne) and cultured in kidney organoid medium (Advanced F-12 medium supplemented with *N*-acetilcysteine, 1.25 mM; B-27 supplement, 1,5X; R-spondin3 conditioned medium, 2X; EGF 50 ng/mL; FGF-10, 100 ng/mL; Rho-kinase inhibitor Y-27632, 10 µM and A83-01, 5 µM. Medium was changed every 2–3 days and organoids were passaged every 1–3 weeks.

To plate organoids for the cytotoxicity assays, organoids were passaged using TryPLE Express (Invitrogen) and between 5000 and 10,000 cells per well were seeded in 5 µL of BME-2 matrix in ultra-low attachment MW96 plates with dark walls (Inycom). Cultures were maintained in 100 µL of the kidney organoids medium for 48 h and then different concentrations of tested compounds were added. Cell viability was measured by luminescence after 3 days of drug incubation using Cell Titer-Glo (Promega) according to the manufacturer's instructions. DMSO was used as negative control. All the results were referred to the control sample (untreated organoids), which was considered as 100% viability.

Statistical analyses

All biological experiments were performed by triplicate. All data are reported as mean ± Standard Deviation and two-way ANOVA test was used to statistically analyse

them (GraphPad Prism 9, San Diego, CA, USA). Statistically significant differences were considered when $p \leq 0.01$.

Abbreviations

A. baumannii	*Acinetobacter baumannii*
Boc	*tert*-butyloxycarbonyl
CFU/mL	Colony forming units per mL
Dab	2,4-diaminobutyric acid
DCC	*N,N'*-dicyclohexylcarbodiimide
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N,N*-dimethylformamide
DOSY	Diffusion-ordered spectroscopy
DPBS	Dulbecco's Phosphate Buffered Saline
DPTS	Dimethylpyridinium *p*-toluenesulfonate
E. coli	*Escherichia coli*
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
HaCat	Keratinocytes
ID-CAMHB	Iron-depleted broth
K. pneumoniae	*Klebsiella pneumoniae*
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
NHS	*N*-hydroxysuccinimide
P. aeruginosa	*Pseudomonas aeruginosa*
PMB	*para*-methoxybenzyl
pEtN	Phosphoethanolamine
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
THF	Tetrahydrofuran
Thr	Threonine
TSA	Trypticase in soy agar

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13065-025-01538-7.

Supplementary Material 1

Acknowledgements

S. Bescós-Ramo acknowledges Gobierno de Aragón for her PhD grant. We want particularly acknowledge the patients and the Biobank of the Aragon Health System (PT20/00112) integrated in the Platform ISCIII Biobanks and Biomodels for their collaboration. Conexión Nanomedicina (CSIC) is also acknowledged.

Author contributions

S.B-R. synthetized, purified and characterized the tested compounds. E.G. performed the microbiological experiments. M.M.E-B. performed experiments with human-derived organoids. S.B-R., E.G. and M.A. wrote the manuscript with contributions and edits from all authors. S.B-R., E.G., L.O., M.P. and M.A. designed the experiments and analyzed the resulting data. L.O., M.P. and M.A. supervised the project and acquired the funding. All authors have given approval to the final version of the manuscript.

Funding

This work was financially supported by Spanish Ministry of Science and Innovation (MICINN) (Grants: PID2021-126132NB-I00 and PID2023-146091OB-I00), the "Grant CEX2023-001286-S funded by MICIU/AEI/10.13039/501100011033"https://doi.org/10.13039/501100011033" and Gobierno de Aragón-FSE (E47_23R research group).

Data availability

The corresponding author will provide the datasets created and/or analyzed during the current study upon reasonable request.

Declarations

Ethics approval and consent to participate

Our study and the request for biobank samples (SA23-10) underwent rigorous evaluation and received approval from the Research Ethics Committee of the Autonomous Community of Aragon (CEICA) (https://www.iacs.es/investigacion/comite-de-etica-de-la-investigacion-de-aragon-ceica/) with code protocol aproval nr. PI23-300. CEICA is attached to the Department of Health of the Government of Aragon and supported by the Aragon Institute for Health Sciences (IACS) (https://www.iacs.es/).

The Biobank of the Aragon Health System (BSSA), was established in 2013 by Decree 146/2013 of the Government of Aragon, and operates in accordance with Spanish legislation on the use of samples of human origin for biomedical research purposes (Royal Decree 1716/2011). The biobank (National Registry of Biobanks B. B.0000873), is also integrated into Spanish National Biobanks Network, Instituto de Salud Carlos III (Madrid, Spain) with the code PT23/00146. Informed consent was obtained from all participants allowing the use of their tissue samples for medical research after pseudoanonymization, and all experiments involving human samples were carried out in strict adherence to the principles of the Helsinki Declaration.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 24 February 2025 / Accepted: 30 May 2025
Published online: 09 June 2025

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