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1            **Dextrin-Colistin Conjugates as a Model Bioresponsive Treatment for Multi-drug Resistant**  
2                                    **Bacterial Infections.**

3  
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20  
21 **Key words:** Nanomedicines; PUMPT; polymer therapeutics; infection; bioresponsive; Gram-  
22 negative bacteria

23 **Abstract:** Polymer therapeutics offer potential benefits in the treatment of multidrug resistant  
24 (MDR) infections; affording targeted delivery of biologically active agents to the site of  
25 inflammation, potential decreases in systemic toxicity and the retention of antimicrobial  
26 activity at the target site. As a prototype model, these studies developed and characterized a  
27 library of dextrin-colistin conjugates (dextrin molecular weight: 7,500 - 48,000 g/mol) as a  
28 means of targeting the delivery of colistin. Optimum colistin release kinetics (following dextrin  
29 degradation by physiological concentrations of amylase (100 IU/L)) were observed in  
30 conjugates containing low molecular weight (~7,500 g/mol) dextrin with ~1 mol%  
31 succinylation (~80% drug release within 48 h, compared to ~33% from sodium colistin  
32 methanesulfonate (CMS, Colomycin<sup>®</sup>)). These conjugates exhibited comparable antimicrobial  
33 activity to CMS in conventional MIC assays against a range of Gram-negative pathogens, but  
34 with significantly reduced *in vitro* toxicity towards kidney (IC<sub>50</sub> = CMS, 15.4 µg/mL; dextrin-  
35 colistin, 63.9 µg/mL) and macrophage (IC<sub>50</sub> = CMS, 111.3 µg/mL; dextrin-colistin, 303.9  
36 µg/mL) cells. *In vivo* dose-escalation studies in rats demonstrated improved pharmacokinetics  
37 of the conjugates, with prolonged plasma levels of colistin (t<sub>1/2</sub> 135-1271 min vs. 53 min) and  
38 decreased toxicity, compared to colistin sulfate. These studies highlight the potential utility of  
39 'nanoantibiotic' polymer therapeutics to aid the safe, effective and targeted delivery of colistin  
40 in the management of MDR infections.

## 41 **Introduction**

42 The prevalence of infections by Gram-negative, multi-drug resistant organisms, including  
43 *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*, represents a world health  
44 problem and a significant clinical challenge in patients with chronic disease e.g. cystic fibrosis, in  
45 whom aminoglycoside and fluoroquinolone resistance is common<sup>1</sup>. Increasing resistance to currently-  
46 available antibiotics has been mirrored by decreases in the design/development of new antibiotic  
47 entities<sup>1</sup>. To overcome these issues clinicians are employing antimicrobial agents (e.g. colistin and  
48 polymyxin B) which are effective against Gram-negative organisms, but have previously been  
49 restricted in clinical use due to the potential for associated toxicity<sup>2</sup>.

50 Polymer therapeutics are being increasingly investigated for the treatment of cancer, arthritis,  
51 viral infections<sup>3,4</sup>, and, more recently, tissue repair<sup>5-8</sup>. Indeed, two of the US top 10 selling drugs in  
52 2013 were polymer therapeutics (Neulasta<sup>®</sup> and Copaxone<sup>®</sup>)<sup>9</sup>. Whilst this technique has been  
53 extensively employed to reduce toxicity in the delivery of cytotoxic therapies in the treatment of  
54 malignancy<sup>3</sup>, surprisingly, little attention has been directed at their use in the treatment of bacterial  
55 infection. The conjugation of antibiotics to polymers offers the ability to improve biodistribution,  
56 enhance stability and reduce toxicity<sup>10,11</sup>. Polymer masked-unmasked protein therapy' (PUMPT) is a  
57 technique, which employs conjugation of a biodegradable polymer to "mask" a protein or peptide's  
58 activity in the circulation<sup>12</sup>. The circulating conjugate accumulates at the site of inflammation (or  
59 infection) due to the enhanced permeability and retention (EPR) effect<sup>11</sup> and "locally-triggered"  
60 degradation of the polymer regenerates bioactivity. The application of PUMPT, to safely deliver  
61 antibiotic agents otherwise associated with toxicity or pharmacokinetic problems (e.g. colistin) may,  
62 therefore, offer not only the ability to design and deliver novel targeted therapies, but also to improve  
63 the safe treatment of patients with Gram-negative MDR infections.

64 In this study we sought to develop a novel, nanomedicine-based, delivery system to target the  
65 delivery of colistin. Colistin was chosen as a model antibiotic since it is an amphiphilic peptide  
66 antibiotic (Mw ~1,400 g/mol) which is highly active towards multi-resistant Gram-negative bacteria,  
67 but is not readily absorbed orally and the free drug is nephro- and neurotoxic<sup>13</sup>. Consequently, it is  
68 commonly administered intravenously as sodium colistin methanesulfonate (CMS), a less toxic pro-  
69 drug, that is readily hydrolyzed to partially sulfomethylated derivatives and colistin in aqueous  
70 solution. This hydrolysis is however, neither controlled or triggered, and may even occur before patient  
71 administration, which has resulted in fatality.

72 We hypothesized that polymer conjugation would not only limit systemic toxicity, but passively  
73 target the protein to sites of infection/inflammation by the effect, which has been widely observed in  
74 cancer and, more recently, to a lesser extent, in inflammation<sup>11</sup> (Figure 1). Dextrin was employed here  
75 as the model polymeric carrier as we have previously shown its suitability as a partner polymer in the  
76 context of PUMPT<sup>12</sup>, as it is rapidly degraded by  $\alpha$ -amylase<sup>14,15</sup>. We, and others, have shown how the  
77 conjugation can be tailored (employing dextrans of different molecular weights and degrees of  
78 succinylation) to optimize the release and activity of the protein for particular clinical applications e.g.  
79 wound healing<sup>12,14,15</sup>.

80 In the present work, we hypothesize that conjugation of dextrin to colistin will improve disease-  
81 specific targeting and reduce the toxic effects of colistin, thus re-establishing its value in Gram-  
82 negative infections and supporting the development of further polymer therapeutic nanoantibiotics.  
83 Here, the synthesis and characterization of a series of dextrin-colistin conjugates is reported and the  
84 ability of the conjugates to release colistin in the presence and absence of amylase at human  
85 physiological concentrations, their toxicity towards mammalian cells (erythrocytes, human kidney  
86 cells) and their *in vitro* antimicrobial activity is described. As dextrin conjugation is hypothesized to  
87 extend the serum half-life of colistin, an *in vivo* assessment of plasma drug concentration following a  
88 single bolus injection was performed in a dose-escalating study.

## 90 **Materials and Methods**

91 **Materials.** Type 1 dextrin from corn (Mw ~ 48,500 g/mol) was from ML laboratories (Keele,  
92 UK). Type I dextrin from corn (Mw ~ 7,500 g/mol), dextrin from maize starch (Mw ~28,000 g/mol),  
93 colistin sulfate,  $\alpha$ -amylase from human saliva, N-hydroxysulfosuccinimide (sulfo-NHS), copper (II)  
94 sulfate pentahydrate 4% w/v solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide  
95 (MTT), bicinchoninic acid solution (BCA), ethanolamine, ninhydrin, hydrindantin, lithium acetate  
96 dihydrate, acetic acid and dimethyl sulfoxide (DMSO) were all from Sigma-Aldrich (Poole, UK).  
97 Dextrin from potato starch (Mw ~10,500 g/mol) was from Fluka (Gillingham, UK). 1-ethyl-3-(3-  
98 dimethylaminopropyl carbodiimide hydrochloride) (EDC) was from Pierce (Rockford, USA).  
99 Disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium chloride, 4-  
100 dimethylaminopyridine (DMAP) and sodium chloride were from Fisher Scientific (Loughborough,  
101 UK). Pullulan gel filtration standards (Mw = 11,800–210,000 g/mol) were from Polymer Laboratories  
102 (Church Stretton, UK). Lactate dehydrogenase (LDH)-cytotoxicity assay kit was from abcam  
103 (Cambridge, UK). MaxSignal<sup>®</sup> colistin ELISA test kit was from Bioo Scientific Corp. (Austin, USA).  
104 Unless otherwise stated, all chemicals were of analytical grade. All solvents were of general reagent  
105 grade (unless stated) and were from Fisher Scientific (Loughborough, UK).

106 **Cell culture.** Mouse macrophage cells (RAW 264.7) and human kidney proximal tubule cells  
107 (HK-2) were from ATCC (Manassas, USA). Cells were screened and found to be free of mycoplasma  
108 contamination before use. Dulbecco's Modified Eagle's medium (DMEM) with GlutaMAX<sup>™</sup>, fetal calf  
109 serum (FCS), keratinocyte serum-free medium (K-SFM) with L-glutamine, epidermal growth factor  
110 (EGF), bovine pituitary extract (BPE) and 0.05% w/v trypsin-0.53 mM EDTA were obtained from  
111 Invitrogen Life Technologies (Paisley, UK).

112 **Bacterial culture.** The strains used for susceptibility testing include both culture collection

113 strains and clinical isolates (Table 1). Their known relevant genotypes and origin have been described  
114 by Khan et al.<sup>16</sup>. Bacterial colonies were grown on tryptone soya agar (TSA) and liquid cultures were  
115 suspended in tryptone soya broth (TSB) for overnight culture or Mueller-Hinton broth (MHB) for MIC  
116 determination (from Oxoid; Basingstoke, UK).

117 **Synthesis and purification of dextrin-colistin conjugates.** First, succinoylated dextrans (1-60  
118 mol%) were synthesized as previously described<sup>15</sup> (Figure 2(a)). Briefly, with dextrin of molecular  
119 weight 7,500 g/mol, 5 mol% succinoylation was achieved as follows. Dextrin (1000 mg,  $6.17 \times 10^{-3}$   
120 mol) was dissolved in anhydrous DMF (10 mL) in a 50 mL round-bottomed flask. Succinic anhydride  
121 (61.7 mg,  $6.17 \times 10^{-4}$  mol) and DMAP (28.6 mg,  $2.34 \times 10^{-4}$  mol) were subsequently added, the  
122 reaction purged with nitrogen, sealed and left to stir at 50 °C for 18 h. Then, the reaction mixture was  
123 poured into vigorously stirring diethyl ether (250 mL) and stirred overnight. The ether was removed by  
124 filtration under vacuum and the residual solid was dissolved in minimal distilled water (dH<sub>2</sub>O), poured  
125 into a dialysis membrane (molecular weight cut-off 2,000 g/mol) and dialysed against 4 x 5 L dH<sub>2</sub>O.  
126 The resultant solution was freeze-dried to yield succinoylated dextrin. The degree of succinoylation  
127 was quantified by titration against a standard solution of NaOH ( $5 \times 10^{-4}$  M) with bromophenol blue as  
128 indicator, and the product was further characterized by FT-IR (Avatar 360 ESP spectrometer with EZ  
129 OMNIC ESP 5.2 software; Thermo Nicolet, Loughborough, UK) to confirm identity and by gel  
130 permeation chromatography (GPC) (TSK G4000PW<sub>XL</sub> and G3000PW<sub>XL</sub> columns (Polymer  
131 Laboratories, Church Stretton, UK) in series, mobile phase PBS (pH 7.4), flow rate of 1 mL/min) to  
132 measure approximate molecular weight and polydispersity (compared to pullulan standards). Samples  
133 for GPC were prepared in PBS (3 mg/mL) and the eluate was monitored using a differential  
134 refractometer (Gilson 153). PL Caliber Instrument software, version 7.0.4, from Polymer Laboratories  
135 (Church Stretton, UK) was used for data analysis.

136 Succinoylated dextrans (1-30 mol% succinoylation) were then conjugated to colistin (Figure  
137 2(b)). Briefly, for 10 mol% succinoylated dextrin, succinoylated dextrin (200 mg,  $1.11 \times 10^{-4}$  mol

138 COOH;  $2.50 \times 10^{-5}$  mol dextrin) was dissolved under stirring in dH<sub>2</sub>O (1 mL) in a 10 mL round-  
139 bottomed flask. To this, EDC (21.3 mg,  $1.11 \times 10^{-4}$  mol) and sulfo-NHS (24.1 mg,  $1.11 \times 10^{-4}$  mol)  
140 were added, and the mixture was left stirring for 30 min. Subsequently, colistin (35 mg,  $2.50 \times 10^{-5}$   
141 mol) dissolved in dH<sub>2</sub>O (1 mL) was added, followed by NaOH (0.5 M) drop-wise to raise the pH to  
142 ~8.0. The reaction mixture was left stirring for up to 18 h, typically for 2 h. The conjugate was then  
143 purified from the reaction mixture by fast protein liquid chromatography (FPLC) (ÄKTA FPLC;  
144 Amersham Pharmacia Biotech, UK) using a pre-packed Superdex 75 10/300 GL column with a UV  
145 detector and data analysis using Unicorn 4.0 software (Amersham Pharmacia Biotech, UK). Samples of  
146 the reaction mixture (0.5 mL) were injected into a 500 µL loop using PBS (pH 7.4), pH 7.4 at 0.5  
147 mL/min as a mobile phase. Fractions (1 mL) were collected, desalted using Vivaspin tubes (5,000  
148 g/mol cut-off) and assayed for protein content (BCA assay) before pooling fractions containing  
149 conjugate (typically fractions 6-13). The final conjugate was lyophilised and stored at -20 °C.

150 **Characterization of dextrin-colistin conjugates.** Dextrin-colistin was characterized by FPLC  
151 and GPC to assess purity and estimate molecular weight, and the total protein content of the conjugate  
152 was determined by the BCA assay using colistin standards.

153 The FPLC system described above for purification was used again for final conjugate  
154 characterization. Samples (200 µL) were dissolved in PBS (pH 7.4) and injected into a 100 µL loop at  
155 0.5 mL/min. The molecular weight was estimated using GPC relative to pullulan standards.

156 **Ninhydrin assay.** Prior to conjugation of polymer to protein, a ninhydrin assay was used to  
157 confirm the number of available amine groups in colistin for conjugation and how many amine groups  
158 were subsequently used for binding to dextrin. First, a 4 M lithium acetate buffer solution was prepared  
159 by dissolving lithium acetate dihydrate (40.81 g) in 60 mL dH<sub>2</sub>O. Sufficient acetic acid (glacial) was  
160 added until pH 5.2 was reached. The volume was made up to a final volume of 100 mL with dH<sub>2</sub>O.  
161 Next, ninhydrin (0.2 g) and hydrindantin (0.03 g) were dissolved in 7.5 mL DMSO and 2.5 mL lithium



162 acetate buffer. Buffered ninhydrin reagent (86  $\mu\text{L}$ ) was added to an equal quantity of sample/ standard  
163 solution (1.5 mL eppendorf) and heated in a water bath at 100  $^{\circ}\text{C}$  for 15 min. The mixture was  
164 subsequently cooled to room temperature and 130  $\mu\text{L}$  of 50% v/v ethanol was added. The solution was  
165 mixed before adding 200  $\mu\text{L}$  of the final solution into wells of a 96-well plate. Spectrophotometric  
166 analysis was performed at 570 nm. Calibration of the assay was achieved using ethanolamine (0-0.1158  
167 mM).

168 **Degradation of dextrin, succinoylated dextrin and dextrin-colistin conjugates by amylase.**

169 To compare the rate of amylase degradation of dextrin, succinoylated dextrin and dextrin-colistin  
170 conjugates, solutions (3 mg/mL in PBS, pH 7.4) of each sample was prepared containing amylase (100  
171 IU/L in PBS) and incubated at 37  $^{\circ}\text{C}$  for up to 48 h. At various time points, samples (300  $\mu\text{L}$ ) were  
172 taken, immediately snap-frozen in liquid nitrogen to stop the reaction and stored at  $-20^{\circ}\text{C}$  until  
173 analysis by GPC and FPLC (conjugates only). Prior to analysis, samples were placed in a water bath  
174 (100  $^{\circ}\text{C}$ ) for 5 min to denature the enzyme activity of the amylase and stop polymer degradation. The  
175 supernatant was then analyzed by GPC to determine the change in molecular weight over time and by  
176 FPLC to determine the change in free colistin over time.

177 ***In vitro* toxicity assay: MTT assay.** An MTT assay was used to assess cell viability in a mouse  
178 macrophage (RAW 264.7) cell line (24 h incubation) and a human kidney (HK-2) cell line (72 h  
179 incubation). RAW 264.7 cells were seeded into sterile, 96-well microtiter plates ( $2 \times 10^5$  cells/mL) in  
180 0.1 mL/well of media (DMEM) containing heat-inactivated FCS (1% v/v) and HK-2 cells were seeded  
181 into sterile 96-well microtiter plates ( $1 \times 10^5$  cells/mL) in 0.1 mL/well of media (K-SFM) containing L-  
182 glutamine, EGF and BPE. They were allowed to adhere for 24 h. The medium was then removed and  
183 test compounds (0.2  $\mu\text{m}$  filter-sterilized) were added to the cells. To study the effect of colistin sulfate,  
184 CMS and dextrin-colistin conjugate (7,500 g/mol, 1.1 mol% succinoylation) on cell viability, complete  
185 media was supplemented with a range of concentrations of each. To study the effect of ‘unmasked’

186 dextrin-colistin on cell viability, complete media was supplemented with a range of concentrations of  
187 conjugate and amylase (100 IU/L). After a further 67 h incubation, MTT (20  $\mu$ L of a 5 mg/mL solution  
188 in PBS) was added to each well and the cells were incubated for a further 5 h. The medium was then  
189 removed and the precipitated formazan crystals solubilized by addition of optical grade DMSO (100  
190  $\mu$ L) over 30 min. Absorbance was measured at 540 nm using a microtiter plate reader. Cell viability  
191 was expressed as a percentage of the viability of untreated control cells. The IC<sub>50</sub> values were  
192 expressed as mean  $\pm$  SEM (n=18).

193 ***In vitro* toxicity assay: LDH assay.** The LDH assay was used to assess cell membrane damage  
194 (24 h incubation) in a HK-2 cell line. Cells were seeded into sterile 96-well microtiter plates (1 x 10<sup>5</sup>  
195 cells/ mL) in 0.1 mL/well of media (K-SFM) containing L-glutamine, EGF and BPE. They were  
196 allowed to adhere for 24 h. The medium was then removed and test compounds (0.2  $\mu$ m filter-  
197 sterilized) were added to the cells. To study the effect of colistin sulfate, CMS and dextrin-colistin  
198 conjugate on cell membrane integrity, complete media was supplemented with a range of  
199 concentrations of each. To study the effect of 'unmasked' dextrin-colistin on cell viability, complete  
200 media was supplemented with a range of concentrations of conjugate and amylase (100 IU/L). After 24  
201 h, microtiter plates were centrifuged (600 g, 10 min), the supernatant was transferred to a clean 96-well  
202 plate and stored at -20 °C until determination of LDH content. LDH content in the cell supernatant was  
203 determined using a commercial LDH-cytotoxicity assay kit following the manufacturer's protocol.  
204 Absorbance was measured at 450 nm using a microtiter plate reader. The absorbance values were  
205 expressed as mean  $\pm$  SEM (n=6).

206 **Measurement of antimicrobial activity.** Antimicrobial activity was measured using broth  
207 micro-dilution in a standard MIC assay<sup>17</sup>. Test organisms were suspended in Mueller Hinton cation-  
208 adjusted broth (100  $\mu$ L, 1 - 5 x 10<sup>4</sup> CFU/mL) and incubated in 96-well microtiter plates in serial two-  
209 fold dilutions of the test compounds.

210 The antimicrobial activity of dextrin-colistin conjugates was similarly determined following  
211 incubation (3 mg/mL colistin equiv.) with amylase (100 IU/L for up to 48 h at 37 °C in PBS buffer (pH  
212 8.2)).

213 ***In vivo* dose escalating studies.** Sprague-Dawley rats (390-470 g) (Charles River (UK)  
214 Limited) with a jugular vein cannulation were housed in an animal care facility and acclimatised for 1  
215 week prior to experimentation. During the on-study period, rats were housed individually in a  
216 metabolism cage with food and water *ad libitum*.

217 On the day of the experiment, colistin sulfate, dextrin-colistin conjugate (1.4 mol%  
218 succinoylated 7,500 g/mol dextrin) and dextrin-colistin conjugate (7.2 mol% succinoylated 7,500 g/mol  
219 dextrin) were dissolved in 0.9% w/v sterile saline (0.1 mg/mL colistin equiv.), filtered (0.22 µm) and  
220 injected via the tail vein as a bolus to 2 rats per test item (0.1 mg/kg colistin equiv.). Following  
221 administration, blood was collected (5 and 30 min, 1, 4, 8 and 24 h post-dose). Blood samples were  
222 placed into tubes containing lithium heparin and centrifuged (3000 rpm, for 10 min at 4 °C)  
223 immediately, before storing the resulting plasma samples at -20 °C prior to assay. The concentration of  
224 “total” colistin in plasma was assessed using a commercial colistin ELISA test kit, according to the  
225 manufacturer’s instructions.

226 **Pharmacokinetic Analysis.** Non-compartmental analysis of the pharmacokinetics of colistin  
227 sulphate and dextrin-colistin conjugates was performed using GraphPad Prism, version 6.0d for  
228 Macintosh, 2014. The following pharmacokinetic parameters were calculated: peak plasma colistin  
229 concentration after administration ( $C_{max}$ ), time to reach ( $t_{max}$ ), plasma half-life ( $t_{1/2}$ ), area under the  
230 concentration-time curve to 24 h ( $AUC_{0-24 h}$ ), volume of distribution ( $V_d$ ) and total body clearance (CL).  
231 Plasma half-life was calculated following linear regression analysis of log-transformed plasma  
232 concentration–time points. The  $AUC_{0-24 h}$  of colistin was calculated with the presumption of the initial  
233 concentration being zero.

234 **Statistical Analysis.** Data are expressed as mean  $\pm$  the error, calculated as either standard

235 deviation (SD) where  $n = 3$ , or standard error of the mean (SEM) where  $n > 3$ . Statistical significance  
236 was set at  $p < 0.05$  (indicated by \*). Evaluation of significance was achieved using a one-way analysis  
237 of variance (ANOVA) followed by Bonferroni *post hoc* tests that correct for multiple comparisons. All  
238 statistical calculations were performed using GraphPad Prism, version 6.0d for Macintosh, 2014.

239

## 240 **Results**

241 **Synthesis and characterization of dextrin-colistin conjugates.** The characteristics of the  
242 library of succinoylated dextrans synthesized are summarized in Table 1. Succinoylated dextrin  
243 intermediates were synthesized having a degree of modification of 1-29 mol% and showed increased  
244 FT-IR signal strength of the ester peak ( $\sim 1,720\text{ cm}^{-1}$ ) relative to degree of modification (Figure 3(a)  
245 and Figure SI 1(a, c, e)). GPC using pullulan standards suggested an increase in dextrin molecular  
246 weight following succinoylation with little change in polydispersity.

247 Using these succinoylated dextrin intermediates, a series of dextrin-colistin conjugates were  
248 prepared (Table 1). Typical FPLC elution profiles of the purified conjugates and free colistin are shown  
249 (Figure 3(b)), with the conjugate typically eluting in the void volume of the column, enabling the  
250 separation of free and bound colistin. The protein content was 3-23% w/w. FPLC analysis confirmed  
251 the presence of a high molecular weight conjugate, however, the free colistin content was always  $< 4\%$ .  
252 The ninhydrin assay indicated that colistin has 4.8  $\text{NH}_2$  groups per molecule and that dextrin typically  
253 bound to  $\sim 3$  of these groups in dextrin-colistin conjugates.

254 **Degradation of dextrin and dextrin-colistin conjugate by amylase.** GPC analysis of dextrin  
255 degradation by amylase revealed a decrease in molecular weight with time (Figure 4(a) and Figure SI  
256 1(b,d,f)). While dextrin degraded rapidly ( $t_{1/2} < 30\text{ min}$ ) in the presence of amylase, chemical  
257 modification by succinoylation slowed the rate of degradation ( $t_{1/2} \geq 4\text{ h}$ ).

258 When degraded dextrin-colistin conjugates were analyzed by FPLC, a peak corresponding to

259 free colistin appeared (~16 mL), which increased in intensity with time (Figure 4(c) and Figure SI 2).  
260 In parallel, the peak corresponding to dextrin-colistin conjugate (~7.5 mL) decreased and GPC analysis  
261 revealed a reduction in conjugate molecular weight over the incubation timecourse (Figure 4(b)). This  
262 corresponds to an increasing concentration of free colistin in parallel to reducing concentrations of  
263 dextrin-colistin conjugate; indicative of release of colistin from the conjugate due to amylase  
264 degradation of dextrin. In these experiments, dextrin-colistin conjugates containing low molecular  
265 weight dextrin (7,500 g/mol) with a low level of polymer modification (1.1 mol%) released the most  
266 free colistin (~80%) after 48 h incubation (Table SI 1). Amylase-triggered dextrin-colistin conjugates  
267 synthesized using low molecular weight (7,500 g/mol) dextrin released more than twice as much free  
268 colistin within 48 h than the commercially available CMS (Figure 4(d)).

269 **Stability of dextrin-colistin and CMS *in vitro*.** Dextrin-colistin conjugates (7,500 g/mol  
270 conjugates at 1.1, 2.5, 4.7 and 8.3 mol%) were stable in water and PBS at 4 °C (Figure 5, Table SI 1).  
271 After 48 h, the percentage of liberated colistin was typically <5%. Dextrin-colistin conjugates were less  
272 stable in water at 37°C in the absence of amylase, and increased colistin was evident in PBS at 37°C in  
273 the absence of amylase. Release of colistin by hydrolysis was also greatest for conjugates containing  
274 low degrees of succinylation. CMS was less stable in PBS than water, releasing 33.0% free colistin  
275 after 48 h in PBS at 37°C. After incubation of CMS in PBS or water at 4°C for 48 h, more free colistin  
276 was detected than for any of the dextrin-colistin conjugates.

277 ***In vitro* toxicity assay.** The concentration-dependent cytotoxicity of colistin sulfate, CMS and  
278 dextrin-colistin conjugates (with and without amylase) is shown in Figure 6. The dextrin-colistin  
279 conjugate caused less metabolic changes and membrane damage than free colistin and CMS in RAW  
280 264.7 and HK-2 cells. Cell viability, measured by MTT assay, was greatest in HK-2 (kidney) cells  
281 ( $IC_{50} = 11.0 \pm 1.0 \mu\text{g/mL}$  (colistin sulfate) >  $15.4 \pm 1.0 \mu\text{g/mL}$  (CMS) >  $35.5 \pm 1.3 \mu\text{g/mL}$  (dextrin-  
282 colistin with amylase) >  $63.9 \pm 1.6 \mu\text{g/mL}$  (dextrin-colistin)), compared to RAW 264.7 (macrophage)

283 cells ( $IC_{50} = 111.3 \pm 1.1 \mu\text{g/mL}$  (CMS)  $> 180.5 \pm 1.3 \mu\text{g/mL}$  (dextrin-colistin with amylase)  $> 187.4 \pm$   
284  $1.1 \mu\text{g/mL}$  (colistin sulfate)  $> 303.9 \pm 1.4 \mu\text{g/mL}$  (dextrin-colistin)) (Figure 6(a,b)). Similarly, the LDH  
285 assay in HK-2 cells demonstrated that dextrin-colistin conjugate caused significantly less (~50%)  
286 membrane damage than colistin sulfate or CMS (Figure 6(c)), however, amylase unmasking of dextrin-  
287 colistin conjugate restored the membrane permeabilization activity of colistin. Likewise, dextrin-  
288 colistin conjugates induced significantly less erythrocyte lysis after a 24 h incubation than colistin  
289 sulfate or CMS, even in the presence of amylase (Figure 6(d)).

290 **Measurement of antimicrobial activity.** Conjugation of dextrin masked the antimicrobial  
291 activity of colistin in a panel of Gram-negative bacteria to some extent, typically increasing the MIC to  
292 2-fold that of CMS. Pre-incubation with amylase (at physiological concentrations) generally caused a  
293 1-fold reduction in MIC value, compared to untreated conjugate (Tables 2, SI 2). Antimicrobial activity  
294 was greatest for conjugates containing low molecular weight dextrin (7,500 g/mol) with a low degree  
295 of succinoylation (1.1 mol%).

296 ***In vivo* pharmacokinetics.** Figure 7 shows the mean concentrations of colistin in plasma as a  
297 function of time in 2 rats after an IV dose of 0.1 mg/kg colistin sulfate or dextrin-colistin conjugates. In  
298 all cases, there was a short distribution phase (5-60 min) followed by an elimination phase. After 8 h,  
299 the concentration of colistin sulfate was below the limit of quantification, but for both conjugates, drug  
300 was detectable in the plasma 48 h post-dose. Colistin sulfate had a significantly shorter half-life than  
301 the dextrin-colistin conjugates (53.2 min vs. 135.3 min (1.1 mol%) and 1,270.9 min (7.2 mol%)) (Table  
302 3).

303

## 304 **Discussion**

305 While previous applications of PUMPT, in cancer and wound repair<sup>5,18</sup>, require prolonged  
306 release of the bioactive protein over several days, acute bacterial infections need much more rapid and

307 complete release of the antibiotic at sites of infection. Here we have optimized the release of colistin by  
308 varying the degree of dextrin functionalization. It has previously been shown that dextrin's degradation  
309 rate, and consequent drug release, can be extended by increasing its molecular weight and degree of  
310 succinylation<sup>12</sup>. In these studies, dextrin-colistin conjugates were synthesized using dextrans with  
311 molecular weights between 7,500 and 48,500 g/mol and 1-29 mol% succinylation. Typically, dextrin-  
312 colistin conjugates contained 1-2 dextrin chains per colistin molecule, bound via ~3 primary amine  
313 groups. Dextrin degradation by amylase breaks the glycosidic bonds within the polymer chain, leaving  
314 oligosaccharides and/or maltose linked to colistin. These cationic amine groups play an important role  
315 in the interaction of colistin with bacterial lipopolysaccharides, as well as permeabilization of the  
316 kidney's proximal tubule, so the number of binding sites was optimized to sufficiently 'mask' colistin  
317 during transit but allow sufficient antimicrobial activity to be regenerated at the target site.

318 Couet et al.<sup>19</sup> recently reported that only 30% of the CMS dose administered intravenously to  
319 healthy volunteers was converted into colistin after 24 h. Here, we demonstrated, using FPLC, that *in*  
320 *vitro* hydrolysis of CMS in PBS at 37 °C released just 23.6% free colistin after 24 h. In contrast,  
321 amylase-triggered release of colistin from dextrin-colistin conjugates released up to 80% free drug in  
322 the same period. While amylase triggered significant release of free colistin from conjugates, stability  
323 experiments showed that drug may also be released at a slower rate in the absence of amylase. This is  
324 important as some patients, such as those with cystic fibrosis, display reduced physiological levels of  
325 amylase<sup>20</sup>, and 'enzyme-free' liberation of drug may be an advantage in these patients. In all cases,  
326 however, the release of free colistin from dextrin-colistin conjugates was less than from amylase-  
327 activated conjugates. While *in vivo* liberation of colistin from dextrin-colistin conjugates is important  
328 for antibacterial activity, release of drug during storage may be harmful. Storage of the dextrin-colistin  
329 conjugates in solution at 4°C resulted in minimal ( $\leq 5.3\%$  after 48 h) colistin release, however, up to  
330 13.2% colistin was liberated from CMS under the same conditions.

331 Colistin exerts its bactericidal activity by permeabilizing bacterial membranes<sup>21,22</sup>. However,

332 since this mechanism is only partially selective, polymyxins can also increase the permeability of  
333 mammalian cells- leading to an increased influx of cations, anions, and water, which eventually result  
334 in cell swelling and lysis<sup>23,24</sup>. Permeabilization of renal proximal tubule cells has been attributed to  
335 colistin's dose-dependent nephrotoxicity, affecting up to 36% of patients receiving intravenous CMS<sup>25</sup>.  
336 Biocompatibility of dextrin-colistin conjugates is essential for safe systemic administration. HK-2 cells  
337 were chosen for these preliminary *in vitro* toxicity studies since they are derived from the proximal  
338 cells of human kidneys and are known to retain the functional characteristics of proximal tubular  
339 epithelium<sup>26</sup>. The MTT and LDH assays demonstrated that dextrin conjugation significantly reduced  
340 the cytotoxicity of colistin. Since colistin is only weakly cytotoxic (compared to an anti-cancer agent),  
341 and toxicity normally ensues from long-term use, treatment incubation times for the toxicity assays  
342 were chosen to replicate the prolonged exposure observed from the conjugates due to extended plasma  
343 half-life observed in the *in vivo* studies. Given that hemocompatibility would be critical to ensure safe  
344 systemic administration of dextrin-colistin conjugates following IV injection, the finding that the  
345 concentration-dependent erythrocyte toxicity exhibited by colistin was effectively inhibited by dextrin  
346 conjugation, but only partially restored after amylase unmasking, was also encouraging. In these  
347 experiments, dextrin conjugation via colistin's cationic amine groups, and the inability of amylase  
348 degradation to restore these positively charged groups, could explain the reduced toxicity of dextrin-  
349 colistin conjugates in comparison to colistin and CMS and support the 'masking' effect of polymer  
350 conjugation. Previous attempts to reduce the toxicity of polymyxin antibiotics, by eliminating two of  
351 the five cationic groups, have been described by Vaara *et al*, resulting in a 6-7-fold lower affinity for  
352 the brush border membrane of the renal cortex<sup>27</sup>.

353 Having confirmed that dextrin conjugation could mask colistin's cytotoxicity, it was important  
354 to confirm that conjugates retained antimicrobial activity prior to future clinical testing. Drug resistance  
355 often occurs when bacteria are exposed to sub-optimal concentrations of antibiotic, while high doses  
356 can cause unpleasant or harmful side effects. Masking of antibiotic activity in transit followed by



357 efficient reinstatement of antibiotic activity after passive localisation at sites of infection/ inflammation  
358 by the EPR effect, provides an ideal means of optimising drug dosing to reduce the emergence of  
359 resistance. The Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoints for  
360 colistin have been identified in *Acinetobacter baumannii* (susceptible,  $\leq 2$  mg/L; resistant,  $\geq 4$  mg/L)  
361 and *Pseudomonas aeruginosa* (susceptible,  $\leq 2$  mg/L; intermediate, 4 mg/L; resistant,  $\geq 8$  mg/L)<sup>28</sup>, while  
362 the general MIC breakpoints for CMS susceptibility are typically higher (susceptible,  $\leq 4$  mg/L;  
363 resistant,  $\geq 8$  mg/L)<sup>29</sup>, Despite their higher *in vitro* cytotoxicity, colistin sulfate and CMS were very  
364 active against Gram-negative bacteria (with MIC values as low as 0.016 mg/L and 0.25 mg/L,  
365 respectively) (Table 2). As expected, dextrin conjugation reduced the antimicrobial activity of colistin,  
366 with dextrans of higher molecular weight and degrees of succinoylation causing an almost quantitative  
367 decrease in activity. Dextrin-colistin conjugates were not always below the susceptibility breakpoints,  
368 even after amylase unmasking with equivalent concentrations of amylase as that found in human  
369 serum. However, since dextrin-colistin conjugates are hypothesized to accumulate in areas of  
370 inflammation and infection by the EPR effect<sup>11</sup>, serum concentration will not predict the drug  
371 concentration at disease sites and pharmacokinetic studies will be important to characterize the  
372 distribution and local concentration of the conjugates. This will represent an important future series of  
373 experiments in pre-clinical studies.

374 The pharmacokinetics of a single IV bolus dose of two “prototype” conjugates with contrasting  
375 degrees of succinoylation (1.4 and 7.2 mol%) was tested in comparison to colistin sulfate, to determine  
376 if conjugation increased the plasma half-life of colistin, and to help identify a suitable dosing interval  
377 for subsequent repeated-dose studies. These studies employed a commercial colistin ELISA test kit  
378 since high-performance liquid chromatography (HPLC), the standard method for colistin detection in  
379 plasma and urine<sup>30-33</sup>, was not possible as dextrin conjugation inhibits colistin binding to the HPLC  
380 column (data not shown). In accordance with our hypothesis, preliminary experiments showed that,  
381 after IV administration, the blood clearance of dextrin-colistin conjugates was markedly inhibited by

382 conjugation. Similarly, conjugation of dextrin (6,600 g/mol) to zidovudine also extended the plasma  
383 half-life from 1.3 to 19.3 h and an increase in the area under the plasma concentration-time curve<sup>34</sup>.  
384 The effect of degree of dextrin succinoylation on *in vivo* pharmacokinetics has not previously been  
385 reported, however, as hypothesized, increasing the degree of modification led to extended plasma half-  
386 life of colistin. Given that serum amylase concentration in rats is in excess of 20 times higher than in  
387 human serum<sup>35,36</sup>, we predict that unmasking of the conjugate in humans would be slower, thereby  
388 increasing plasma retention further. Rats in these studies had a plasma amylase concentration of 3,068  
389  $\pm$  119 IU/L (measured by Phadebas<sup>®</sup> assay), compared to the normal human plasma amylase range of  
390 40-125 IU/L. Interestingly, whilst this study was not designed to measure toxicity, the administration of  
391 0.5 mg/kg colistin sulfate, induced signs of systemic toxicity which prevented sampling until 2 h post-  
392 dose, which was not evident at equivalent concentrations of the dextrin-colistin conjugates.

393         These studies demonstrate the clear potential of bioresponsive polymer therapeutics-based  
394 “nanoantibiotics”, in which colistin is conjugated to dextrin, a clinically approved biodegradable  
395 polymer. Having established the optimum composition of dextrin-colistin conjugates which show  
396 comparable antimicrobial activity to commercially available CMS, but with reduced toxicity, ongoing  
397 work is determining *in vivo* distribution and clinical effectiveness.

398  
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403  
404 **Supporting Information:** Additional experimental details as noted in the text. This material is  
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- 498

**Table 1** Characteristics of succinoylated dextrans and dextrin-colistin conjugates.

Compound	Dextrans		Dextrin-colistin conjugates				
	Mw <sup>a</sup> (g/mol) (M <sub>w</sub> /M <sub>n</sub> )	Succinoylation (mol%)	Mw <sup>a</sup> (g/mol) (M <sub>w</sub> /M <sub>n</sub> )	Protein content (% w/w)	Molar ratio (dextrin:colistin)	Conjugated NH <sub>2</sub> per molecule <sup>b</sup>	Free protein (%)
<i>Colistin</i>	1,408						
<i>Dextrin</i>	7,500 (1.8)						
<i>Succ. dextrin (7,500 g/mol)</i>	8,500 (1.9)	1.1	9,000 (1.5)	10.1	1.6:1	3.3	3.6
<i>Succ. dextrin (7,500 g/mol)</i>	9,000 (1.9)	2.5	14,500 (1.5)	17.0	0.9:1	3.2	3.7
<i>Succ. dextrin (7,500 g/mol)</i>	9,500 (2.0)	4.7	15,500 (1.7)	22.2	0.6:1	3.0	3.0
<i>Succ. dextrin (7,500 g/mol)</i>	12,000 (1.8)	8.3	22,000 (1.6)	21.6	0.6:1	3.5	0.7
<i>Dextrin</i>	10,500 (2.6)						
<i>Succ. dextrin (10,500 g/mol)</i>	13,500 (2.0)	2.2	35,000 (2.7)	10.6	0.8:1	1.4	2.8
<i>Succ. dextrin (10,500 g/mol)</i>	17,500 (2.2)	4.3	165,000 (4.3)	11.5	0.7:1	1.6	2.4
<i>Succ. dextrin (10,500 g/mol)</i>	17,500 (2.0)	7.0	180,000 (6.4)	14.5	0.4:1	1.4	2.7
<i>Dextrin</i>	28,000 (2.6)						
<i>Succ. dextrin (28,000 g/mol)</i>	28,500 (1.9)	2.0	95,000 (4.3)	3.3	1.4:1	2.2	1.3
<i>Succ. dextrin (28,000 g/mol)</i>	39,000 (2.0)	3.4	140,000 (3.8)	3.0	1.5:1	1.4	1.2
<i>Succ. dextrin (28,000 g/mol)</i>	45,000 (1.9)	6.1	270,000 (5.5)	6.6	0.7:1	1.0	0.8
<i>Dextrin</i>	48,500 (2.2)						
<i>Succ. dextrin (48,500 g/mol)</i>	98,000 (1.9)	17.4	55,000 (1.8)	5.1	1:1	1.2	0.1
<i>Succ. dextrin (48,500 g/mol)</i>	97,000 (1.7)	28.6	66,500 (1.7)	7.3	0.7:1	1.3	0.1

<sup>a</sup> M<sub>w</sub> was estimated by GPC using pullulan standards. <sup>b</sup> usually 4.81 NH<sub>2</sub> per free colistin.

**Table 2** Antimicrobial activity of colistin sulfate, CMS and dextrin-colistin conjugates\* (containing 1.1 mol% succinoylated dextrin with and without amylase pre-exposure), measured by MIC assay. Data is expressed as mode ( $n=3$ ). \*MIC value represents equivalent colistin concentration of conjugates.

Isolate	MIC ( $\mu\text{g/L}$ )					
	Colistin sulfate	CMS	Conjugate at indicated amylase pre-incubation (h)			
			0	3	6	24
V4 <i>A. baumannii</i> MDR ACB	0.125	4	8	8	8	8
V9 <i>A. baumannii</i>	0.25	4	16	16	16	8
V19 <i>A. baumannii</i> 7789	0.25	2	8	16	8	16
V20 <i>A. lwoffii</i> 8065	0.063	1	8	4	4	4
V22 <i>A. lwoffii</i> 6056	0.125	2	16	8	8	8
V5 <i>E. coli</i> AIM-1	0.016	0.25	1	0.5	0.5	0.125
V11 <i>E. coli</i> 5702	0.5	1	8	4	8	2
V24 <i>E. coli</i> 7273	0.25	2	16	32	16	8
V12 <i>K. pneumoniae</i> 5725	0.5	1	4	4	4	2
V6 <i>K. pneumoniae</i> IR25	0.5	2	8	8	8	8
V8 <i>K. pneumoniae</i> K3	0.5	2	8	16	16	8
V3 <i>K. pneumoniae</i> KP05 506	0.25	2	4	16	16	16
V13 <i>P. aeruginosa</i> PA01	0.25	1	64	64	128	64
V1 <i>P. aeruginosa</i> R22	0.5	0.5	128	128	128	128
V2 <i>P. aeruginosa</i> MDR 301	0.5	1	512	512	512	512
V7 <i>P. stuartii</i> IR57	0.25	1	4	16	8	8



**Table 3** Mean estimates of pharmacokinetic parameters for colistin sulfate or dextrin-colistin conjugates in rats (n = 2).

<b>Pharmacokinetic parameter</b>	<b>Colistin sulfate</b>	<b>Dextrin-colistin (1.4 mol%)</b>	<b>Dextrin-colistin (7.2 mol%)</b>
<i>C<sub>max</sub></i> (ng/mL)	96.1	106.7	101.6
<i>t<sub>max</sub></i> (min)	5	30	60
<i>t<sub>1/2</sub></i> (min)	53.2	135.3	1270.9
<i>AUC<sub>0-24 h</sub></i> (mg.min/mL)	0.0082	0.0342	0.0913
<i>V<sub>d</sub></i> (mL/kg)	669.7	676.4	925.7
<i>CL</i> (mL/min/kg)	13.4	2.8	1.1

## Legends to Figures

- Figure 1** Schematic showing the proposed mechanism of action of dextrin-colistin conjugates.
- Figure 2** Synthesis of (a) succinoylated dextrin; and (b) dextrin-colistin conjugates.
- Figure 3** Characterization of succinoylated dextrin intermediates and dextrin-colistin conjugates. (a) FT-IR spectra showing amplification of peak intensity at  $1720\text{ cm}^{-1}$  with increasing incorporation of carboxyl groups; and (b) FPLC chromatogram of dextrin-colistin conjugates containing dextrans ( $7,500\text{ g/mol}$ ) with different degrees of succinoylation ( $V_0 = \text{void volume (}7.7\text{ mL)}$ )).
- Figure 4** Characterization of the degradation of dextrin, succinoylated dextrin and dextrin-colistin conjugates ( $3\text{ mg/mL}$ ) in the presence of amylase ( $100\text{ IU/L}$  in PBS at  $37\text{ }^\circ\text{C}$ ). Panels (a) and (b) show the change in relative molecular weight in the presence of amylase by GPC of (a) native dextrin ( $7,500\text{ g/mol}$ ) and its succinoylated intermediates; and (b) dextrin-colistin conjugates containing dextrin ( $7,500\text{ g/mol}$ ) with different degrees of succinoylation. Panel (c) shows a typical elution profile of dextrin-colistin conjugate (containing  $7,500\text{ g/mol}$  dextrin,  $4.7\text{ mol\%}$  succinoylation) from a Superdex 75 FPLC column, following incubation with amylase ( $V_0 = \text{void volume (}7.7\text{ mL)}$ )), and panel (d) shows the release of colistin from dextrin-colistin conjugates

(containing 7,500 g/mol dextrin; 3 mg/mL) in the presence of amylase (100 IU/L in PBS) and CMS (3 mg/mL) in PBS at 37 °C (measured by FPLC). Data is expressed as the percentage of total colistin. ( $n=1$ ).

Where  $\blacklozenge$  = dextrin or dextrin-colistin conjugate;  $\circ$  = 1.1 mol% dextrin or dextrin-colistin conjugate;  $\blacktriangledown$  = 2.5 mol% dextrin or dextrin-colistin conjugate;  $\ast$  = 4.7 mol% dextrin or dextrin-colistin conjugate;  $\bullet$  = 8.3 mol% dextrin or dextrin-colistin conjugate;  $\blacksquare$  = 21.3 mol% dextrin or dextrin-colistin conjugate and  $\square$  = CMS.

**Figure 5** Stability of dextrin-colistin conjugates (containing 7,500 g/mol dextrin; 3 mg/mL) in dH<sub>2</sub>O and PBS at pH 7.4 (37 °C) in the absence of amylase (measured by FPLC), and in comparison with amylase-treated conjugates. Data is expressed as the percentage of total colistin. Panels show conjugates containing (a) 1.1 mol% succinylation, (b) 2.5 mol% succinylation, (c) 4.7 mol% succinylation, and (d) 8.3 mol% succinylation. Where  $\bullet$  = dextrin-colistin conjugate with amylase (100 IU/L) in PBS at 37 °C;  $\blacktriangledown$  = dextrin-colistin conjugate in PBS at 37 °C;  $\blacklozenge$  = dextrin-colistin conjugate in PBS at 4 °C;  $\blacksquare$  = dextrin-colistin conjugate in dH<sub>2</sub>O at 37 °C and  $\triangle$  = dextrin-colistin conjugate in dH<sub>2</sub>O at 4 °C.

**Figure 6** *In vitro* cytotoxicity of colistin sulfate, CMS and dextrin-colistin conjugates. Panels (a) and (b) show cell viability by MTT assay of RAW 264.7 (24 h incubation) and HK-2 (72 h incubation) cells, respectively, following incubation with colistin sulfate, CMS or

dextrin-colistin conjugate with and without amylase (100 IU/L) at 37 °C. Data is expressed as mean % untreated control  $\pm$  SEM, n=18. Panel (c) shows membrane integrity by LDH assay of HK-2 cells incubated for 24 h with colistin, CMS or dextrin-colistin with and without amylase (100 IU/L) at 37 °C. Data is expressed as mean  $\pm$  SEM, n=6. Panel (d) shows hemolysis of rat erythrocytes following incubation for 24 h with colistin, CMS or dextrin-colistin conjugate with and without amylase (100 IU/L) at 37 °C. Data is expressed as mean % triton X-100 control  $\pm$  SEM, n=18. Where ● = colistin sulfate; × = CMS; ◇ = dextrin-colistin conjugate and ▲ = dextrin-colistin conjugate with amylase. \* indicates significance ( $p < 0.05$ ) compared to colistin sulfate; \*\* indicates significance ( $p < 0.01$ ) compared to colistin sulfate; \*\*\* indicates significance ( $p < 0.001$ ) compared to colistin sulfate.

**Figure 7** Mean plasma concentration of colistin following an IV dose of colistin sulfate, dextrin-colistin conjugate (1.4 mol%) and dextrin-colistin conjugate (7.2 mol%) (0.1 mg/kg). Data is expressed as colistin concentration  $\pm$  S.D. (n=2). Concentrations of colistin following administration of colistin sulfate were not quantifiable beyond 4 h. Where ● = colistin sulfate; □ = dextrin-colistin conjugate (with 1.4 mol% succinoylation) and ◆ = dextrin-colistin conjugate (with 7.2 mol% succinoylation). \* indicates significance ( $p < 0.05$ ) compared to colistin sulfate; \*\* indicates significance ( $p < 0.01$ ) compared to colistin sulfate; \*\*\* indicates significance ( $p < 0.001$ ) compared to colistin sulfate.

