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Citation for final published version:

Rizzo, Siân, Varache, Mathieu , Sayers, Edward J. , Jones, Arwyn T. , Tonks, Alex , Thomas, David W. and Ferguson, Elaine L. 2024. Modification of the antibiotic, colistin, with dextrin causes enhanced cytotoxicity and triggers apoptosis in myeloid leukemia. *International Journal of Nanomedicine*

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1 ORIGINAL RESEARCH

2 Rizzo *et al*

3 **Modification of the antibiotic, colistin, with dextrin causes**
4 **enhanced cytotoxicity and triggers apoptosis in myeloid**
5 **leukemia**

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18 **Abstract:**

19 **Introduction.** Acute myeloid leukemia (AML) remains difficult to treat due to its heterogeneity in
20 molecular landscape, epigenetics and cell signaling alterations. Precision medicine is a major goal
21 in AML therapy towards developing agents that can be used to treat patients with different
22 'subtypes' in combination with current chemotherapies. We have previously developed dextrin-
23 colistin conjugates to combat the rise in multi-drug resistant bacterial infections and overcome dose-
24 limiting nephrotoxicity. Recent evidence of colistin's anticancer activity, mediated through inhibition
25 of intracellular lysine-specific histone demethylase 1 (LSD1/KDM1A), suggests that dextrin-colistin
26 conjugates could be used to treat cancer cells, including AML. This study aimed to evaluate
27 whether dextrin conjugation (which reduces *in vivo* toxicity and prolongs plasma half-life) could
28 enhance colistin's cytotoxic effects in myeloid leukemia cell lines and compare the intracellular
29 uptake and localization of the free and conjugated antibiotic.

30 **Results.** Our results identified a conjugate (containing 8,000 g/mol dextrin with 1 mol%
31 succinylation) that caused significantly increased toxicity in myeloid leukemia cells, compared to
32 free colistin. Dextrin conjugation altered the mechanism of cell death by colistin, from necrosis to
33 caspase 3/7-dependent apoptosis. In contrast, conjugation via a reversible ester linker, instead of
34 an amide, had no effect on the mechanism of the colistin-induced cell death. Live cell confocal
35 microscopy of fluorescently-labelled compounds showed both free and dextrin-conjugated colistin
36 were endocytosed and co-localized in lysosomes and increasing the degree of modification by
37 succinylation of dextrin significantly reduced colistin internalization.

38 **Discussion.** Whilst clinical translation of dextrin-colistin conjugates for the treatment of AML is
39 unlikely due to the potential to promote antimicrobial resistance (AMR) and the relatively high
40 colistin concentrations required for anticancer activity, the ability to potentiate the effectiveness of
41 an anticancer drug by polymer conjugation, while reducing side effects and improving biodistribution
42 of the drug, is very attractive, and this approach warrants further investigation.

43

44 **Keywords:** polymer therapeutics; biodegradable; internalization; polysaccharide; antibiotic

45 Introduction

46 Acute myeloid leukemia (AML) is characterized by a block in hemopoietic differentiation where the
47 hemopoietic stem cell (HSC) fails to develop into mature myeloid cells resulting in bone marrow
48 failure.¹ AML arises due to a wide range of molecular abnormalities and genetic mutations, making
49 the disease difficult to treat.² Although AML accounts for fewer than 1% of all new cancer cases,³
50 recurrence after complete remission is common. Importantly, infection, due to treatment-related
51 immunosuppression, expression of immunosuppressive molecules or specific defects in the immune
52 response, remains a major cause of death in patients with AML, requiring antibacterial or antiviral
53 therapies to treat infections during first-line treatment.⁴ Given the high costs, high risk of failure and
54 protracted development times of new chemotherapy drugs, exploiting the off-target effects of existing
55 antimicrobial agents is an attractive approach to improve survival rates in cancer patients.

56 Polymer therapeutics are an important class of drugs that use conjugation of a water-soluble
57 polymers to improve the therapeutic index of drugs, including for oncology. Oncaspar[®], a PEG-L-
58 asparaginase conjugate was one of the first polymer therapeutics to receive FDA approval, for the
59 treatment of acute lymphoblastic leukemia (ALL).⁵ There are currently several nanomedicines in
60 clinical use or undergoing clinical trials for the treatment of AML, including liposomes (e.g. VYXEOS[®];
61 liposomal daunorubicin and cytarabine),⁶ polymeric nanoparticles (e.g. AZD2811; aurora kinase B
62 inhibitor-loaded Accurin[™] polymeric nanoparticles)⁷ and polymer-drug conjugates (e.g. N-(2-
63 hydroxypropyl) methacrylamide (HPMA) copolymer-cytarabine and GDC-0980 conjugates).⁸ We
64 have previously developed bio-triggered polymer therapeutics, based on the attachment of the
65 polysaccharide, dextrin, to a bioactive molecule, including phospholipase A2 (PLA2),⁹ epidermal
66 growth factor¹⁰ and colistin (also known as polymyxin E),¹¹ as treatments for breast cancer, chronic
67 wounds and bacterial infection, respectively. Dextrin is an attractive polymeric carrier for protein and
68 peptide drugs as it is non-toxic, non-immunogenic and biodegradable. Varying dextrin's molecular
69 weight and degree of modification by succinylation can control the rate of amylase-mediated
70 degradation, and hence payload release rate.¹²⁻¹⁴

71 Recently, it has been shown that the polymyxin antibiotics, polymyxin B and colistin, can kill
72 some types of cancer cells,¹⁵ including the ALL cell line REH.¹⁶ Polymyxin's anticancer activity is
73 believed to be mediated through inhibition of lysine-specific histone demethylase 1 (LSD1, also
74 referred to as lysine-specific demethylase 1A: KDM1A),¹⁷ a nuclear histone-modifying enzyme which
75 is overexpressed in AML leading to poorer clinical outcomes.¹⁸ Recently, it has been shown that
76 inhibition of LSD1/KDM1A activity results in inhibition of AML cell proliferation and enhanced
77 differentiation.¹⁸ Thus, several LSD1/KDM1A inhibitors, including GSK2879552 and tranylcypromine,
78 are currently in clinical trials for cancer treatment,¹⁹ including AML, as a combination therapy.²⁰ Given
79 the efficacy of colistin as a prophylactic treatment of neutropenia in AML,²¹ polymyxin antibiotics have
80 the potential to simultaneously act as antibacterial and anticancer agents. However, dose-limiting
81 neuro- and nephrotoxicity prevent their routine use,²² which may be overcome by polymer
82 conjugation, as we have shown in our previous work, where dextrin-colistin conjugates reduced
83 toxicity *in vitro* and *in vivo*, and extended plasma half-life.^{11,23,24} Our recent studies have demonstrated
84 that conjugation of dextrin to colistin reduced cellular internalization of the antibiotic by kidney cells
85 and caused reduced cytotoxicity.²⁵ In parallel, dextrin conjugation to colistin dramatically diminished
86 both proximal tubular injury and renal accumulation of colistin in mice receiving twice-daily doses of
87 the antibiotic. Therefore, we hypothesized that dextrin-colistin conjugates may be repurposed as an
88 anticancer therapy, offering improved biodistribution, sustained drug exposure and reduced side-
89 effects.

90 Polymer conjugation has been widely used as a means of improving drug delivery.^{26,27}
91 However, while polymer-protein and -peptide conjugation has shown particular success for their
92 delivery to extracellular targets, intracellular delivery has proved to be more challenging.²⁸ Our
93 previous studies have shown that dextrin conjugation has a varying effect on intracellular uptake of a
94 drug; while attachment of dextrin to PLA2 significantly increased the proportion of internalized PLA2
95 by MCF-7 breast cancer cells,²⁹ less colistin internalization in HK-2 proximal tubule cells was
96 observed when conjugated to dextrin.²⁵ Therefore, it was important to establish whether conjugation
97 of colistin to dextrin would alter its endocytic properties in AML cells.

98 The aim of this study was to investigate and quantify the intracellular delivery of 4 different
99 dextrin-colistin conjugates (containing 8,000 or 51,000 g/mol dextrans, having 1 or 10 mol%
100 succinylation) to demonstrate whether dextrin conjugation can enhance colistin's anticancer activity.
101 To achieve this, *in vitro* cytotoxicity, cellular uptake, and localization of fluorescently labelled
102 conjugates were assessed in three leukemia cell lines.

103 **Material and methods**

104 **Materials**

105 Type I dextrin prepared from corn (Mw = 8,000 g/mol, degree of polymerization (DP = 50), colistin
106 sulfate, N-hydroxysulfosuccinimide (sulfo-NHS), dimethyl sulfoxide (DMSO), dimethylformamide
107 (DMF), Hoechst 33342 solution and staurosporine were purchased from Sigma-Aldrich (Poole, UK).
108 Dextrin (Mw 51,000 g/mol, DP = 315) was from ML Laboratories (Liverpool, UK). Disodium hydrogen
109 phosphate, potassium dihydrogen phosphate, potassium chloride, 4-dimethylaminopyridine (DMAP),
110 BCA protein assay kit, N,N'-dicyclohexyl carbodiimide (DCC), 1-ethyl-3-(3-(dimethylamino)propyl
111 carbodiimide hydrochloride) (EDC), AlexaFluor® 594 (AF594) cadaverine, AF594 succinimidyl ester,
112 sodium chloride, Gibco™-branded keratinocyte serum-free medium (K-SFM) with L-glutamine,
113 epidermal growth factor (EGF), bovine pituitary extract (BPE), 0.05% w/v trypsin-0.53 mM EDTA,
114 Iscove's modified Dulbecco's medium (IMDM) with GlutaMAX™, fetal bovine serum (FBS) and
115 Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX™ were obtained from
116 ThermoFisher Scientific (Loughborough, UK). CellTiter-Blue® (CTB) cell viability assay kit, CytoTox-
117 One™ homogeneous membrane integrity assay kit and Caspase-Glo® 3/7 assay system kit were
118 from Promega, WI, USA. Recombinant human granulocyte-monocyte colony stimulating factor
119 (rhGM-CSF) was from Peprotech (London, UK). Pullulan gel filtration standards (M_w = 180–788,000
120 g/mol) were purchased from Polymer Laboratories (Church Stretton, UK) and Shodex (Tokyo, JP).
121 Unless otherwise stated, all chemicals were of analytical grade and used as received. All solvents
122 were of general reagent grade (unless stated) and were from Fisher Scientific (Loughborough, UK).

123 **Synthesis and characterization of dextrin-colistin conjugates and** 124 **AF594-labelled probes**

125 Amide-linked dextrin–colistin conjugates, containing dextrin ($M_w = 8,000$ or $51,000$ g/mol) with 1 and
126 10 mol% succinylation, were synthesized using EDC and sulfo-NHS and characterized as described
127 previously.¹¹ To synthesize ester (E)-linked conjugates, succinoylated dextrans (1, 2.5 and 10 mol%
128 succinylation) were conjugated to colistin using DCC and DMAP. Briefly, for 1 mol% succinoylated
129 dextrin, succinoylated dextrin (1000 mg, 0.125 mmol), DCC (25.5 mg, 0.25 mmol) and DMAP (7.5
130 mg, 0.125 mmol) were dissolved under stirring in anhydrous DMSO (10 mL) in a 50 mL round-
131 bottomed flask, and left stirring at 21°C for 15 min. Subsequently, colistin sulphate (176 mg, 0.25
132 mmol) was added, and the mixture was left stirring overnight at 21°C. To stop the reaction, the mixture
133 was poured into excess chloroform (~100 mL). Resulting precipitates were isolated by filtration and
134 dissolved in ultrapure water (H_2O ; Milli-Q® filtered to 18.2 M Ω cm^{-1}) (10 mL), then stored at $-20^\circ C$
135 before purification by fast protein liquid chromatography (FPLC) (AKTA Purifier; GE Healthcare, UK)
136 using a pre-packed HiLoad Superdex 30 26/600 column equipped with a UV detector, using Unicorn
137 5.31 software (GE Healthcare, Amersham, UK) for data analysis.

138 To enable visualization of conjugates by flow cytometry and confocal microscopy,
139 succinoylated dextrin, colistin and dextrin-colistin conjugate were fluorescently labelled with
140 AlexaFluor® 594 (AF594) and characterized according to previously published methods,³⁰⁻³² including
141 spectrophotometric and fluorometric analysis. Briefly, to prepare AF594-labelled colistin, the antibiotic
142 (16.5 mg) was solubilized under stirring in PBS (1 mL, pH 7.4) in a 10 mL round-bottomed flask.
143 AF594 succinimidyl ester (9.5 mg, from a stock solution of 10 mg/mL in anhydrous DMF, stored at -
144 $20^\circ C$ until use) was added dropwise. Then, the reaction mixture was stirred at room temperature,
145 protected from light. After 1 h, the solution was purified by FPLC (as above) using a prepacked
146 Superdex 30 26/600 column coupled with a UV detector set at 210, 280 and 550 nm. The reaction
147 mixture (5 mL) was injected into a 5 mL loop and eluted using 0.1 M ammonium acetate (pH 6.9, 0.22
148 μm filter-sterilized) at a flow rate of 2.5 mL/min. Fractions (15 mL) containing colistin-AF594 (typically

149 between 230 and 290 mL) were identified by HPLC-fluorescence, then pooled and desalted by freeze-
150 drying ($\times 5$) to remove ammonium acetate. The final compound was stored at -20°C until use. To
151 prepare AF594-labelled dextrin, succinoylated dextrin (160 mg, 8,000 g/mol, 10 mol%) was dissolved
152 under stirring in PBS buffer (5 mL, pH 7.4) in a 10 mL round-bottomed flask. To this, EDC (18.9 mg)
153 and sulfo-NHS (21.4 mg) were added, and the mixture stirred for 15 min before addition of AF594
154 cadaverine (8 mg, from a stock solution of 10 mg/mL in anhydrous DMF, stored at -20°C until use).
155 The reaction mixture was stirred in the dark for 5 h prior to purification by size exclusion
156 chromatography (SEC, disposable PD-10 desalting column containing Sephadex G-25). To prepare
157 the AF594-labelled dextrin-colistin conjugate with 10 mol% succinoylation, a dextrin-AF594
158 intermediate was prepared, as described above, conjugated to colistin and purified by FPLC, as
159 previously described.¹¹ As 1 mol% succinoylated 8,000 g/mol dextrin would not contain sufficient
160 reactive groups to attach both, colistin and AF594, to prepare AF594-labelled dextrin-colistin
161 conjugate with 1 mol% succinoylation, a colistin-AF594 intermediate was initially prepared, as above,
162 and subsequently conjugated to 1 mol% succinoylated dextrin and purified as previously described¹¹.

163 The total AF594 content of AF594-labelled dextrin and dextrin-colistin conjugates was
164 determined by measuring absorbance at 485 nm. Free AF594 content was assessed by measuring
165 fluorescence ($\lambda_{\text{ex}} = 588 \text{ nm}$, $\lambda_{\text{em}} = 612 \text{ nm}$, gain 1000) of fractions (1 mL) eluting from a PD-10 column,
166 according to previously described methods.³² Analysis of colistin-AF594 was performed using LC-MS
167 on a Synapt G2-Si quadrupole time-of-flight (QTOF) mass spectrometer (Waters, U.K.), operating in
168 the positive electrospray ionization mode, coupled to an ACQUITY H-Class UPLC system (Waters,
169 Wilmslow, UK). Separation was accomplished using an ACQUITY UPLC BEH column (1.7 μm , 2.1 x
170 100 mm, Waters) inside a column oven at 40°C . A multistep gradient method was used (0-2 min, 98%
171 A; 2-20 min, 2% A; flow rate 0.3 mL/min), where mobile phase A is water (0.1% formic acid), and
172 mobile phase B is acetonitrile (0.1% formic acid). Analysis of the purity of AF594-labelled dextrin and
173 dextrin-colistin conjugates was performed using PD-10 columns (Cytiva, Little Chalfont, UK), by
174 analysis of SEC fractions for fluorescence and protein content (by BCA reagent).

175 **Hemolytic activity**

176 All animal experiments were conducted according to the United Kingdom Use of Animals (Scientific
177 Procedures) Act 1986. Animal work was reviewed by the Animal Welfare and Ethical Review Body
178 under the Establishment License held by Cardiff University and authorized by the UK Home Office.
179 Fresh blood was extracted from recently euthanized male Wistar rats (~250 g body weight) by cardiac
180 puncture and added to 4 mL PBS (pH 7.4) in a heparin/lithium blood tube. The tube was centrifuged
181 at 400 x *g* for 10 min at 4°C to extract red blood cells (RBCs), then washed a further two times.
182 Following the final wash, the RBC pellet was diluted to 2% w/v with PBS. Subsequently, this diluted
183 RBC suspension was added to a 96-well plate (100 µL/well, replicates n=6) containing an equal
184 volume of test compounds, PBS (negative control) or Triton X-100 (1% v/v) (positive control).
185 Following incubation for 1 h at 37°C, the plate was centrifuged at 400 x *g* for 10 min at 4°C. The
186 supernatant (100 µL) of each well was transferred to a 96-well plate, and absorbance at 550 nm was
187 read using a Fluostar Omega microtiter plate reader. Cells were plated with 6 technical replicates per
188 plate (n=6) and each experiment was performed three times. The negative control (PBS) absorbance
189 was subtracted, and the results were expressed as mean percentage of maximum (Triton X-100)
190 hemoglobin released ± 1SD (n=6).

191 **Cell culture**

192 Human kidney proximal tubule cells (HK-2) and the leukemia cell lines MV-4-11 and TF-1 were
193 obtained from ATCC (Manassas, USA). The leukemia cell line THP-1 was from ECACC (UK Health
194 Security Agency). Characteristics of the cell lines used are shown in Table S1. Cells were screened
195 to be free of mycoplasma contamination upon thawing and monthly thereafter using a Venor GeM
196 Classic Mycoplasma Detection Kit from Minerva Biolabs (Berlin, Germany). Cell lines were
197 maintained in log-phase proliferation at 37°C with 5% CO₂/air and cultured in their respective culture
198 medium (Table S1). HK-2 cells were passaged using 0.05% w/v trypsin-0.53 mM EDTA.³³

199 **Evaluation of *in vitro* cytotoxicity**

200 Cytotoxicity of colistin sulfate and the dextrin-colistin conjugates was assessed in the above cell lines
201 using a multiplexed assay system, to measure cell viability, membrane integrity (lactate
202 dehydrogenase (LDH) release), caspase activity/apoptosis and DNA content as described
203 previously.³⁴ In summary, cells were seeded into sterile black, clear-based 96-well microplates (HK-
204 2 at 2,500 cells/ well: all other cell lines at 10,000 cells/ well in 0.1 mL of complete media). Cultures
205 were incubated at 37°C with 5% CO₂ for 1 h (MV-4-11, THP-1, TF-1) or, to allow adherent cells to
206 adhere, for 24 h (HK-2). Test compound stock solutions were prepared in PBS (0.22 µm filter-
207 sterilized) and used to supplement the complete media. Test compounds were evaluated in triplicate
208 at concentrations up to 1 mg/mL colistin base with respective vehicle-only control (PBS), while 1-10
209 µM staurosporine (apoptosis) and 100 µM Triton-X100 (necrosis) were used as positive controls.

210 Following a further 24 or 72 h incubation, plates were processed as follows, protected from
211 light throughout. To measure necrosis, 25 µL of supernatant from each well was transferred to a fresh
212 black 96-well microplate, containing 25 µL of CytoTox-One™ assay reagent. Cultures were gently
213 mixed then incubated at 21°C for 10 min, then a “stop solution” was added and fluorescence
214 measured at $\lambda_{\text{ex}} = 560 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$ using a Fluostar Omega microplate reader. Total cellular
215 LDH activity was measured after the addition of LDH lysis solution to cells, prior to addition of the
216 assay reagent. Next, to measure cytotoxicity and DNA content, CTB reagent was supplemented with
217 Hoechst 33342 (100 µg/mL) and added to the wells of the original microplate (10 µL/well). Cultures
218 were gently mixed followed by incubation at 37°C with 5% CO₂ in air for 1 h and fluorescence
219 measured at $\lambda_{\text{ex}} = 560 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$ and $\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$ (for cytotoxicity and DNA
220 content, respectively) using a Fluostar Omega microplate reader. Finally, 60 µL of Caspase-Glo 3/7
221 assay reagent was added to each well of the original microplate. Plates were gently agitated then
222 incubated at 20°C with 5% CO₂ in air for 1 h, followed by luminescence assays using a Fluostar
223 Omega microplate reader. Cells were plated with 3 technical replicates per plate (n=3) and each
224 experiment was performed twice. Data was corrected for no-cell background, then expressed as
225 mean percentage of the response of vehicle-only control cells $\pm 1 \text{ SD}$ (n=3). Relative IC₅₀ values were

226 determined using the non-linear regression analysis of dose-response-inhibition using a 4-parameter
227 logic model in GraphPad Prism (version 9.3.1, 2021; San Diego, CA, USA).

228 To monitor apoptosis and necrosis continuously over 72 h, a RealTime-Glo™ Annexin V
229 apoptosis and necrosis assay (Promega, Southampton, UK) was used. Briefly, MV-4-11 cells were
230 plated into sterile black, clear-based 96-well microplates (4,500 cells/ well in 45 µL of complete
231 media). Test compound solutions (10 µL) and 2x detection reagent (55 µL) were added to each well,
232 in triplicate, and the plate was gently agitated before placing inside a Fluostar Omega plate reader
233 (maintained at 37°C with 5% CO₂). Fluorescence ($\lambda_{ex} = 485 \text{ nm}$, $\lambda_{em} = 525\text{-}530 \text{ nm}$) and luminescence
234 were measured at regular timepoints for up to 72 h. Data was corrected for no-cell background and
235 expressed as a percentage of the response of vehicle only control cells. Staurosporine and Triton-
236 X100 were used as positive controls for apoptosis and necrosis, respectively, as described above.
237 Cells were plated with 3 technical replicates per plate (n=3) and each experiment was performed
238 once.

239 **Cell cycle analysis**

240 Cell cycle analysis of MV-4-11 cells following incubation with colistin sulfate, DC8/1 or DC8/10 at the
241 previously determined IC₅₀ value was performed according to a modified version of Ormerod's
242 protocol.³⁵ Briefly, 500 µL cells at 10⁵ cell/ mL per well of a 24-well plate were incubated in complete
243 media supplemented with test compounds (in duplicate) at their IC₅₀ value at 37°C with 5% CO₂ for
244 24 h. The contents of each well were diluted with 3 mL PBS, then centrifuged (350 x g for 5 min at
245 20°C), the cell pellet was resuspended in ice-cold PBS then centrifuged again prior to removing the
246 supernatant. Subsequently, ice-cold 70% v/v ethanol was added dropwise under vortex to resuspend
247 the cells, then incubated on ice. Following 30 min, cells were centrifuged (450 x g for 10 min at 4°C)
248 then washed twice with ice-cold PBS, vigorously resuspending the pellet each time. To the cell pellet,
249 50 µL of RNase (100 µg/mL) solution was added then incubated at 37°C for 15 min. Finally, 200 µL
250 of propidium iodide (PI; 50 µg/mL) solution was added to each tube 20 min prior to data acquisition
251 with a Becton Dickinson FACS CANTO II Cell Analyser flow cytometer equipped with a 488 nm blue

252 laser and 584/42 nm emission filter. Data were collected for 25,000 events per sample and data
253 analyzed using FlowJo™ software, v10.8.1. MV-4-11 cells incubated with medium only were used to
254 determine background fluorescence. Cell fragments, clumps and debris were excluded using
255 sequential gating on a forward-scatter (FSC)-height vs side-scatter (SSC)-height cytogram and a
256 DNA (FL2)-area vs DNA (FL2)-width cytogram, then the remaining single cells were displayed in a
257 histogram of DNA-area (FL2), as described by Ormerod; the Watson (pragmatic) algorithm, a
258 univariate cell cycle model, was employed to assess the proportion of cells in each cell cycle phase:
259 G1, S and G2(M)³⁶ using FlowJo™ software (version 10.8.1). Cells were plated with 2 technical
260 replicates per plate (n=2) and each experiment was performed four times.

261 **Colony formation**

262 Colony-forming ability of MV-4-11 and THP-1 cells following incubation with colistin sulfate, DC8/1 or
263 DC8/10 at the previously determined IC₅₀ value was measured using a human Colony Forming Cell
264 (CFC) assay in methylcellulose-based media, according to manufacturer's instructions (R&D
265 Systems, Abingdon, UK). Briefly, cells (10⁵ cells/mL) were incubated in complete media
266 supplemented with 10% v/v test compounds at their IC₅₀ value or with vehicle (PBS) at 37°C with 5%
267 CO₂ for 24 h. Then, the equivalent of 667 THP-1 or 400 MV-4-11 cells were added in duplicate to 1
268 mL human methylcellulose base media supplemented with recombinant human granulocyte-
269 monocyte colony stimulating factor (10 ng/mL), interleukin-3 (10 ng/mL) and stem cell factor (20
270 ng/mL) in a 35 mm culture dish (Nunclon, Fisher Scientific). Following incubation at 37°C with 5%
271 CO₂ for 10 days, colonies containing >32 cells were enumerated by inverted light microscopy. The
272 results were expressed as mean percentage colony-forming units (CFU) of vehicle-only control (n=2).

273 **Determination of cellular uptake**

274 To investigate cell association at 4 or 37°C, leukemia cells were first resuspended at 200,000
275 cells/well of a 96-well plate in 75 µL complete medium (without phenol red). Experiments at 37°C
276 were conducted with standard cell culture conditions, but for low temperature experiments, cells were

277 pre-incubated for 30 min at 4°C prior to the addition of the probe. Solutions of fluorescent probes
278 were freshly prepared in complete medium at sub-toxic, equivalent concentrations of AF594 base
279 (1µg AF594 base/mL), filter-sterilized (0.22 µm), then equilibrated to either 37 or 4°C for 30 min.
280 Probe solutions were added to each well (75 µL) containing cells and incubated at 4 or 37°C for 2 h.
281 Subsequently, plates were placed on ice before transferring the cell suspension into individual tubes
282 and washing twice by centrifugation (350 x *g*, 5 min) with ice-cold PBS (2 x 3 mL). Finally, cells were
283 resuspended in ice-cold PBS (200 µL) prior to data acquisition using a Becton Dickinson LSR
284 Fortessa Cell Analyser flow cytometer equipped with a yellow-green laser (561 nm) and emission
285 filter for 585/15 nm. For each sample, 10,000 events were collected and analyzed using FlowJo™
286 software v 10.8.1. Control cells incubated with medium only were used to determine background
287 fluorescence. Throughout, results were corrected for cell autofluorescence and expressed as
288 (geometric mean × % positive cells)/100, where % positive cells was calculated as 100 – % cells in
289 M1. Internalization was calculated by subtracting the cell-associated fluorescence at 4°C
290 (extracellular binding) from that at 37°C (intracellular uptake plus extracellular binding) and expressed
291 as mean ± SD. All uptake studies were performed using colistin concentrations that have previously
292 been shown to be non-toxic. Cells were plated with three technical replicates per plate (n=3); each
293 experiment was performed three times. Results are expressed as mean fluorescence ± 1SD (n=3).

294 **Intracellular localization**

295 Cells were incubated in complete media (without phenol red, at 6.67 x 10⁵ cells/mL) containing
296 dextran-AF488 (150 µg/mL) at 37°C with 5% CO₂ /in air. After 3 h, excess dextran-AF488 was
297 removed by addition of complete media (10x labelling volume) followed by centrifugation at 350 x *g*
298 for 5 min. The cell pellet was then resuspended in complete media (10⁵ cells/150 µL) with and without
299 AF594 probes (5 µg AF594 base/mL) and incubated at 37°C with 5% CO₂ for 2 h in 96-well plates.
300 In all cases, colistin concentration was below IC₅₀. The cells were washed twice with 10x volume
301 complete medium, as described above. After the final wash, 10⁵ cells/mL cells were cultured in fresh

302 medium at 37°C with 5% CO₂ for a further 2 h (short chase) or overnight (16 h, long chase). Control
303 cells (not incubated with a probe) were used to account for autofluorescence.

304 Confocal laser scanning microscopy was performed using a Leica SP5 system (37°C, 5%
305 CO₂). Confocal imaging was performed sequentially with the 405 nm (Hoechst 33342) and 543 nm
306 (AF594) lasers captured concurrently and 488 nm (AF488) laser captured sequentially between lines.
307 Images were captured using a HCX PL APO CS 100.0x1.40 oil immersion objective at 400 Hz with a
308 line average of 3 (bi-directional scanning), with the pinhole set to 1 airy unit. Images were acquired
309 with a raster size of 1024 x 1024 and a zoom of 1.12 to give an apparent pixel size of 138 x 138 nm
310 (XY). At least eight representative images (single section) were obtained from each sample; typical
311 results are shown. Images were analyzed and processed using ImageJ.³⁷

312 **Statistical analysis**

313 GraphPad Prism (version 9.3.1, 2021; San Diego, CA, USA) software was used to perform statistical
314 analyses. Statistical significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.
315 One-way analysis of variance (ANOVA) was used to evaluate multiple group comparisons ($n \geq 3$)
316 followed by Dunnett's post hoc test to account for multiple comparisons, unless otherwise stated.

317 **Results**

318 Amide- and ester-linked dextrin-colistin conjugates (containing 8,000 or 51,000 g/mol dextrans, having
319 1 or 10 mol% succinylation) were synthesized to compare their *in vitro* cytotoxicity and colony
320 forming ability of treated cells. Fluorescent dextrin, colistin and dextrin-colistin conjugate were
321 subsequently obtained by chemical attachment of Oregon Green (OG) to enable measurement of
322 cellular uptake and localization in leukemia cell lines.

323 **Synthesis and characterization of dextrin-colistin conjugates**

324 The characteristics of dextrin-colistin conjugates used in these studies are summarized in Table 1.
325 Typical FPLC chromatograms of conjugate reaction mixtures are shown in Figures S1, and overlaid

326 with the protein content of fractions in Figure S2, to confirm successful conjugation. Free colistin
327 content of purified conjugates, analyzed by FPLC, was determined to be <3%.

328 **Hemolytic activity**

329 The hemolytic activity of colistin and dextrin-colistin conjugates was measured with fresh rat RBCs to
330 assess the effect of free and dextrin-conjugated colistin on normal blood cells. After exposure of RBCs
331 to treatments for 24 h, only the highest dose of free colistin sulfate induced significant lysis, compared
332 to vehicle alone (Figure 1). No hemolysis was observed for any of the dextrin-colistin conjugates up
333 to 500 µg/mL. Control experiments with dextrin and succinoylated dextrans showed <10% hemolysis
334 up to 10 mg/mL (data not shown).

335 ***In vitro* cytotoxicity**

336 Cytotoxicity was determined in three representative myeloid leukemia cells lines, in comparison to a
337 non-cancerous human kidney proximal tubular cell line. As expected, cell viability decreased with
338 longer incubation times in all cell lines. Where inhibition at 24 h achieved at least 50%, cell viability
339 data was used to generate relative IC₅₀ values, summarized in Table 2.

340 Colistin sulfate exhibited greatest cytotoxicity against HK-2 cells (Figure 2a and S3a); amide-
341 linked dextrin conjugation reduced toxicity of colistin in HK-2 by at least 1.4-fold, compared to the free
342 drug, and was most effective in conjugates containing longer dextrin chains and higher degrees of
343 succinoylation. Similarly, in the leukemia cell lines, conjugation to dextrin reduced cytotoxicity for
344 three of the four amide-linked conjugates tested: DC8/10, DC51/1 and DC51/10 (Table 2, Figures 2b
345 and S3b) by varying degrees. However, in all leukemia cell lines tested, DC8/1 was between 1.3-3.7-
346 fold more potent than the free antibiotic (Table 2).

347 Colistin sulfate induced dose-dependent necrosis in all cell lines at 24 and 72 h (Figures 2c,d
348 and S3c,d), with no evidence of caspase 3/7-dependent apoptosis (Figures 2e,f and S3e,f). In
349 contrast, amide-linked conjugates showed dose-dependent caspase 3/7 activity (DC8/1 > DC51/1 >
350 DC8/10 > DC51/10), in all leukemia cell lines at 24 h post-treatment (3.8-fold increase in caspase 3/7

351 activity in TF-1 cells treated with DC8/1 at 0.25 mg/mL colistin base, Figure 2f). However, by 72 h, no
352 caspase 3/7 activity was seen. No comparable caspase 3/7 upregulation was seen in HK-2 cells for
353 any of the treatments tested (Figures 2e and S3e). Overall, patterns of concentration-dependent
354 cytotoxicity were similar in all leukemia cell lines tested, in contrast to non-cancerous HK-2 cells
355 (Figure 2 and Figure S3).

356 Differences in concentration-dependent cell viability and apoptosis were observed between
357 amide- and ester-linked dextrin-colistin conjugates (Figure 3). Ester-linked conjugates exhibited a
358 similar effect on cell viability as the amide-linked DC8/1, but the latter caused the greatest increase
359 in caspase 3/7 activity (Figure 3a,b). When cell viability and apoptosis data were plotted against the
360 dextrin content of the respective dextrin-colistin conjugate, the cytotoxic effects became more
361 congruent (Figure S4). DC8/1 remained the most potent amide-linked conjugate, however there was
362 no difference in cytotoxicity between the ester- and amide-linked conjugates. Amide-linked
363 conjugates, however, showed more caspase 3/7 activity at equivalent dextrin concentrations, than
364 the ester-linked conjugates. Annexin V binding occurred within a few hours of treatment and a
365 subsequent peak in necrosis was detected (Figure 3c,d). Ester-linked DC8/1e conjugate caused less
366 apoptosis, but more necrosis, than the amide-linked equivalent (DC8/1). Ester-linked conjugates were
367 not tested in further experiments, due to the high batch-to-batch variability in conjugation efficiency
368 and excessive amounts of unconjugated polymer, compared to amide- linked conjugates.

369 Cells treated with DC8/10 and DC51/10 in the multiplex assay exhibited concentration-
370 dependent increases in Hoechst 33342 staining (Figures 2d,h and S3d,h), which suggested DNA
371 accumulation. Subsequent cell cycle analysis using PI failed to show any significant changes in phase
372 distribution associated with different treatments, apart from a limited, but statistically significant,
373 reduction in the G2M population of cells treated with DC8/1, compared to colistin sulfate (Figure 4a).

374 **Colony formation**

375 A colony formation assay was employed to determine the potential inhibitory effects of dextrin-colistin
376 conjugates on tumor cell phenotype. Following exposure of leukemia cells to colistin sulfate at IC₅₀

377 for 24 h, colony formation was reduced, compared to control, in both cell lines (MV-4-11: 69±10%,
378 THP-1: 55±1%) (Figure 4b,c). Inhibition of colony formation was most pronounced in cells treated
379 with DC8/1 at the equivalent colistin dose (MV-4-11: 4.7±1.6%, THP-1: 19±2%), while DC8/10 did not
380 alter colony formation in either cell line tested.

381

382 **Evaluation of cellular uptake and intracellular fate**

383 To study cellular uptake, AF594-labelled colistin, dextrin and dextrin-colistin conjugates were
384 synthesized, containing <2% free AF594 (Figure S5). The characteristics of all AF594-labelled probes
385 used in these studies are summarized in Table 3.

386 Flow cytometry demonstrated that all AF594-labelled conjugates exhibited cell association
387 after 1 h at 37°C (Figure 5 and Figure S6). In all instances, the cell-associated fluorescence was
388 reduced at 4°C, indicating >98% calculated internalization of fluorescent probes. Colistin-AF594
389 cellular levels were significantly greater than observed for the AF594-labelled dextrin-colistin
390 conjugates. However, DC8/1-AF594 displayed significantly more uptake by MV-4-11 cells than
391 DC8/10-AF594 at 37°C ($p>0.0001$, 2-way ANOVA, Tukey's multiple comparison test).

392 In line with flow cytometry data, confocal microscopy showed that colistin-AF594 and AF594-
393 labelled dextrin-colistin conjugates were internalized, with punctate labelling resembling
394 endolysosomal structures (Figures 6, 7). There was clear evidence that colistin-AF594 was reaching
395 the lysosomes, manifesting as colocalization with the pulse chased dextran. However, dextrin
396 conjugation significantly reduced colistin internalization, exacerbated by increased succinylation.

397 **Discussion**

398 Targeted therapies, which interfere with specific proteins involved in tumorigenesis, survival and
399 growth, are promising precision medicines for cancer.³⁸ The clinical development of targeted
400 therapies has yielded few FDA-approved treatments for AML,³⁹ in part, due to unacceptable side
401 effects and an inability to reach their intracellular targets. Drug delivery strategies, such as polymer

402 therapeutics, offer the ability to overcome these issues.⁴⁰ LSD1/KDM1A is overexpressed in many
403 cancers, including AML and has a significant role in regulating differentiation, proliferation and
404 invasion of cancer cells, making it an attractive target for AML treatment.⁴¹ Speranzini *et al*¹⁷ reported
405 non-covalent inhibition of LSD1/KDM1A by polymyxin antibiotics, which supported a serendipitous
406 observation by Vertsovsek *et al*,¹⁶ that polymyxin B was cytotoxic to a range of cancer cell lines,
407 including the human acute lymphocytic leukemia cell line, REH (IC₅₀ = ≥20 µg/mL).

408

409 Here, we showed that colistin sulfate was less cytotoxic to leukemia cell lines than a normal
410 kidney cell line. HK-2 cells were used as non-cancerous cells for comparison due to their consistent
411 response to dextrin-colistin conjugates in previous studies.²⁵ However, it would also be useful, in
412 future studies, to perform these experiments in CD34+ cells from healthy donors to further
413 substantiate our findings in leukemia and healthy kidney cells. When Speranzini *et al*¹⁷ treated MV-
414 4-11 cells with colistin, they did not observe any significant alteration in cell growth or H3-Lys⁴/H3-
415 Lys⁹ methylation. However, this study used a relatively low concentration of colistin sulfate (1.2
416 µg/mL, 0.85 µM), given that our study found an IC₅₀ of this agent in the same cells to be 888 µg/mL
417 (768.5 µM). In contrast, other LSD1 inhibitors have shown substantially higher activity towards AML
418 cell lines, for example, tranylcypromine-based compounds developed by Fioravanti *et al* had IC₅₀
419 values of 0.4 and 2.5 µM in MV-4-11 cells (48 h incubation)⁴² and the LSD1 inhibitor, IMG-7289 had
420 an IC₅₀ values of 0.007 µM in the same cell line (48 h incubation).⁴³

421 Speranzini *et al* hypothesized that colistin was unable to cross the plasma membrane
422 efficiently, however, we showed here that fluorescently-labelled colistin was rapidly endocytosed by
423 all 3 leukemia cell lines tested, with compelling evidence of further traffic to lysosomes. Contrary to
424 our initial hypothesis, however, dextrin conjugation reduced internalization of colistin by leukemia
425 cells, but did not alter its intracellular localization or promote nuclear localization.

426 Although all leukemia cell lines tested had similar mRNA expression of KDM1A mRNA
427 expression (summarized in Table S1)⁴⁴, the relative cytotoxicity of colistin sulfate or dextrin-colistin
428 conjugates varied between the leukemia cell lines tested; MV-4-11 showing the largest reduction in

429 IC₅₀ for DC8/1 (4-fold lower). Cellular uptake of fluorescently-labelled compounds were similar for all
430 three leukemia cell lines tested, suggesting that inconsistent internalization across the cell lines was
431 not the cause of sensitivity differences.

432 When dextrin was conjugated to colistin, variable effects on cytotoxicity were observed, which
433 were dependent on dextrin chain length, degree of succinylation and type of linker used. The most
434 potent effect seen in leukemia cell lines was from dextrin-colistin conjugates containing 8,000 g/mol
435 dextrin, with 1 mol% succinylation and attached to colistin by an amide bond (DC8/1). Although
436 ester-linked conjugates showed similar potency, when their effects were compared to dextrin content
437 of the conjugate, they caused less apoptosis than the amide-linked conjugates. Varache *et al*⁴⁵ have
438 previously demonstrated that increasing the degree of dextrin succinylation typically leads to a
439 greater number of linkers bound to colistin; corresponding to the conjugate's antimicrobial activity.
440 Even after degradation by amylase, residual linker groups, with differing lengths of glucose units
441 attached, prevent complete restoration of colistin activity.

442 Compared to the amide-linked dextrin-colistin conjugates, colistin sulfate and ester-linked
443 dextrin-colistin conjugates induced less annexin V binding and caspase 3/7 dependent cell death.
444 This suggests that the residual linker groups remaining attached to colistin may play a key role in the
445 activation of caspase 3/7 apoptosis.³⁰ Conjugation efficiency of ester-linked conjugates was generally
446 lower than those containing amide linkers, meaning that conjugates contained more unreacted
447 dextrin. The presence of free dextrin in polymer conjugates has been previously reported.⁴⁶ Removal
448 of unreacted dextrin using size exclusion chromatography is challenging, since its size is relatively
449 similar to the dextrin-colistin conjugate. At the concentrations used for antibacterial activity (typically
450 2-16 µg colistin base/mL), the succinoylated dextrin concentration contained in the conjugates is not
451 toxic. However, to achieve the high concentrations of colistin required to cause cell death in leukemia
452 cells lines, the dextrin content of conjugates exceeded safe levels and masked the effects of colistin.

453 The mechanism of polymyxin nephrotoxicity has been widely studied and is related to colistin's
454 interaction with oxidative stress and death-related pathways such as necrosis, apoptosis and
455 autophagy.⁴⁷ Here, necrosis, but not apoptosis, was the main mode of cell death by colistin sulfate in

456 both, kidney and leukemia cell lines. However, DC8/1 caused caspase-dependent apoptosis in
457 leukemia cells, suggesting that this chemical modification of colistin alters the mechanism of cell
458 death. Induction of annexin V binding, and thus apoptosis, occurred within a few hours of exposure
459 to DC8/1, and cell death was extensive by 24 h, suggesting that the mechanism of cytotoxicity may
460 not be mediated through induction of differentiation, for example, by LSD1 inhibition. Preliminary
461 studies found no evidence of upregulation of CD11b or CD86 following 48 h treatment with DC8/1
462 and 24 h recovery (data not shown).

463 Whilst at higher concentrations, some of the dextrin-colistin conjugates caused an apparent
464 increase in DNA content, subsequent analysis did not show any cell cycle disruption. As a
465 characterized efflux pump substrate (e.g. ATP-binding cassette (ABC) transporter proteins) we
466 propose that the increased Hoechst 33342 uptake that was greatest for dextrin-colistin conjugates
467 containing 10 mol% succinoylated dextrin, may be due to altered activity or expression of these
468 proteins in the leukemia cell lines, rather than G2 cell cycle accumulation. Cell cycle disruption has
469 been linked to LSD1/KDM1A inhibition, for example, Nicosia *et al*⁴⁸ demonstrated cell cycle inhibition,
470 via downregulation of a LSD1/KDM1A target in the acute promyelocytic leukemia cell line, NB-4.
471 Interestingly, although none of the colistin treatments tested here altered the cell cycle in leukemia
472 cells, Dai *et al*⁴⁷ did observe cell cycle arrest in a murine nephrotoxicity model and Eadon *et al* found
473 that homogenized kidney tissue of mice who were administered colistin had altered expression of
474 genes that regulate the cell cycle,⁴⁹ which suggests a tissue-specific effect. Reduced colony forming
475 ability of leukemia cell lines correlated with cell viability at 24 h, suggesting that colony forming cells
476 were not preferentially targeted by either colistin sulfate or the dextrin-colistin conjugates.

477 Here, we were unable to establish the exact mechanism of enhanced cytotoxicity of DC8/1 and
478 we did not observe any evidence of LSD1/KDM1A inhibition. However, Smitheman⁵⁰ observed peak
479 LSD1/KDM1A inhibition of leukemia cell lines (including MV-4-11 and THP-1) treated with sub-lethal
480 concentrations of LSD1/KDM1A inhibitors (GSK2879552 and GSK-LSD1) at 3-6 days, with no
481 evidence of apoptosis. Colony formation results suggested that the main impact on functionality of

482 surviving cells occurred within 24 h of treatment, which supports the timescales used in our
483 cytotoxicity experiments.

484 **Clinical potential of dextrin-colistin conjugates to treat AML**

485 Drug repurposing (also known as drug repositioning, reprofiling or re-tasking) is a process of
486 identifying new uses for existing drugs beyond the scope of the original medical indication.⁵¹ The
487 advantages of this approach include lower risk of failure, shorter time to market and reduced drug
488 development costs.⁵² Recently, several antibiotics have been repurposed to treat cancer.⁵³ For
489 example, levofloxacin was shown to inhibit proliferation and induce apoptosis of lung cancer cells in
490 preclinical studies⁵⁴ and doxycycline has shown anti-proliferative and pro-apoptotic activity in cervical
491 cancer and breast cancer cell lines.^{55,56} Of the 371 drugs currently being evaluated in clinical trials as
492 potential treatments for various cancers, 10 are antibiotics.⁵⁷ Nevertheless, antibiotic repurposing
493 remains controversial, due to the potential impact on the development of antimicrobial resistance
494 (AMR),⁵⁸ which correlates strongly with increased consumption.^{59,60} AML is an aggressive condition
495 that requires rapid and effective treatment that can last for several years. Prolonged antibiotic use
496 may induce drug resistance among gut microbes or create antibiotic resistance that could reduce the
497 effectiveness of treatments for common infections or render the patients more prone to infection.

498 When intravenous colistin (usually administered as the prodrug, colistimethate sodium (CMS))
499 is used to treat patients with Gram-negative bacterial infections, a target average colistin plasma
500 concentration at steady-state ($C_{ss,avg}$) of 2 mg/L is usually used,⁶¹ corresponding to the minimum
501 inhibitory concentration clinical breakpoint for several Gram-negative pathogens. However, at plasma
502 colistin concentrations of >2.5 mg/L, the risk of concentration-dependent nephrotoxicity increases
503 substantially.^{62,63} In agreement with previous studies,¹¹ dextrin-colistin conjugates were ~3-fold less
504 toxic to the kidney cell line. While this is a key development in tackling the cytotoxicity of colistin to
505 normal tissues, ultimately, it is probably unfeasible that the IC_{50} values seen for colistin and dextrin-
506 colistin conjugates in leukemia cell lines (exceeding 235 mg/L) could be achieved clinically without
507 causing severe nephrotoxicity. Although therapeutic concentrations of dextrin-colistin conjugate are

508 unlikely to be achieved clinically, this approach could be applied to modify other, more potent, non-
509 antibiotic therapeutics that have shown promise for the treatment of AML to reduce toxic side effects
510 and boost potency.

511 **Conclusion**

512 This study has demonstrated a potentiation of colistin's anti-leukemic activity by covalent, irreversible
513 attachment of dextrin, which was unlikely to be dependent on LSD1/KDM1A. Our results show that,
514 despite cellular uptake being effectively reduced by dextrin attachment, polymer conjugation can
515 enhance the biological activity of a drug, alter its mechanism of action and localize drugs to
516 endolysosomes. Whilst clinical translation of dextrin-colistin conjugates for the treatment of AML is
517 unlikely due to the potential to promote AMR and the relatively high colistin concentrations required
518 for anticancer activity *in vivo*, the ability to potentiate the effectiveness of an anticancer drug by
519 polymer conjugation, while reducing side effects and improving biodistribution of the drug, is very
520 attractive, and this approach warrants further investigation. The future design of polymer conjugates
521 bearing alternative LSD1/KDM1A inhibitors should consider incorporating specific ligands or
522 antibodies to enhance endocytosis and organelle targeting. Future studies should also include toxicity
523 testing in CD34+ cells from healthy human donors.

524 **Funding**

525 This research was funded in whole, or in part, by the Wellcome Trust [204824/Z/16/Z]. For the
526 purpose of open access, the author has applied a CC BY public copyright license to any Author
527 Accepted Manuscript version arising from this submission. This research was also funded by Cardiff
528 University and UK Medical Research Council (MR/N023633/1).

529 **Acknowledgments**

530 This article is dedicated to the memory of Dr Konrad Beck, who presented the idea of investigating
531 dextrin-colistin conjugates as an LSD1 inhibitor to treat cancer. The authors acknowledge Miss Gilda
532 Pourbahram and Miss Amanda Gilbert for their contributions to literature review and method
533 development, respectively. We also acknowledge the technical cell culture support provided by Dr
534 Sarah Youde and Dr Maria Stack and thank Dr Catherine Naseriyan from Central Biotechnology
535 Services, Cardiff University for their technical expertise in FACS.

536 **Disclosure**

537 The author reports no conflicts of interest in this work. The funders had no role in the design of the
538 study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the
539 decision to publish the results. This paper has been uploaded to bioRxiv as a preprint:
540 <https://www.biorxiv.org/content/10.1101/2023.11.09.565276v1>.

541 **Supplementary materials**

542 The following supporting information can be downloaded at <https://www.dovepress.com/>.

543 **Data Sharing Statement**

544 All the data contained in the article is available upon reasonable request from the corresponding
545 author.

546 **Author Contributions**

547 All authors made a significant contribution to the work reported, whether that is in the conception, study
548 design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting,
549 revising or critically reviewing the article; gave final approval of the version to be published; have agreed

550 on the journal to which the article has been submitted; and agree to be accountable for all aspects of the
551 work.

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716

717 Tables

718 **Table 1** Summary of the properties of the dextrin-colistin conjugates used in this study

Compound	Dextrin/ succinylation	Abbreviation	Mw ^a (g/mol) (Mw/Mn)	Colistin content (% w/w)	Molar ratio of dextrin (per colistin)
Dextrin-amide- colistin conjugate	8,000 g/mol, 1 mol%	DC8/1	11,500 (2.3) 13,800 (2.3)	4.89 6.03	3.65 2.93
	8,000 g/mol, 10 mol%	DC8/10	35,000 (3.2) 39,500 (3.7)	14.21 16.41	1.13 0.96
	51,000 g/mol, 1 mol%	DC51/1	49,000 (1.8) 48,200 (2.9)	6.08 2.21	0.43 1.22
	51,000 g/mol, 10 mol%	DC51/10	52,500 (2.4) 59,000 (2.6)	13.07 12.41	0.18 0.19
Dextrin-ester- colistin conjugate	8,000 g/mol, 1 mol%	DC8/1e	7,200 (2.0)	2.50	7.32
	8,000 g/mol, 10 mol%	DC8/10e	19,200 (4.9)	3.24	5.61

719 ^a relative to pullulan standards, using TSK G4000PW_{XL} and G3000PW_{XL} columns in series.

720 **Abbreviations:** g/mol, gram per mole; mol, mole; Mw, weight average molecular weight; g/mol,
721 gram per mole; Mn, number average molecular weight; Mw/Mn, polydispersity; w/w, weight in
722 weight; DC, dextrin-colistin; e, ester

723

724

725 **Table 2** Half maximal inhibitory concentration (IC₅₀) values and fold-change (CTB assay) of colistin
 726 sulfate and dextrin-colistin conjugates after 24 h incubation

Compound	IC ₅₀ (µg/mL colistin base, ± SD) ^a			
	THP-1	TF-1	MV-4-11	HK-2
Colistin sulfate	500 ± 89	670 ± 17	888 ± 37	447 ± 67
DC8/1	396 ± 13 (0.8)	315 ± 57 (0.5)	239 ± 19 (0.3)	653 ± 78 (1.5)
DC8/10	>1000	>1000	>1000	>1000
DC51/1	724 ± 161 (1.5)	819 ± 2 (1.2)	880 ± 64 (1.0)	638 ± 19 (1.4)
DC51/10	>1000	>1000	>1000	>1000

727 ^a Values in parentheses correspond to fold-change, compared to colistin sulfate.

728 **Abbreviations:** IC₅₀, half-maximal inhibitory concentration; µg/mL, microgram per milligram; SD,
 729 standard deviation; DC, dextrin-colistin

730

731

732 **Table 3** Characteristics of AF594-labelled conjugates

Compound	Degree of labelling (moles AF594 per mole colistin/ dextrin/ dextrin-colistin conjugates)	Labelling efficiency (µg AF594/mg conjugate)
Colistin-AF594	0.273	135.12
Dextrin (8 kDa, 10 mol%)-AF594	0.026	2.79
DC8/1-AF594	0.036	2.29
DC8/10-AF594	0.092	2.11

733 **Abbreviations:** AF594, AlexaFluor 594; µg, microgram; mg, milligram, kDa, kilodalton; DC, dextrin-

734 colistin

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736

737 **Figures**

738 **Figure 1.** Hemolysis of rat erythrocytes following incubation for 24 h with colistin sulfate and dextrin-
739 colistin conjugates at 37°C. Data is expressed as mean % Triton X-100 control \pm 1SD, n = 6,
740 where * indicates significance $p < 0.05$, ** indicates significance $p < 0.01$, *** indicates significance
741 $p < 0.001$ and **** indicates significance $p < 0.0001$ compared to colistin sulfate. Where significance is
742 not shown, $p > 0.05$ (ns, not significant).

743

744 **Figure 2.** Detection of resazurin reduction (metabolic activity), LDH leakage (cell-membrane
745 integrity, necrosis), caspase 3/7 activity (apoptosis) and Hoechst 33342 staining (DNA content)
746 under multiplex conditions of (a,c,e,g) HK-2 and (b,d,f,h) TF-1 cells incubated for 24 h with colistin
747 sulfate and dextrin-colistin conjugates. Data represent mean (\pm 1SD, n =3). Where error bars are
748 invisible, they are within size of data points.

749

750 **Figure 3.** Panels (a) and (b) show detection of resazurin reduction (metabolic activity) and caspase
751 3/7 activity (apoptosis), respectively, under multiplex conditions of MV-4-11 cells incubated for 24 h
752 with colistin sulfate and dextrin-colistin conjugates containing amide or ester linkers. Data represent
753 mean (\pm 1SD, n =3). Panels (c) and (d) show time course of annexin V binding and LDH activity
754 (necrosis), respectively, during incubation of MV-4-11 cells with colistin sulfate or ester- and amide-
755 linked dextrin-colistin conjugates at 750 μ g/mL colistin base (\pm 1SD, n=3). Where error bars are
756 invisible, they are within size of data points.

757

758 **Figure 4.** Cell cycle analysis of (a) MV-4-11 cell, and colony-forming ability of (b) MV-4-11 and (c)
759 THP-1 cells following incubation with colistin sulfate or dextrin-colistin conjugates at IC_{50} for 24 h.
760 Data in panel (a) are presented as mean % gated population (\pm 1SD, n=8). Data in panels (b) and
761 (c) are presented as mean % CFU, relative to control (n = 2). ** indicates significance $p < 0.01$,

762 compared to untreated control. Where significance is not shown, $p>0.05$ (ns, not significant). Panels
763 (b) and (c) do not show error bars as data is $n=2$.

764

765 **Figure 5.** Binding and internalization of AF594 (control) and AF594-labelled colistin, succinoylated
766 dextrin (8k, 10 mol%) and dextrin-colistin conjugates by MV-4-11 cells after 1 h incubation at 4°C
767 and 37°C. Panels (a-d) show histograms for cell-associated fluorescence at 4°C ((a) and (c)) and
768 37°C ((b) and (d)). Panels (a) and (b) show comparative uptake of DC8/10 compared to AF594-
769 labelled colistin and dextrin alone. Panels (c) and (d) show a second experiment comparing the up-
770 take of DC8/1 vs. DC8/10, with appropriate controls. Panel (e) shows cell-associated fluorescence
771 at 37°C (total association) and 4°C (external binding) of AF594-labelled colistin, dextrin and dextrin-
772 colistin conjugates ($\pm 1SD$, $n = 6$). Where **** indicate significance $p<0.0001$, and ns (not
773 significant) indicates $p>0.05$, compared to colistin-AF594.

774

775 **Figure 6.** Uptake of Colistin-AF594, DC8/10-AF594 and Dextrin-AF594 (8k/10%) following a 2 h
776 pulse and either 2 h (short) or 16 h (long) chase in MV-4-11 cells. Dextran-AF488 (green) was used
777 to identify late endolysosomes and Hoechst 33342 (blue) was used as a nuclear marker. Scale bar
778 shows 10 μm . Arrows indicate colocalised cargo and lysosomal dextran, solid arrow heads indicate
779 non-colocalised cargo and hollow arrow heads indicate non-colocalised lysosomal dextran. Image
780 intensities have been adjusted so that differences in colocalisation can be more evenly compared.
781 All images are single sections.

782

783 **Figure 7.** Uptake of colistin-AF594, DC8/1-AF594 and DC8/10-AF594 following a 2 h pulse and
784 either 2 h (short) or 16 h (long) chase in MV-4-11 cells. Dextran-AF488 (green) was used to identify
785 late endolysosomes and Hoechst 33342 (blue) was used as a nuclear marker. Scale bar shows
786 10 μm . Arrows indicate colocalised cargo and lysosomal dextran, solid arrows indicate non-
787 colocalised cargo and hollow arrow heads indicate non-colocalised lysosomal dextran. Image

788 intensities have been adjusted so that differences in colocalisation can be more evenly compared.

789 All images are single sections.