Development and Validation of an *In Vitro* Pharmacokinetic/Pharmacodynamic Model To Test the Antibacterial Efficacy of Antibiotic Polymer Conjugates

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This study describes the use of a novel, two-compartment, static dialysis bag model to study the release, diffusion, and antibacterial activity of a novel, bioresponsive dextrin-colistin polymer conjugate against multidrug resistant (MDR) wild-type *Acinetobacter baumannii*. In this model, colistin sulfate, at its MIC, produced a rapid and extensive drop in viable bacterial counts ($<2 \log_{10}$ CFU/ml at 4 h); however, a marked recovery was observed thereafter, with regrowth equivalent to that of control by 48 h. In contrast, dextrin-colistin conjugate, at its MIC, suppressed bacterial growth for up to 48 h, with $3 \log_{10}$ CFU/ml lower bacterial counts after 48 h than those of controls. Doubling the concentration of dextrin-colistin conjugate (to $2 \times$ MIC) led to an initial bacterial killing of $3 \log_{10}$ CFU/ml at 8 h, with a similar regrowth profile to $1 \times$ MIC treatment thereafter. The addition of colistin sulfate ($1 \times$ MIC) to dextrin-colistin conjugate ($1 \times$ MIC) resulted in undetectable bacterial counts after 4 h, followed by suppressed bacterial growth ($3.5 \log_{10}$ CFU/ml lower than that of control at 48 h). Incubation of dextrin-colistin conjugates with infected wound exudate from a series of burn patients ($n = 6$) revealed an increasing concentration of unmasked colistin in the outer compartment (OC) over time (up to 86.3% of the initial dose at 48 h), confirming that colistin would be liberated from the conjugate by endogenous α -amylase within the wound environment. These studies confirm the utility of this model system to simulate the pharmacokinetics of colistin formation in humans administered dextrin-colistin conjugates and further supports the development of antibiotic polymer conjugates in the treatment of MDR infections.

In an attempt to meet the challenge of the global epidemic of antibiotic-resistant infections, treatment strategies are increasingly employing nanomedicine-based approaches, which have been used in the treatment of a number of human diseases with considerable success (1, 2). The adoption of these approaches as antimicrobial therapies has seen the preclinical development of novel agents, including antibiotic-bearing nanoparticles, liposomes, dendrimers, and polymer therapeutics (3, 4). These agents may offer potential benefits over conventional systemic delivery in minimizing toxicity and overcoming drug resistance by affording selective targeting of systemically delivered antibiotics to sites of infection and inflammation, thus maximizing local bioavailability of the chemotherapeutic agent (3, 5). The employment of polymer therapeutics, in which an antibiotic is covalently attached to a water-soluble polymer, may afford a number of important practical advantages, including increased solubility and bioavailability, decreased toxicity, and a chemically modifiable controlled-release system that can be truly customized (2, 4, 6).

In this respect, we recently described a nanoantibiotic polymer therapeutic, based on dextrin conjugated to colistin, whereby colistin is controllably released from the conjugate by amylase-triggered degradation of dextrin, according to the polymer masked-unmasked protein therapy (PUMPT) principle (7), and in so doing, reinstates the antibiotic activity of colistin (8, 9). These dextrin-colistin conjugates demonstrated antibacterial activity across a range of Gram-negative bacteria that was comparable to colistin methanesulfonate (CMS) but exhibited reduced *in vitro* and *in vivo* toxicity and prolonged plasma half-life.

In the preliminary design and development of any novel chemical entity, it is pivotally important to understand its pharmacokinetic/pharmacodynamic (PK/PD) profile before moving to *in vivo*

efficacy studies. This is particularly important in nanoantibiotic delivery systems, as the incorporation of conventional small molecules into nano-sized structures is associated with PK/PD properties that are substantially altered from the original antibiotic (10, 11). However, the complex release or formation of drug from nanoantibiotics renders conventional PK/PD models inappropriate in the preclinical development of these controlled-release systems (10, 11). Conventional single-compartment static models, while extensively utilized to study the effect of fixed antibiotic doses on fixed bacterial loads (12, 13), are typically viable for only ≤ 24 h (14, 15) and are of limited value in investigating drug release and the real-time antimicrobial effects of controlled-release drugs (16, 17). Single-compartment time-to-kill (TTK) models are also unable to prevent denaturation of the macromolecule's activating enzyme (e.g., α -amylase) by artificial media used to sustain bacterial growth (16, 17). Multiple-chamber dynamic

Received 23 June 2014 Returned for modification 31 August 2014

Accepted 7 December 2014

Accepted manuscript posted online 15 December 2014

Citation Azzopardi EA, Ferguson EL, Thomas DW. 2015. Development and validation of an *in vitro* pharmacokinetic/pharmacodynamic model to test the antibacterial efficacy of antibiotic polymer conjugates. *Antimicrob Agents Chemother* 59:1837–1843. doi:10.1128/AAC.03708-14.

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doi:10.1128/AAC.03708-14

FIG 1 Schematic illustration of the two-compartment static dialysis bag PK/PD model under infinite sink conditions (a) and the hypothesized distribution of dextrin-colistin conjugates (b).

models may be used to produce concentration gradients, although they require considerable technical expertise and specialist software and are associated with high costs (18). In the development and screening of nanoantibiotics, a need thus exists for a suitable *in vitro* model to define the PK/PD profiles and optimize the characteristics of these novel agents prior to *in vivo* testing.

This study employed dextrin-colistin conjugates as prototypical nanoantibiotics to develop a model system which would afford the opportunity to study the *in vitro* degradation, diffusion, and activation of the conjugate alone and in the presence of amylase or infected wound fluid. The study sought to define an *in vitro* model in which the PK/PD parameters of biodegradable antibiotic polymer conjugates can be reliably assessed.

MATERIALS AND METHODS

Materials. Colistin sulfate, ethanol, human salivary α -amylase, 4-dimethylaminopyridine (DMAP), anhydrous *N,N*-dimethylformamide (DMF), succinic anhydride, diethyl ether, type 1 dextran from cornstarch (molecular weight, $\sim 8,100$ g/mol), and carbonic anhydrase (molecular weight, 29,000 g/mol) were purchased from Sigma-Aldrich (Poole, United Kingdom). Sodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, potassium chloride, sodium hydroxide (NaOH), copper (II) sulfate pentahydrate, bicinechonic acid (BCA) assay kit, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Thermo Fisher Scientific (Loughborough, United Kingdom). Sterile 0.9% sodium chloride solution was obtained from Baxter (Berkshire, United Kingdom). *N*-hydroxysulfosuccinimide (sulfo-NHS) was purchased from Fluka (Buchs, Switzerland). Trypticase soy agar (TSA), Trypticase soy broth (TSB), and cation-adjusted Mueller-Hinton broth (CAMHB, lot 43145) were purchased from Oxoid (Basingstoke, United Kingdom). Polyvinylidene fluoride sterile membrane syringe filters (0.22 μ m) were from Elkay (Basingstoke, United Kingdom). Tegaderm semipermeable dressings were purchased from 3M Healthcare (Neuss, Germany). Sterile graduated plastic syringes were from BD Plastipak (Madrid, Spain). The Phadebas amylase test was from Magle Life Sciences (Lund, Sweden), and the MaxSignal colistin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bioo Scientific Corp. (Austin, TX, USA). All chemicals were of analytical grade.

Escherichia coli (*E. coli*) National Collection of Type Cultures (NCTC)

10418 and *Acinetobacter baumannii* 7789 (v19) from clinically infected wounds (19) were donated by R. Howe (Public Health Wales Microbiology Laboratory, University Hospital of Wales, Cardiff, United Kingdom). The sensitivities of these strains were reported previously (19). MIC values of *A. baumannii* 7789 were previously reported as 0.0625 and 8 μ g/ml for colistin sulfate and dextrin-colistin conjugate (plus amylase), respectively (20).

Synthesis and characterization of dextrin-colistin conjugates. Dextrin-colistin conjugates were synthesized and characterized as previously described (8, 9). The dextrin-colistin conjugate used in these studies contained dextran with 1.0 mol% succinylation and had a molecular weight of $\sim 10,300$ g/mol (gel permeation chromatography [GPC] with pullulan standards) and a colistin content of 11.2% wt/wt (BCA assay) with $<3\%$ free colistin (fast protein liquid chromatography [FPLC]).

***In vitro* PK/PD model.** The unmasking of dextrin-colistin conjugates was simulated using a two-compartment static dialysis bag model under sink conditions (total volume, 20 ml; inner-compartment volume, 5 ml), as shown schematically in Fig. 1. Dialysis membrane (10,000 g/mol molecular weight cutoff [MWCO]) was presoaked for 15 to 30 min in distilled water (dH_2O), secured with dialysis clips, and suspended from an injection port to separate the inner compartment (IC) from the outer compartment (OC) in a sterilized 25-ml beaker sealed with a sterile medical-grade polyurethane membrane (Tegaderm). The model system was prepared under aseptic conditions in a class 2 laminar airflow cabinet and transferred to a shaking incubator set at 37°C in ambient air and constant orbital agitation at 70 rpm for 48 h.

Validation of *in vitro* PK/PD model. To validate the ability of the dialysis membrane to retain intact dextrin-colistin conjugate in the IC while permitting unrestricted diffusion of unmasked colistin to the OC, the protein content in both compartments was measured over time. Briefly, the IC was spiked with dextrin-colistin conjugate (equivalent to 10 μ g/ml colistin) in phosphate-buffered saline (PBS; pH 7.4, 37°C) in the absence and presence of α -amylase (100 IU/liter), while the OC contained sterile PBS (15 ml). The sealed beaker was incubated as described previously. Samples (150 μ l) were extracted from each compartment at various intervals (0, 4, 8, 12, 24, 36, and 48 h) using sterile, medical-grade, single-use vascular catheters and immediately frozen on dry ice and stored at -20°C prior to analysis. Protein content was determined using a standard BCA assay, and the antimicrobial susceptibility of *E. coli* NCTC 10418 isolates was assessed using an MIC assay (see below). At the end of the 48

h of incubation, FPLC was used to estimate the proportion of unmasked colistin in both compartments by calculating the area under the chromatographic curve. At the end of the experiment, the integrity of the dialysis membrane to α -amylase was confirmed using carbonic anhydrase as a molecular weight marker. The contents of both the IC and OC were removed. The dialysis membrane was flushed with several volumes of sterile dH_2O and then resuspended in sterile PBS as described previously. The IC was spiked with carbonic anhydrase ($2\text{ }\mu\text{g/ml}$) in PBS. After a further 17 h of incubation with PBS in the OC, the ratio of carbonic anhydrase in both compartments was quantified by UV-visible (UV-vis) spectrophotometry ($n = 3$).

Susceptibility testing. Antimicrobial activity in the OC was measured in *E. coli* NCTC 10418 isolates in a standard MIC assay (21). Isolates were grown overnight in TSB and then diluted in sterile PBS to an optical density at 625 nm (OD_{625}) of between 0.08 and 0.10 (equivalent to $0.5\text{ McFarland standard}$; $\sim 10^8\text{ CFU/ml}$). Serial 2-fold dilutions were prepared for each sample in flat-bottom 96-well microtiter plates ($100\text{ }\mu\text{l/well}$). The diluted bacterial cultures were then diluted 10-fold in CAMHB, and $5\text{ }\mu\text{l}$ was added to each well of the microtiter plate. Plates were incubated at 37°C in ambient air for 18 to 20 h, and the lowest concentration at which there was no visible growth was taken as the MIC. In addition, on each plate, triplicate wells were prepared containing no antibiotic and no bacterial inoculum as growth and sterility controls, respectively.

Time-to-kill assay. A modified TTK assay was used to investigate the concentration- and time-dependent antimicrobial activity of colistin sulfate, dextrin-colistin conjugate, and a mixture of colistin sulfate plus dextrin-colistin conjugate against *A. baumannii* 7789. In these experiments, the OC contained $\sim 5\text{ log}_{10}\text{ CFU/ml}$ of *A. baumannii* 7789 in MHB, and the IC contained α -amylase (100 IU/liter) in sterile PBS in combination with (i) colistin sulfate ($0.25\text{ }\mu\text{g/ml}$, $1\times\text{ MIC}$), (ii) dextrin-colistin conjugate ($32\text{ }\mu\text{g/ml}$ colistin equivalent, $1\times\text{ MIC}$), (iii) dextrin-colistin conjugate ($64\text{ }\mu\text{g/ml}$ colistin equivalent, $2\times\text{ MIC}$), or (iv) a mixture of colistin sulfate ($0.25\text{ }\mu\text{g/ml}$, $1\times\text{ MIC}$) and dextrin-colistin conjugate ($32\text{ }\mu\text{g/ml}$ colistin equivalent, $1\times\text{ MIC}$). Colony counts (CFU/ml) from samples taken as described previously were performed in triplicate according to the method of Miles et al. (22). Briefly, samples ($50\text{ }\mu\text{l}$) were diluted 10-fold in PBS and centrifuged. Pellets were resuspended in an equivalent volume of fresh PBS. Serial 10-fold dilutions were performed in triplicate across a 96-well plate. Samples ($5\text{ }\mu\text{l}$) from each dilution were dropped onto the surface of the dried TSA plates in triplicate. The TSA plates were left undisturbed for 30 min, inverted, incubated for 18 to 20 h (37°C in ambient air), and then visually inspected for growth. The drop position displaying the highest number of full-size discrete colonies (range, 2 to 20 colonies) was quantified and multiplied by the dilution factor. The resulting colony count (CFU/ml) was plotted versus the time to produce a TTK curve. Growth and sterility controls in the absence of antibiotic or *A. baumannii* 7789, respectively, were performed in triplicate.

Ex vivo sample collection. Following research ethics committee approval and informed consent, wound fluid samples were collected from adult infected burn wounds ($n = 6$) being treated at the Welsh Burns Centre (Swansea, United Kingdom). Burn wound infection was diagnosed according to the American Burn Association diagnostic criteria (23). Patients with recorded pancreatic or salivary disease were excluded. After dressing removal, the wounds were lightly washed with sterile 0.9% sodium chloride solution, and a sterile Teak graft board was used to collect the exudate. Samples were then transferred to a sterile syringe, sealed, frozen on dry ice, and stored at -80°C until required. Samples were centrifuged ($15,000\times g$ for 5 min at 4°C), and then the α -amylase content was determined in triplicate using a Phadebas amylase test according to the manufacturer's instructions.

In vitro modeling of dextrin-colistin conjugate unmasking in infected wound fluid. The same PK/PD setup was used to evaluate the feasibility of dextrin-colistin conjugate unmasking in infected burn wound samples. Each wound fluid sample was diluted 2-fold in PBS to make up the IC volume (5 ml), and dextrin-colistin conjugate ($64\text{ }\mu\text{g/ml}$

FIG 2 Distribution and antimicrobial activity of dextrin-colistin conjugates in a two-compartment dialysis bag model under infinite sink conditions. (a) Protein concentrations in the IC and OC over time (mean $\pm$ SD, $n = 3$). (b) The corresponding MIC for each time point as a measure of antimicrobial susceptibility (mode, $n = 3$).

colistin equivalent, $2\times\text{ MIC}$) was added. For each wound sample, the procedure was repeated in triplicate. Total colistin content in the OC was quantified by ELISA according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microtiter plate reader, and these values were used to calculate the colistin concentration in the OC (mean $\pm$ standard deviation [SD], $n = 3$) according to the calibration curve (assay detection range, 0.5 to 50 ng/ml). The proportion of unmasked conjugate in the OC was calculated using FPLC, as previously described.

RESULTS

Method validation. Protein concentration in the IC decreased in a logarithmic fashion over time, while protein concentration in the OC increased reciprocally (Fig. 2). A corresponding increase in antimicrobial activity of the OC over the 48 h of incubation was also observed (Fig. 2). FPLC demonstrated that by 48 h, significant unmasking had occurred ($P < 0.05$), with unmasked conjugate accounting for $\sim 68\%$ of total colistin content in the system (Fig. 3). At 48 h, there was no significant difference in the distribution of unmasked conjugate between the two compartments ($P > 0.05$). Correspondingly, there was a significantly higher proportion of high-molecular-weight (masked) dextrin-colistin conjugate in the IC ($\sim 80\%$; $P < 0.05$) than in the OC (Fig. 3b). When the experiment was conducted using dextrin-colistin conjugate in the absence of α -amylase, the OC protein content was $< 10\%$ after 17 h. Membrane integrity testing confirmed that the presence of carbonic anhydrase in the OC was always below the lower limit of quantification (data not shown).

FIG 3 Distribution of masked and unmasked dextrin-colistin conjugates at 48 h in a two-compartment dialysis bag model under infinite sink conditions. (a) The relative amounts of masked and unmasked dextrin-colistin conjugates in the system at 48 h. (b) The distribution of masked and unmasked dextrin-colistin conjugates in each compartment at 48 h. Data shown represent means $\pm$ SD ($n = 3$). *, Statistical significance ($P < 0.05$).

PK/PD modeling against *A. baumannii* 7789 isolates. Colistin sulfate, at its MIC (0.0625 $\mu\text{g/ml}$), showed rapid initial killing, and viable bacterial counts were below the assay's lower limit of quantification at 4 h. However, early and significant bacterial regrowth, to the level seen for controls, was observed from 8 h on (Fig. 4). Dextrin-colistin conjugate was bactericidal at $2\times$ MIC, where the maximum reduction in viable bacterial counts ($\sim 3 \log_{10}$ CFU/ml) occurred at 8 h. Thereafter, regrowth was slower than in the presence of colistin sulfate. In contrast, the colistin sulfate plus dextrin-colistin conjugate combination retained optimal characteristics of both components, with rapid initial bacterial killing, maximum reduction in viable bacterial counts at 4 h, and sustained reduction in viable bacteria throughout the experiment. The control growth curves confirmed that bacterial growth can be maintained under the experimental conditions, while sterility controls verified that sterility conditions were maintained throughout. Culture onto TSA plates at the end of each experiment confirmed the presence of a homogeneous culture.

α -Amylase activity in clinical samples. Wound fluid was collected from a consecutive series of six burn patients (Table 1). The total protein content in the supernatant was 50.7 ± 15.1 mg/ml (mean $\pm$ SD). These studies revealed that α -amylase activity was significantly increased in infected wound fluid (mean $\pm$ SD, 408.4 ± 168.3 IU/liter) compared to that in patient-matched serum (mean $\pm$ SD, 60.0 ± 25.3 IU/liter; $P < 0.05$) (Fig. 5).

Unmasking of dextrin-colistin conjugate in infected wound fluid. A time-dependent increase in colistin concentrations in the OC was observed in all cases (Fig. 6). Similarly, concentrations of colistin in the OC also increased in the presence of higher α -amylase activity, equating to a mean OC colistin content of 68.1% to 86.3% of the theoretical concentration of the original dose concentration at equilibrium (16 $\mu\text{g/ml}$) at 48 h.

DISCUSSION

We hypothesized that a static, two-compartment dialysis bag model under infinite sink conditions would offer a simple and cost-effective method for the initial PK/PD characterization of biodegradable antibiotic polymer conjugates. Using dextrin-colistin conjugate, we showed that the model can be employed to study

degradation, diffusion, and activity across a biological membrane. Moreover, we demonstrated how these processes may be modified by local enzyme activity at sites of infection.

The use of a two-compartment system permitted the discrete

FIG 4 TTK curves against *A. baumannii* 7789 clinical isolate. (a) Bacterial viability count following a challenge with colistin sulfate or dextrin-colistin conjugate in the presence of α -amylase (100 IU/liter). (b) Bacterial viability count following a challenge with dextrin-colistin conjugate at $2\times$ MIC or colistin sulfate plus dextrin-colistin conjugate in the presence of α -amylase (100 IU/liter). Data shown represent mean CFU values ($n = 3$). ●, growth control; ◇, colistin sulfate (1 $\times$ MIC); ▼, dextrin-colistin conjugate (1 $\times$ MIC); ×, dextrin-colistin (2 $\times$ MIC); □, colistin sulfate (1 $\times$ MIC) plus dextrin-colistin conjugate (1 $\times$ MIC). Lower limit of quantification, $2 \log_{10}$ CFU/ml.

TABLE 1 Basic demographic characteristics of patients included in the study

Parameter	Data
Etiology (<i>n</i>)	Domestic scald, 2; flame, 4
Mean age (yr [range])	50.6 (22–65)
Total body surface area burnt (% range)	5–90
Mean wt on admission (kg [range])	80.6 (65.0–105.4)
Predominant burn depth (<i>n</i>)	Partial thickness, 4; full thickness, 2
Mean $\pm$ SD time of sample collection postburn (days)	17 $\pm$ 6

sampling and detailed analysis of the individual compartments over time. Furthermore, this model enabled the composition of the two compartments to be modified to represent the environmental conditions of the disease of interest, as demonstrated here using simulated and real biological fluids containing physiological concentrations of α -amylase. The *ex vivo* model described here can easily be adapted to allow modeling of disease- and target site-specific levels of enzymatic activity in the assessment of enzyme-triggered pharmacokinetics and pharmacodynamics using alternative biological fluids (e.g., wound fluid or bronchoalveolar lavage fluid). The model utilized Spectra/POR 7 membrane to prevent nonspecific drug binding to the membrane and heavy metal contamination (24–26). The MWCO of the dialysis membrane serves as a size exclusion barrier and can be selected according to the molecular weight of the drug carrier, antibiotic, or activating enzyme. Thus, the dialysis membrane can also effectively prevent premature denaturation of the activating enzyme by artificial media in the OC. The dextrin-colistin conjugates studied here were activated by α -amylase-mediated degradation of dextrin, resulting in a reduction in its molecular weight and diffusion of the unmasked conjugate along a concentration gradient to the OC (Fig. 1), where bioactivity can be assayed under various conditions. In this regard, an IC-to-OC ratio of 1:4 vol/vol and a total volume of 20 ml in the system maintained infinite sink conditions, provided a concentration gradient, and prevented OC saturation with unmasked conjugate. The infinite sink strategy presented a convenient, cost-effective means of controlling localized drug concentrations (18, 27) and monitoring drug activation in the IC and the resulting bioactivity in the OC. Considering the controlled-release nature of the dextrin-colistin conjugate, it was essential to provide an incubation environment that resembled *in vivo* conditions, while ensuring bacterial viability. Therefore, 37°C was selected to mimic *in vivo* conditions; constant agitation reduced the risk of boundary-layer effects at the dialysis membrane (27) and contributed to the maintenance of bacterial growth. Indeed, this two-compartment model demonstrated colistin sulfate's rapid bacterial killing and the subsequent bacterial regrowth observed in previously validated one-compartment models (28–30).

The ability of the dialysis membrane to retain the intact conjugate within the IC until it is unmasked was confirmed during validation, where, in the absence of α -amylase, OC protein content remained at <10% at 17 h. The addition of α -amylase led to increases in the protein content and antimicrobial activity of the OC, which were mirrored by decreased bacterial counts in the TTK study and were in agreement with previous *in vitro* physicochemical data (8, 9).

The analysis of bacterial counts in the presence of colistin sulfate was interesting and showed that bacterial recovery occurred earlier than the shortest currently recommended dosing interval for colistin (31, 32), demonstrating the clinical benefit that these models may offer. In contrast, with the dextrin-colistin conjugates, the recovery of bacterial counts was significantly delayed, presumably due to the sustained release of colistin from dextrin-colistin conjugates by α -amylase. Since colistin is a concentration-dependent antibiotic (33) with a modest postantibiotic effect (34), the ability of the polymer conjugates to prolong exposure of bacteria to colistin may be more beneficial to antibacterial efficacy than the achievement of high peak concentrations (25, 35, 36).

Several concentration-dependent antibiotics, including azithromycin, amikacin, and ciprofloxacin, have been incorporated into controlled-release polymeric carriers, such as nanoparticles and liposomes (37–39), which can readily be tested in our *in vitro* system. For instance, Wang et al. (40) used a similar two-compartment model to characterize the *in vitro* release kinetics of colistin sulfate-loaded liposomes. In this study, a combination of colistin sulfate and dextrin-colistin conjugate provided a rapid decline in viable counts, followed by a sustained suppression of bacterial growth rate. The conjugate unmasking by α -amylase was shown to be time dependent, meaning that in clinical use, administration of an initial dose of conventional antibiotic may be required when treating acute disease. In addition to improving the therapeutic efficacy of nanoformulated drugs, incorporating antibiotics into nano-sized structures also converts conventional small-molecule antibiotics into macromolecules, which may benefit from passive size-based targeting and accumulation at sites of inflammation/infection via the enhanced permeability and retention (EPR) effect (4, 41). A potential limitation of this model is its inability to replicate the potential benefits of the EPR effect, representing an avenue for future development.

Modification of the IC to incubate dextrin-colistin conjugate directly in *ex vivo* infected wound fluid represented an adaptation of the PK/PD model to a higher level of complexity to enable *ex vivo* testing. In these studies, the α -amylase concentration in burn wound fluid was markedly higher than that in plasma. These results cannot be simply explained by the effects of thermal injury, as the mean time of sample collection was >2 weeks postburn, when vascular permeability and interstitial fluid leakage have largely

FIG 5 Patient-matched α -amylase activity in infected wound fluid (Phadebas assay) (mean $\pm$ SD, *n* = 3) and plasma from clinical reports. The average data set represents mean α -amylase activity in wound fluid and plasma samples (mean $\pm$ SD, *n* = 6). *, Statistically significant difference (*t* test, *P* < 0.05).

Sample	Amylase activity in undiluted wound fluid (IU/L)	Amylase activity in IC (IU/L)	Colistin concentration in OC at 48 h (µg/mL)	Calculated unmasked conjugate in OC (%)
A	710 ± 26	71	13.8 ± 0.7	86.3 (81.3-91.2)
B	322 ± 13	64	13.2 ± 0.5	82.5 (79.0-86.0)
C	256 ± 3.9	25.6	10.9 ± 1.3	68.1 (58.9-77.3)

FIG 6 Estimation of unmasked colistin release after incubation of masked conjugate in infected burn wound fluid. (a) OC colistin content over time. Data shown represent means ± SD (*n* = 3). (b) Wound fluid α-amylase concentration determined by the Phadebas assay and the final α-amylase activity in the IC after dilution. In the amylase activity in undiluted wound fluid and colistin concentration columns, values represent means ± SD (*n* = 3). In the calculated unmasked conjugate column, values represent mean unmasked dextrin-colistin conjugate (95% confidence interval) by FPLC.

subsided (42, 43). Incubation of dextrin-colistin conjugates in infected wound fluid demonstrated that colistin would be readily released from the conjugate locally at infected sites due to local α-amylase activity. The conjugate unmasking rate (68.1% to 86.2% at 48 h) compared favorably to published values for CMS, for which conversion to colistin in *ex vivo* human plasma samples after 48 h was ~60% (44).

Recent advances in the design and development of nanoantibiotics, with complex drug-release profiles, warrant the establishment of a novel PK/PD model for monitoring their drug release and antibiotic efficacy. This two-compartment model system permits the building in of increasing levels of complexity, providing a versatile tool to describe the effect of bioresponsive dextrin-colistin conjugates by characterization of *in vitro* drug release (in simulated and *ex vivo* biological fluids) and bacterial killing over time. It is anticipated that these results will assist in designing optimal dosing strategies for dextrin-colistin conjugates *in vivo*.

ACKNOWLEDGMENTS

The research work disclosed by E.A. in this publication was funded by the Strategic Educational Pathways Scholarship (Malta) and the European Social Fund.

E.A. acknowledges the Welsh clinical academic training scheme.

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