

Short Communication

Revisiting aminocoumarins for the treatment of melioidosis

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ABSTRACT

Burkholderia pseudomallei causes melioidosis, a potentially lethal disease that can establish both chronic and acute infections in humans. It is inherently recalcitrant to many antibiotics, there is a paucity of effective treatment options and there is no vaccine. In the present study, the efficacies of selected aminocoumarin compounds, DNA gyrase inhibitors that were discovered in the 1950s but are not in clinical use for the treatment of melioidosis were investigated. Clorobiocin and coumermycin were shown to be particularly effective in treating *B. pseudomallei* infection *in vivo*. A novel formulation with DL-tryptophan or L-tyrosine was shown to further enhance aminocoumarin potency *in vivo*. It was demonstrated that coumermycin has superior pharmacokinetic properties compared with novobiocin, and the coumermycin in L-tyrosine formulation can be used as an effective treatment for acute respiratory melioidosis in a murine model. Repurposing of existing approved antibiotics offers new resources in a challenging era of drug development and antimicrobial resistance.

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1. Introduction

Burkholderia pseudomallei, a Gram-negative, soil-dwelling bacterium, is the causative agent of the potentially fatal disease, melioidosis. Reports of *B. pseudomallei* being isolated from soil are now commonplace in subtropical climates, including Southeast Asia, Africa and South America, leading to concerns that the incidence of melioidosis will increase in the future [1,2].

Burkholderia pseudomallei can infect humans via two main routes of entry: (i) inhalation of contaminated particles; and (ii) wound site infection. It can then establish itself as either a chronic infection, potentially lasting for many years [3], or as an acute illness owing to bacteraemia. It is also able to disseminate in the host via the circulatory and lymphatic systems. Melioidosis is difficult to treat because *B. pseudomallei* has inherent resistance to many commonly used antibiotics, notably β -lactam compounds, fluoroquinolones and ciprofloxacin [4]. There is currently no effective vaccine that is safe for use in humans and it is estimated that melioidosis was responsible for more than 89 000 deaths in 2016 [5]. Treatment of the acute infection involves intravenous administration of the frontline antibiotic ceftazidime for up to 4 weeks, followed by secondary treatment with oral trimetho-

prim/sulfamethoxazole (co-trimoxazole) for a further 3 months to eradicate persistent intracellular bacteria [6].

Antibiotic resistance in *B. pseudomallei* is mediated by a formidable array of factors encoded by a large genome spread over two chromosomes. The thick polysaccharide capsule can resist complement, aminoglycosides and antimicrobial peptide stress [6]; efflux pumps can reduce susceptibility to chloramphenicol and macrolides; and overproduction of β -lactamases and deletion of penicillin-binding proteins can confer resistance to ceftazidime [6]. A recent study of the related species *Burkholderia cepacia* found that only around one-quarter of >2500 isolates from cystic fibrosis patients were susceptible to any treatment whatsoever, despite testing 23 different antibiotic combinations [7].

Novel, repurposed or reformulated antibiotics are required to provide more effective treatment options for *Burkholderia* infections. The current state of drug development is that no new classes of antibiotic effective against Gram-negative bacteria have been approved since 1962 [8]. Compounding this, many pharmaceutical companies are withdrawing from antibiotic development, in part due to unfavourable economic incentives [9]. In light of this, making better use of antibiotics that are already in clinical use represents a possible cost-effective and expeditious solution.

Aminocoumarins, which include novobiocin, coumermycin and clorobiocin, are a class of antibiotics that target the ATPase activity of the GyrB subunit of DNA gyrase and the ParE subunit of topoisomerase IV, disrupting DNA replication and transcription. They bind

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gyrase with greater affinity than quinolones, which target the GyrA subunit, and they additionally prevent ATP binding [10].

Novobiocin, also known as albamycin, was discovered in the 1950s derived from *Streptomyces niveus* and was shown to be active against *B. pseudomallei* in vitro, particularly in synergy with tetracycline [11]. Despite this early promise, there is a paucity of data on the use of aminocoumarins to treat melioidosis. Commonly cited reasons include their poor solubility as well as concerns over the potential emergence of drug resistance [12]. Efforts to improve the pharmacokinetics of these compounds have sometimes been accompanied by a loss of affinity for the bacterial target site, and such programmes have since been discontinued (reviewed by Bisacchi and Manchester [13]).

Given the high mortality associated with acute melioidosis (>70% without appropriate antibiotic treatment [5]), the difficulties with existing treatment options and the unrealised potential shown by aminocoumarins, we sought to revisit this class of compounds as a potential therapeutic agent against *B. pseudomallei* infection using two different in vivo models. In the current study, the efficacies of commercially available aminocoumarins were assessed and the *Galleria mellonella* (wax moth) larvae model was used to screen for effective formulations that could improve in vivo activity in a murine model. These findings were applied to demonstrate that coumermycin in equimolar solution with L-tyrosine significantly improves survival in a murine infection model of acute melioidosis.

2. Methods

2.1. *Galleria mellonella* infection assay

Different formulations were investigated to improve the bioavailability and efficacy of poorly soluble compounds, using novobiocin as a representative aminocoumarin. The compounds clorobiocin, coumermycin (A1) and novobiocin (all from Sigma-Aldrich, Gillingham, UK) were solubilised in dimethyl sulfoxide (DMSO) carrier at a stock concentration of 10 mg/mL. For injection into *G. mellonella* larvae, the stock solution was diluted in sterile saline to a final concentration of 1 mg/mL. Saline solutions were prepared with either sodium alginate, povidone or methylcellulose at different concentrations ranging from 0–1% [w/v sterile phosphate-buffered saline (PBS)]. Alternatively, and also in combination, the amino acids L-tyrosine, DL-tryptophan or casamino acids (Sigma-Aldrich) were added at various molar ratios relative to the antibiotic. Casamino acids contain all amino acids except tryptophan. For infection, five *Galleria* larvae per group of matching size were injected with ~150 CFU of *B. pseudomallei* in a 10 μ L volume in the uppermost right footpad. *Galleria* larvae were then incubated at 37 °C for 1 h. At this time, *Galleria* were injected in the uppermost left footpad with 10 μ L of antibiotic solution or saline control. *Galleria* larvae were then incubated for 24 h at 37 °C. To enumerate the bacterial load, haemolymph was drained into a sterile 1.5 mL Eppendorf tube. Then, 10 μ L of haemolymph was serially diluted in sterile PBS with 0.1% Tween 80 and was plated onto Luria–Bertani (LB) agar for CFU enumeration of ex vivo isolated *B. pseudomallei*.

2.2. Murine infection

Female BALB/c mice (Charles Rivers Laboratories International, Inc., Margate, UK) aged 6–8 weeks were used. Mice were housed under specific pathogen-free (SPF) conditions with free access to food and water. Animal work was performed in accordance with the Animals (Scientific Procedures) Act of 1986 and the local Ethical Review Committee, under animal biohazard Containment Level 3 conditions. All units were infected intranasally with a total of

500 CFU of *B. pseudomallei* K96243 by pipetting 25 μ L into each nostril. Treatment began at 6 h post-infection, followed by treatment once every 24 h for 4 consecutive days thereafter. Treatment comprised either sterile saline (negative control), 1200 mg/kg ceftazidime (intraperitoneal), 30 mg/kg coumermycin (subcutaneous) or 30 mg/kg coumermycin in solution with equimolar L-tyrosine (subcutaneous). In all cases, mice were checked at least daily for signs of illness and, if determined to have reached the humane endpoint specified in the Project Licence, were euthanised. The experiment was concluded at 30 days post-infection.

2.3. Pharmacokinetics

Pharmacokinetic studies were performed by Domainex (Saffron Walden, UK). Three BALB/c units per group were injected intraperitoneally with 15 mg/kg novobiocin or coumermycin \pm 0.7 mg/mL DL-tryptophan, formulated in 5% DMSO in 95% Kleptose® (8% w/v in PBS). Animals were housed in pre-assigned cages until sampling. Appropriate samples were taken at the defined time points and were stored immediately at –20 °C. Protein was precipitated with acetonitrile and then ultra-high performance liquid chromatography time-of-flight mass spectrometry (UHPLC-TOF/MS) using electrospray ionisation was used to quantify the compound concentration in plasma.

2.4. Statistical analysis

All data analysis was performed using GraphPad Prism 8.1.2 (GraphPad Software Inc., La Jolla, CA, USA). For comparison of the effect of different treatment conditions on the bacterial load in *Galleria* haemolymph, one-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparisons test compared with the untreated condition or unformulated novobiocin as indicated. For murine survival studies, log-rank (Mantel–Cox) test was performed for each treatment condition versus the saline control group.

3. Results and discussion

3.1. The aminocoumarin formulation significantly alters its in vivo activity

Historically, only limited assessment of aminocoumarin activity against *B. pseudomallei* infection in vivo has been performed. In the current study, two different in vivo models were chosen to assess the efficacy of a group of aminocoumarin compounds, initially using *G. mellonella* larvae to screen the effect of different drug formulations before proceeding to an experimental model of acute melioidosis using a murine infection model. Use of *G. mellonella* larvae is an increasingly popular method for host–pathogen virulence studies as well as for screening drug efficacy and safety. They represent a more complex model than any single cell line since they possess multiple different cell types, tissue differentiation and serum factors including host defence peptides and complement-like proteins [14,15]. They are particularly valuable not as a complete replacement of murine studies but in reducing the size of those experiments by defining experimental conditions and testing hypotheses a priori.

To assess compound activity in vivo, *G. mellonella* larvae were treated 1 h after infection with ~150 CFU of *B. pseudomallei*. Novobiocin, whilst highly active in vitro [minimum inhibitory concentration (MIC) <10 μ g/mL], showed no significant killing activity in vivo, whereas clorobiocin and coumermycin significantly reduced the bacterial load (Fig. 1A). Clorobiocin is reportedly a more potent inhibitor of gyrase and topoisomerase IV than novobiocin, although it is difficult to source commercially and is not in clinical

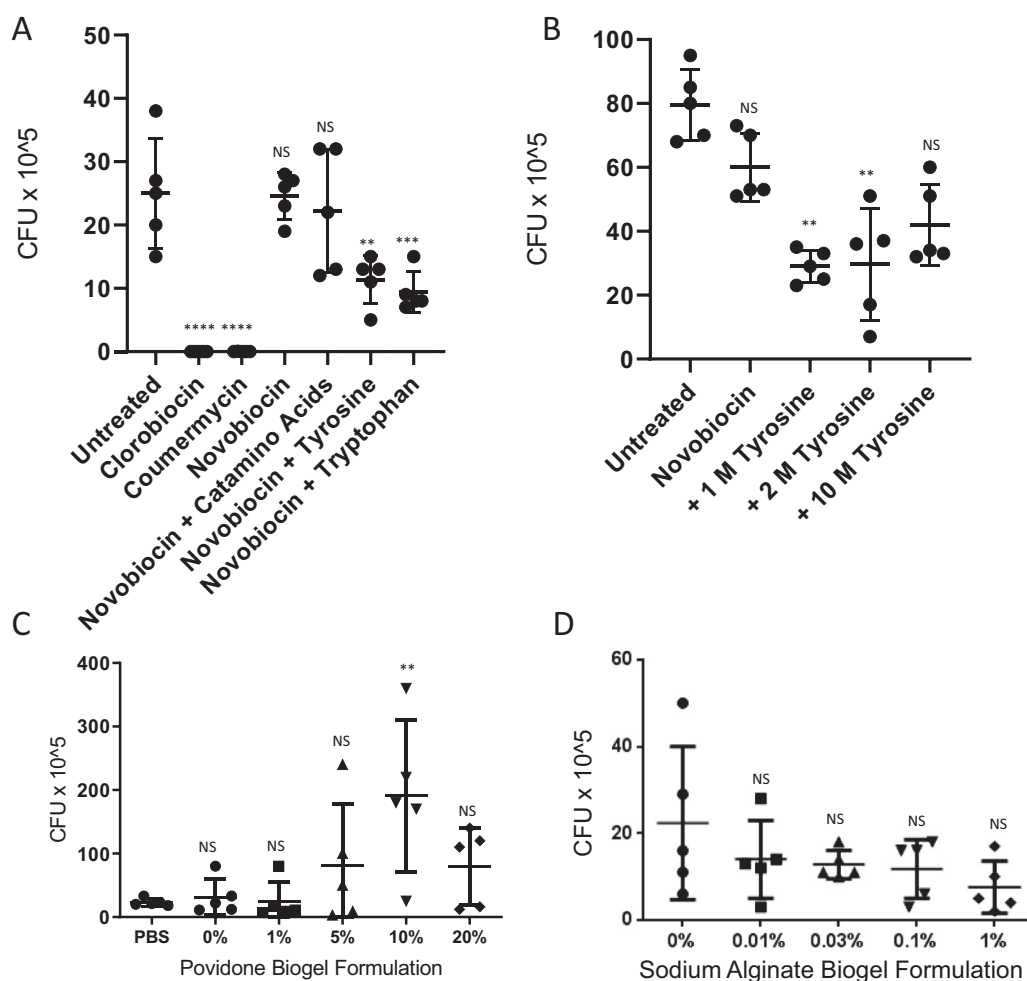


Fig. 1. The novobiocin formulation modifies in vivo potency in *Galleria mellonella* larvae. *Galleria* larvae ($n = 5$ per group) were infected with *Burkholderia pseudomallei* K96243 (110–178 CFU) for 1 h at 37 °C and were then treated with different aminocoumarins (10 μ L of 1 mg/mL stock solution) or novobiocin in different formulations as indicated. One-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparisons test versus (A,B) untreated larvae or (C,D) novobiocin formulation in phosphate-buffered saline (PBS). Representative figures from at least three independent biological replicates are shown. Error bars represent the standard deviation from the mean. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS, not significant.

use [16]. Coumermycin has a structure similar to a dimer of novobiocin and, like clorobiocin, proved to be more potent than novobiocin in vivo at the same dose. Both Clorobiocin and coumermycin are reported to have improved membrane permeability compared with novobiocin [13], which may explain these findings. With these two aminocoumarins, all *Galleria* larvae survived and showed no signs of disease after 24 h of infection, whereas no larvae treated with novobiocin survived longer than 24 h post-infection. Haemolymph from clorobiocin- and coumermycin-treated larvae was also clear from significant amounts of *B. pseudomallei*, and in some cases (100% and 60%, respectively) was sterile of any bacteria.

Use of certain amino acids as co-amorphous partners has been reported to increase the solubility of poorly water-soluble drugs [17,18]. We therefore investigated the effect of addition of either L-tyrosine, DL-tryptophan or casamino acids at various molar ratios on the in vivo efficacy of novobiocin (Fig. 1A,B). Tryptophan and tyrosine, but not casamino acids, improved the activity of novobiocin at equimolar ratios, showing reduced bacterial burden in *Galleria* larvae. Since casamino acids, which contain a mixture of amino acids minus tryptophan, were not effective at enhancing activity, we suggest that a unique property of tyrosine and tryptophan—potentially their non-polar aromatic carbon ring—is responsible for the improved novobiocin activity. The fact that the molar ratio of

amino acid to novobiocin influenced synergy suggests that specific stoichiometry is important for optimal interaction.

To investigate the synergy with amino acid formulation further, novobiocin was chemically conjugated to a fluorescent reporter compound (Supplementary methods; Supplementary Fig. S1A) and its tissue distribution was imaged in histological samples of treated, non-infected *Galleria* larvae (Supplementary Fig. S1C). No significant effect on drug accumulation in adipose or haemolymphatic tissue was observed. The mechanism of in vivo synergy between DL-tryptophan, L-tyrosine and aminocoumarins remains to be established.

Novobiocin exists in solution as an equilibrium between two forms, its less bioactive crystalline form and its more bioactive amorphous form. Unlike the crystalline form, the amorphous form is readily absorbed in vivo via the gastrointestinal tract leading to high blood concentrations [19]. Gelling agents have been previously described to suppress crystallisation of similar compounds, thus in the current study different formulations of povidone and sodium alginate with novobiocin were tested. Whilst povidone significantly increased bacterial CFU during *Galleria* infection, sodium alginate formulations appeared to reduce the bacterial burden, although this was not statistically significant (Fig. 1C,D). Gelling agents can be problematic due to the associated changes in solution viscosity. Techniques such as nanosuspension and prodrug

Table 1
Summary of mean pharmacokinetic parameters.

Parameter	Coumermycin		Novobiocin	
	Alone	+ DL-tryptophan	Alone	+ DL-tryptophan
$t_{1/2}$ (h)	6	6	3	3
T_{max} (h)	2	2	1	1
C_{max} (nM)	102 767	103 297	10 739	10 739
AUC_{last} (h•nM)	957 128	909 773	33 198	33 198
AUC_{all} (h•nM)	957 128	909 773	33 198	33 198
AUC_{inf} (h•nM)	1 016 517	963 025	33 240	33 240

$t_{1/2}$, elimination half-life; T_{max} , time to maximum concentration; C_{max} , maximum concentration; AUC, area under the concentration–time curve (AUC_{last} , AUC from 0 h to last measurable concentration; AUC_{all} , AUC for all values; AUC_{inf} , AUC from 0 h to infinity).

formulation with phosphate esters have been explored by pharmaceutical companies with mixed success. Alternative methods such as polymersome encapsulation [20,21] or microgel formulation may enhance novobiocin efficacy in vivo and remain to be tested.

3.2. Coumermycin shows improved pharmacokinetics in vivo compared with novobiocin

The *G. mellonella* data suggested that while both compounds are effective against *B. pseudomallei* in vitro, coumermycin retains activity in vivo, in contrast to novobiocin. We sought to characterise the pharmacokinetic profile of both compounds in a murine model by injection of either compound into the intraperitoneal cavity and quantification of its absorption into peripheral blood plasma (Fig. 2). Whilst some mild toxicity including skin rash and nausea has been reported with the use of novobiocin, other reports suggest that it is in fact generally well tolerated and that impurities in the formulation may instead be responsible for such side effects [10]. In the present study, no toxicity was observed at the dosage of coumermycin used in the murine experiments.

It was found that while novobiocin reached a peak concentration of 10 739 nM, coumermycin peaked at 102 767 nM and also had a significantly longer half-life in plasma (5.9 h vs. 2.8 h) (Table 1). The half-life values are similar to those reported by others [10,22], whilst the plasma concentrations for coumermycin are substantially higher than in studies that used a subcutaneous rather than intraperitoneal injection route [23], even accounting for differences in treatment dose. The relatively low plasma concentration of novobiocin may explain its loss of activity in vivo since the unbound (free) drug available is likely to be less than the threshold required to inhibit topoisomerase and gyrase [13]. Interestingly, addition of DL-tryptophan, which improved antibiotic activity in *Galleria*, did not influence the pharmacokinetics for either drug in the murine model.

3.3. Treatment with coumermycin protects against acute melioidosis in a murine model

Since coumermycin was highly active in the *Galleria* screen and showed enhanced pharmacokinetics over novobiocin, this compound was selected as the test aminocoumarin for treatment of melioidosis in an acute murine model of infection [24]. Novobiocin has previously shown potential in the treatment of sepsis caused by *Streptococcus pneumoniae* [10]. Since formulation with L-tyrosine enhanced the in vivo activity of novobiocin, we reasoned that it may similarly enhance the potency of coumermycin. Ceftazidime, the currently recommended antibiotic for treating melioidosis, was used as a comparative control.

All treatment groups had a significantly improved survival rate until the end of the experiment compared with the saline-treated control group (Fig. 3A). In agreement with the *Galleria* data, the

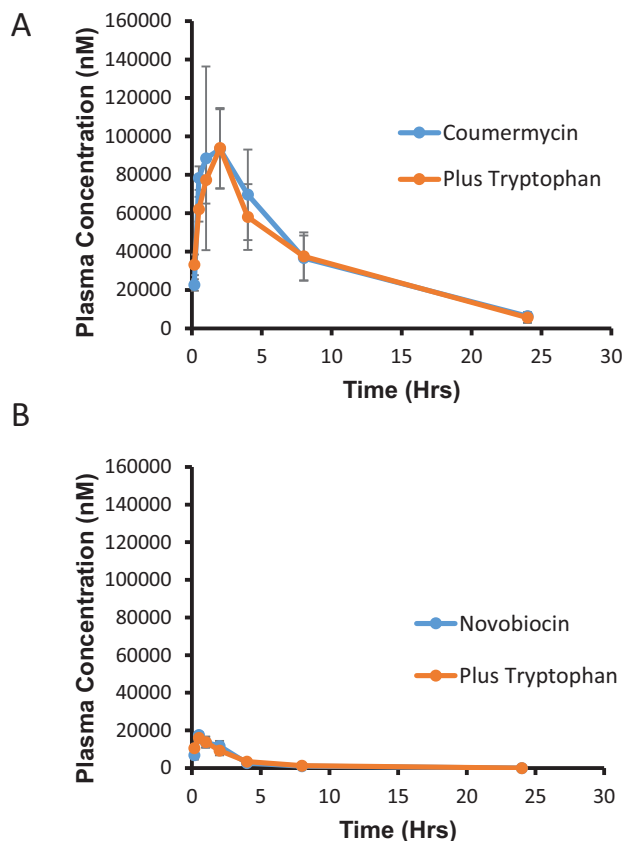


Fig. 2. Pharmacokinetics of novobiocin and coumermycin. Female BALB/c mice ($n = 3$ per group) were intraperitoneally administered (A) 15 mg/kg coumermycin or (B) 15 mg/kg novobiocin with or without formulation in an equimolar concentration of DL-tryptophan (dose volume 10 mL/kg). Error bars represent the standard deviation from the mean. Plasma concentrations of the compound were assessed by mass spectrometry at different time points as indicated.

efficacy of coumermycin was enhanced by formulation with a 1:1 molar ratio L-tyrosine, with 100% survival at 30 days post-infection compared with 60% survival with ceftazidime and 40% survival with coumermycin alone. In general, the coumermycin treatment groups scored better for clinical symptoms of disease than the ceftazidime group and recovered a greater percentage of body weight (Fig. 3B).

It is notable that successful treatment with coumermycin was achieved with one-fortieth the dose of ceftazidime. Of the surviving units at the termination of the experiment, homogenised lung and spleen tissue were examined for pathology and bacterial burden. All surviving ceftazidime-treated mice showed splenomegaly containing $>1 \times 10^7$ CFU bacteria, and two of three also had *B. pseudomallei* bacilli present in their lungs. By comparison, 40%

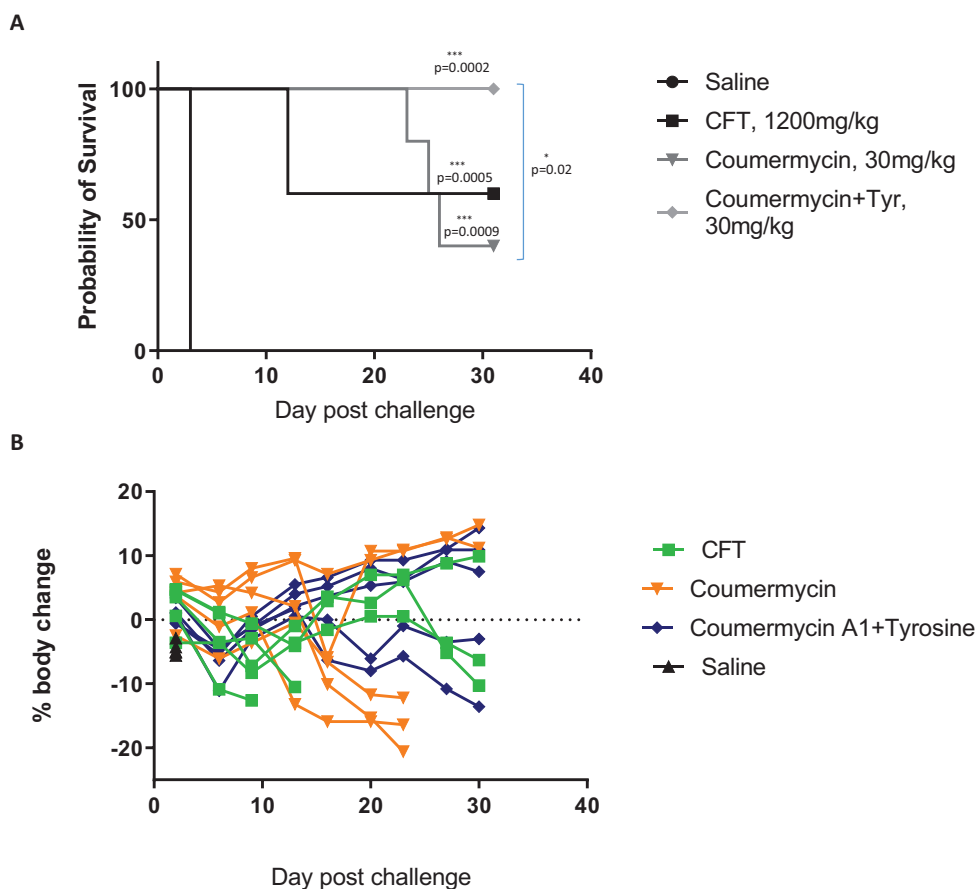


Fig. 3. Coumermycin treatment significantly improved survival in a murine model of melioidosis. Female BALB/c mice were infected intranasally with 500 CFU of *Burkholderia pseudomallei* K96243 ($n = 5$ per group). Ceftazidime (CFT) (1200 mg/kg, intraperitoneal) or coumermycin \pm L-tyrosine (Tyr) (30 mg/kg, subcutaneous) was administered at 6 h post-infection, with subsequent treatments every 24 h for 4 days thereafter. (A) Survival and (B) body weight was assessed until the end of the experiment at 30 days post-infection. Statistical comparison of survival curves was performed by log-rank (Mantel-Cox) test.

of surviving units from the coumermycin plus L-tyrosine group demonstrated splenomegaly, with no bacteria in their lung. At the end of the experiment, 40% of units from the coumermycin treatment group and 60% from the coumermycin plus L-tyrosine group showed complete organ sterility.

The only previous report of coumermycin being tested against *B. pseudomallei* in vivo was from a 1970 study that used pig mucin to deplete host immunity [25]. Six doses of coumermycin over 72 h showed significant activity against multiple strains of *B. pseudomallei* using an intraperitoneal infection model. In the present work, a mode of infection via the aerosol route was simulated, which more closely reflects the clinical situation in endemic countries.

Antimicrobial resistance among many pathogens is a legitimate concern. Success in this regard has been reported by utilising novobiocin combined with rifampicin in a dual-therapy approach that mitigates drug resistance [10]. Novel related antibiotics such as kibdelomycin are also effective against strains of bacteria that are otherwise resistant to aminocoumarins or quinolones [26]. There is also promise in the rational design of novel aminocoumarins through metabolic engineering of the producer strains of *Streptomyces* sp. [27].

4. Conclusions

Taken together, these data suggest that aminocoumarins deserve renewed attention for their potential to treat melioidosis and potentially other diseases. Some doubts over their use have focussed on their pharmacokinetic properties, potential side ef-

fects and the potential for the development of resistance. In the present work, we demonstrate innovative methods for improving their bioactive potential. Given the lack of development of new antibiotics, we cannot afford to ignore the potential benefits that aminocoumarins may have to offer.

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Competing interests: None declared.

Ethical approval: Animal work was performed in accordance with the Animals (Scientific Procedures) Act of 1986 and the local Ethical Review Committee.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijantimicag.2020.106002](https://doi.org/10.1016/j.ijantimicag.2020.106002).

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