Effect of malate on the activity of ciprofloxacin against *Pseudomonas aeruginosa* in different *in vivo* and *in vivo*-like infection models

Xuerui Bao[a], Ellen Goeteyn[a], Aurélie Crabbé[a], Tom Coenye[a]#

[a]Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

Running title: *In vivo* (or -like) effects of malate on ciprofloxacin

#Address correspondence to: Tom Coenye, Tom.Coenye@UGent.be

ABSTRACT

The clinical significance of *Pseudomonas aeruginosa* infections and the tolerance of this opportunistic pathogen to antibiotic therapy makes the development of novel antimicrobial strategies an urgent need. We previously found that D,L-malic acid potentiates the activity of ciprofloxacin against *P. aeruginosa* biofilms grown in a synthetic cystic fibrosis sputum medium by increasing metabolic activity and TCA cycle activity. This suggested a potential new strategy to improve antibiotic therapy in *P. aeruginosa* infections. Considering the importance of the microenvironment on microbial antibiotic susceptibility, the present study aims to further investigate the effect of D,L-malate on ciprofloxacin activity against *P. aeruginosa* in physiologically relevant infection models, aiming to mimic the infection environment more closely. We used *Caenorhabditis elegans* nematodes, *Galleria mellonella* larvae, and a 3-D lung epithelial cell model to assess the effect of D,L-malate on ciprofloxacin activity against *P. aeruginosa*. D,L-malate was able to significantly enhance ciprofloxacin activity against *P. aeruginosa* in both *G. mellonella* larvae and the 3-D lung epithelial cell model. In addition, ciprofloxacin combined with D,L-malate significantly improved the survival of infected 3-D cells compared to ciprofloxacin alone. No significant effect of D,L-malate on ciprofloxacin activity against *P. aeruginosa* in *C. elegans* nematodes was observed. Overall, these data indicate that the outcome of the experiment is influenced by the model system used which emphasizes the importance of using models that reflect the *in vivo* environment as closely as possible. Nevertheless, this study confirms the potential of D,L-malate to enhance ciprofloxacin activity against *P. aeru*ginosa-associated infections.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that is a common cause of (nosocomial) infections, especially in immunocompromised patients (1–4). Pneumonia due to *P. aeruginosa* is associated with high mortality (2, 3, 5) and in cystic fibrosis (CF) patients, colonization of the lungs by *P. aeruginosa* is a leading cause of morbidity and mortality (6). *P. aeruginosa* infections are difficult to treat with antibiotics because of the low outer membrane permeability and the numerous intrinsic and acquired resistance mechanisms (7–9). The growing prevalence of multidrug-resistant and extensively drug-resistant *P. aeruginosa* is of increasing concern worldwide, leading to its designation by the World Health Organization (WHO) as a high-risk organism (10, 11). Besides resistance, also antimicrobial tolerance linked to a biofilm lifestyle contributes to difficulties in finding an effective treatment (12, 13). Treatment options for infections caused by *P. aeruginosa* are limited, and include colistin and aminoglycosides (14, 15). However, the use of these antibiotics is frequently associated with side effects (5, 11, 16, 17) and more effective/less toxic antimicrobial strategies are urgently needed to combat *P. aeruginosa* infections.

Various compounds, including carbohydrates, amino acids, and organic acids, are able to enhance the antimicrobial activity of antibiotics by modulating bacterial metabolism (6, 18, 27, 19–26). For instance, lower tricarboxylic acid cycle (TCA) metabolites, such as fumarate, succinate, α-ketoglutarate, as well as pyruvate, can sensitize stationary phase *P. aeruginosa* cells to tobramycin (24). For fumarate it was shown that this potentiating activity was due to activating the electron transport chain (ETC), leading to an increased proton motive force (PMF) and enhanced cellular respiration (24). Previously we showed that D,L-malic acid and sodium acetate could potentiate the activity of ciprofloxacin and ceftazidime, respectively, against *P. aeruginosa* biofilms in a synthetic sputum medium by modulating bacterial metabolism (26).

The infectious microenvironment, comprising host cells, microorganisms and their extracellular polymeric substances, and host polymers, plays an important role in bacterial physiology and contributes to reduced antibiotic susceptibility in chronic infections (28–30). Consequently, it is important to use physiologically relevant models to study the potentiation of antibiotic activity to narrow the gap between *in vitro* studies and the *in vivo* situation. In the present study, we further investigated the ciprofloxacin-potentiating activity of D,L-malate

against *P. aeruginosa* using several *in vivo* and *in vivo*-like infection models.

Caenorhabditis elegans has been widely used as a model organism to study bacterial virulence and to evaluate various antimicrobial treatments; *C. elegans* has been used in studies with different fungal and bacterial pathogens, including *P. aeruginosa* (31–37). Larvae of the greater wax moth *Galleria mellonella* are also frequently used as an invertebrate infection model; in contrast to *C. elegans, G. mellonella* has an innate immune system and can grow at 37°C (38). *G. mellonella* has been used to study virulence and antimicrobial treatment of a wide range of microorganisms, including *P. aeruginosa* (39–43). Three-dimensional (3-D) *in vivo*-like lung epithelial cell cultures better mimic physiological characteristics of *in vivo* lung epithelium (including 3-D architecture, barrier function, apical-basolateral polarity, and multicellular complexity) than conventional monolayers (44, 45). In addition, *P. aeruginosa* adhesion and subsequent host-secreted cytokine profiles in 3-D lung epithelial cell culture model are more similar to those found *in vivo* than in 2-D monolayers grown on plastic (46, 47).

MATERIALS AND METHODS

Bacterial strains and culture conditions

Pure cultures of *P. aeruginosa* PAO1, LES B58, AA2, AA44, and DK2 (48) were maintained on tryptic soy agar (TSA; Lab M). For imaging purposes, GFP-expressing *P. aeruginosa* PAO1 was used (47). Overnight cultures were grown statically in Luria Bertani broth (LB; Lab M) at 37°C under aerobic conditions. Serial dilutions of *P. aeruginosa* were plated on two different media types: Difco *Pseudomonas* Isolation Agar (PIA; BD Diagnostics) for the *C. elegans* infection assay, and TSA for the quantification of *P. aeruginosa* adhering to 3-D cell models.

Chemicals

Stock solutions of 24 mg/mL ciprofloxacin (Sigma-Aldrich) were prepared in 0.1 M HCl (Sigma-Aldrich) and stored at −20°C. Stock solutions of D,L-malate (Sigma-Aldrich) were stored at 4°C at a concentration of 600 mM. The final pH value of all media was adjusted to 6.8 using 1M NaOH (Merck Life Sciences).

C. elegans nematode infection assay

The *C. elegans* nematode infection assay was done as previously described (49). *C. elegans* strain AU37 (*glp-4; sek-1*) (a temperature-sensitive sterile mutant) was propagated on nematode-growth media (NGM) plates seeded with *Escherichia coli* OP50 at 13°C. Stocks of *C. elegans* were maintained by transferring approx. 1 cm^2 NGM agar pieces with nematodes to fresh NGM plates with *E. coli* OP50 every seven days. Eggs were isolated from adult worms by hypochlorite bleaching, leading to a synchronized *C. elegans* population. The harvested eggs were transferred to NGM seeded with *E. coli* OP50 and were incubated at 25°C for at least 3 days to generate stage L4 sterile animals for further experiments. Synchronized L4 stage worms were suspended in OGM medium, containing 95% M9 buffer, 5% brain heart infusion broth (Oxoid) and 10 µg/ml cholesterol (Sigma-Aldrich); this nematode suspension was transferred to wells of 96-well microtiter plates (approx. 20 worms/well) (49). Bacterial overnight cultures were centrifuged, resuspended and standardized to 10^9 CFU/mL in OGM medium and nematodes were infected with 25 µL of this suspension. D,L-malate (60 mM) and/or ciprofloxacin (0.6, 0.075, or 0.0375 µg/mL) was added to the test wells. Nematodes that were not infected and/or not treated served as controls. The microtiter plates were incubated at 25°C for 3 days and the number of living and dead worms in each well was determined every 24h using an EVOS FL Auto microscope (Life Technologies) at a final magnification of 40x. Worms were considered dead when they were straight and immobile. For each condition, at least five biological replicates were performed and each experiment consisted of three technical replicates. The number of CFU per worm was determined after 72 h incubation. To this end, nematodes were collected and rinsed with M9 buffer, containing 1 mM of sodium azide to prevent the nematodes from vomiting. Subsequently, the nematodes were washed with physiological saline (PS, 0.9% NaCl solution) before counting. The bacteria were released from the nematodes by disrupting the latter by vortexing in microtubes containing 1.0 mm silicon carbide beads (BioSpec Products) for 10 min. Serial dilutions of the supernatants were plated on TSA agar plates and incubated at 37°C for 15 h.

G. mellonella infection assay

G. mellonella (greater wax moth) larvae (Hengelsport De Poorter, Ghent, Belgium) were randomly assigned to five groups (10 larvae/group); four groups were infected by injecting 10

μL of a *P. aeruginosa* PAO1 suspension (containing 2 x 10^4 CFUs per milliliter) at the left posterior gastropod while the uninfected control group was injected with 10 μL of PS. 30 min post-infection, the infected larvae were treated (by injecting 10 μL at the right posterior gastropoda) with PS, ciprofloxacin (1.2 μg/mL), D,L-malate (60 mM), or a combination of ciprofloxacin (1.2 μg/mL) and D,L-malate (60 mM). The uninfected larvae were injected with 10 μL of PS at the right posterior gastropoda. Larvae were incubated at 37°C and survival was monitored after 0 h, 15 h, 16 h, 17 h, 20 h, 24 h, and 48 h. Larvae were considered dead when they failed to respond to external stimuli and when they showed dark pigmentation caused by melanisation. Kaplan-Meier survival curves were plotted using data pooled from six biological replicates (49).

Determination of bacterial growth curves in cell culture medium

Prior to assessing the effect of D,L-malate on ciprofloxacin activity against *P. aeruginosa* in 3-D lung epithelial cell cultures, we evaluated the growth of *P. aeruginosa* PAO1, AA2, and AA44 in GTSF-2 cell culture medium without FBS (47, 50, 51) with or without ciprofloxacin (final concentration: 0, 0.25 or 0.5 μg/mL) and/or 60 mM D,L-malate. Growth was quantified for 50 h at 37°C by measuring the optical density at 600 nm (OD_{600}) using an EnVision spectrophotometer (Perkin Elmer). GTSF-2 medium (HyClone) was supplemented with 1.5 g/L sodium bicarbonate (Sigma-Aldrich), and 2.5 mg/L insulin transferring sodium selenite (ITS, Lonza) (47, 50, 51). All experiments were performed in three biological replicates (with three technical replicates in each biological replicate, i.e. n = 3 x 3).

3-D lung epithelial cell culture

The 3-D *in vivo*-like lung model was generated from the human adenocarcinomic alveolar epithelial cell line A549 (ATCC CCL-185) using the Rotating Wall Vessel (RWV) as described previously (27, 47). On the day of the infection, the 3-D aggregates were transferred to 96-well plates at a concentration of 2.5 x 10^5 cells/well containing the above-described GTSF-2 medium.

3-D lung epithelial model infection assay

The 3-D lung epithelial model was infected with *P. aeruginosa* biofilms as described previously

(47, 51), with some modifications. A targeted multiplicity of infection (MOI) of 30:1 was used for *P. aeruginosa*. The prepared ciprofloxacin and D,L-malate solutions in GTSF-2 medium were added together with *P. aeruginosa* or separately to the 3-D A549 cells at final concentrations of 0.5 or 0.25 μg/mL (ciprofloxacin) and 60 mM (D,L-malate). The uninfected as well as untreated cells were considered as controls. For all infection experiments, plates were incubated for 24 h statically at 37 °C under 5% CO_2 conditions. After 24 h incubation, cells in each well were rinsed with pre-warmed GTSF-2 medium three times. Next, fresh GTSF-2 medium and the same treatments as described above were added to the corresponding wells. Following the addition of fresh medium with different treatments, the test plates were incubated for another 24 h at 37 °C under 5% CO_2 conditions, after which biofilm formation on 3-D cell model and cytotoxicity were determined as described below. At least five biological replicates were performed and in each experiment three technical replicates were performed.

Quantification of *P. aeruginosa* in the 3-D cell model and cytotoxicity assay

For *in vitro* host-pathogen interaction studies, the viability of mammalian cells is often measured using the conventional ("extracellular") lactate dehydrogenase (LDH) assay. However, a recent study indicated *P. aeruginosa* could interfere with the extracellular LDH activity through protease production and therefore developed a modified ("intracellular") LDH assay to avoid this interference (50). Thus, to assess the viability of 3-D lung epithelial cells in this study, the "intracellular" LDH assay was applied as previously described (50). Briefly, after 48-hour infection, 3-D cells were rinsed with pre-warmed HBSS (Hank's Balanced Salt Solution, Life Technologies, Thermo Fisher Scientific). The content of each well was transferred to new 96-well plates without touching the plates' bottom using the wide bore pipette tips. After rinsing three more times with HBSS, the attached 3-D cells were lysed using 0.1% Triton-X100 (Sigma-Aldrich) through vigorously pipetting up and down 30x. To remove cell debris and bacteria, the rest of the resulting mixture was centrifuged. Intracellular LDH release was then quantified using an LDH activity assay kit (Sigma-Aldrich) following the manufacturer's instructions. A standard curve was determined using NADH. The completely lysed uninfected 3-D cells were used as the positive control. Survival of 3-D cells in infected cultures was calculated as a percentage of the positive control. In addition, to determine the

number of *P. aeruginosa* in the 3-D model, the lysate was serially diluted and plated on TSA agar (at 37°C for 15 h)

All experiments were performed at least in five replicates, each containing three technical replicates.

Light- and fluorescence microscopy

The overall morphology and integrity of 3-D epithelial cells after 48 h infection with *P. aeruginosa* PAO1, AA2, AA44, and GFP-expressing *P. aeruginosa* PAO1 was imaged with an EVOS FL Auto Microscope (Life Technologies) equipped with a 10x and 20x objective and appropriate filter cubes (final magnification: 200x or 400x) (47). Two images were taken per condition and fluorescence microscopy images were processed using the image processing application ImageJ software (National Institutes of Health, USA). For each experiment at least five biological replicates were included, with each biological replicate consisting of three technical replicates. For each biological and technical replicate per condition, at least two representative images were taken.

Statistical analysis

Statistical analysis was performed using SPSS version 27 (IBM). The normal distribution of the data was verified by the Shapiro-Wilk test. When the data were normally distributed, an independent sample t-test or one-way ANOVA with Bonferroni correction was used. Data that were not normally distributed were analyzed by nonparametric Mann-Whitney U tests. Kaplan-Meier survival curves of infected *G. mellonella* were analyzed by using the log-rank (Mantel-Cox) test and the significance was Bonferroni corrected for multiple comparisons.

RESULTS

D,L-malate has no effect on ciprofloxacin activity against *P. aeruginosa* in *C. elegans*

The effect of D,L-malate on ciprofloxacin antimicrobial activity was assessed using a *C. elegans* infection model. Ciprofloxacin concentrations were optimized for each *P. aeruginosa* strain (Fig. S1). Concentrations of ciprofloxacin causing the largest difference in survival between infected *C. elegans* treated with ciprofloxacin alone and infected *C. elegans* treated

with the combination after 72 h-incubation were selected for further in depth studies; the selected ciprofloxacin concentrations for *P. aeruginosa* PAO1, AA44, DK2, and LES B58 were 0.0375, 0.6, 0.075, and 0.075 μg/mL respectively (Fig. S1), which were much lower than the final maximum concentration of 33.0 μg/mL in sputum after aerosolized administration of 50 mg dry powder ciprofloxacin (52, 53). The selected concentrations aimed to partially affect *P. aeruginosa* without complete inhibition, thus allowing room for malate to enhance the efficacy of ciprofloxacin.

At the concentrations selected, neither D,L-malate nor ciprofloxacin alone affected the survival of uninfected nematodes (Fig. S2A, F). In the absence of treatment, the survival of nematodes infected with *P. aeruginosa* PAO1, AA44, DK2, or LES B58 began to decrease at 24 h post-infection (Fig. S2B-E).

Compared to the untreated control, the addition of D,L-malate (60 mM) alone caused a significant increase in the survival of nematodes infected with *P. aeruginosa* PAO1 ($p = 0.003$), AA44 ($p = 0.00002$), DK2 ($p = 0.004$), or LES B58 ($p = 0.008$) (Fig. 1). Ciprofloxacin alone (at the selected concentrations, Fig. 1) did not significantly increase the survival of nematodes infected with *P. aeruginosa* PAO1, AA44 or LES B58; a small (16.6%) but significant ($p=0.041$) increase was observed after ciprofloxacin treatment of nematodes infected with DK2. The combination of ciprofloxacin + D,L-malate significantly increased the survival of nematodes infected with *P. aeruginosa* PAO1 ($p = 0.0007$), AA44 ($p = 0.00003$), DK2 ($p = 0.004$), or LES B58 ($p = 0.008$) compared to the untreated control. However, no significant difference in survival was observed between treatment with D,L-malate alone or treatment with D,L-malate + ciprofloxacin, suggesting D,L-malate affected *C. elegans* survival without potentiating ciprofloxacin activity. To further confirm the effect of D,L-malate, higher concentrations of ciprofloxacin (1.2 μg/mL for *P. aeruginosa* PAO1, DK2, and LES B58; 9.6 μg/mL for *P. aeruginosa* AA44) were also tested. There was no significant difference in survival between infected *C. elegans* treated with D,L-malate alone or those treated with the combination of D,L-malate and ciprofloxacin ($p = 0.909$, 0.286, 0.571 and 0.073 for *P. aeruginosa* PAO1, DK2, LES B58 and AA44, respectively).

No significant difference in the number of *P. aeruginosa* recovered from nematodes was observed between groups of infected nematodes without treatment and with the treatment of

D,L-malate alone, ciprofloxacin alone, or the combination (Fig. S3), except for *P. aeruginosa* AA44 for which treatment with ciprofloxacin (alone or in combination with D,L-malate) led to a lower microbial load. Combined these data indicated that the addition of D,L-malate did not significantly increase ciprofloxacin antimicrobial activity in *C. elegans*. A possible explanation for this observation is that D,L-malate affected the nematodes directly, as this compound has been reported to extend the lifespan in *C. elegans* by increasing oxygen consumption, and decreasing ATP levels and mitochondrial membrane potential (54). While it is possible that malate influences the virulence of *P. aeruginosa* in nematodes (potentially leading to an increased survival of infected nematodes), this needs to be investigated further.

D,L-malate potentiates ciprofloxacin activity against *P. aeruginosa* PAO1 in the *G. mellonella* infection model

Subsequently, we assessed the effect of D,L-malate on ciprofloxacin activity in the *G. mellonella* infection model. Neither D,L-malate nor ciprofloxacin was toxic for larvae at the concentration used (Fig. S4). Infection with *P. aeruginosa* PAO1 significantly decreased the survival of larvae ($p < 0.0001$), while D,L-malate alone did not lead to a significant change in the survival of infected larvae (Fig. 2). In contrast, treatment with ciprofloxacin alone significantly increased the percentage of survival ($p = 0.001$) and the combination of ciprofloxacin + D,L-malate further improved survival of infected larvae significantly compared to treatment with ciprofloxacin alone ($p = 0.001$). These data demonstrate D,L-malate possesses the ability to potentiate the activity of ciprofloxacin against *P. aeruginosa* PAO1 in *G. mellonella*.

D,L-malate increases ciprofloxacin activity against *P. aeruginosa* biofilms in a 3-D lung epithelial cell model

To further explore the effect of D,L-malate on ciprofloxacin against *P. aeruginosa* biofilms in a 3-D lung epithelial cell model, we determined the number of CFU in *P. aeruginosa* biofilms that attached to cells and microcarrier bead scaffolds after rinsing (to remove unattached cells and *P. aeruginosa* in the cell supernatant) (Fig. 3). For each strain, ciprofloxacin concentrations were selected that did not completely inhibit bacterial growth in the cell culture medium (Fig.

S5). These selected ciprofloxacin concentrations for *P. aeruginosa* PAO1 (0.25 µg/mL), AA2 (0.25 and 0.5 µg/mL), and AA44 (0.25 and 0.5 µg/mL) were then used in further studies with infected 3-D epithelial cell cultures. D,L-malate alone did not significantly affect the number of attached *P. aeruginosa* cells. Likewise, treatment with 0.25 µg/mL ciprofloxacin alone did not significantly reduce the number of attached PAO1 or AA44 cells, while for *P. aeruginosa* AA2 a reduction of approx. 1 log ($p = 0.008$) was observed. Treatment with 0.5 µg/mL ciprofloxacin alone significantly reduced the number of attached *P. aeruginosa* cells for strain AA44 (approx. 0.96 log, $p = 0.0006$) but not for strain AA2 (approx. 0.51 log, $p = 0.235$) compared to the untreated control. Combined treatment with D,L-malate and either 0.25 or 0.5 µg/mL ciprofloxacin significantly decreased the number of attached *P. aeruginosa* PAO1, AA2, and AA44 cells compared to untreated controls (Fig. 3). The combination of D,L-malate and 0.25 µg/mL ciprofloxacin significantly increased the anti-biofilm effect for strain PAO1 only (approx. 3.7 log reduction compared to 0.25 µg/mL ciprofloxacin alone, $p < 0.0001$). Compared to 0.5 µg/mL ciprofloxacin alone, the combination of D,L-malate and 0.5 µg/mL ciprofloxacin significantly increased the anti-biofilm effect for *P. aeruginosa* AA2 (approx. 5.3 log, $p < 0.0001$) and AA44 (by approx. 2.5 log, $p < 0.0001$). Fluorescence microscopy of 3-D cultures infected with GFP-expressing *P. aeruginosa* PAO1 confirmed the potentiating effect of D,L-malate on ciprofloxacin anti-biofilm activity (Fig. S6).

The combination of ciprofloxacin and D,L-malate increases the viability of infected 3-D epithelial cells compared to ciprofloxacin alone

We used light microscopy and LDH measurements to assess the effect of the combined treatment D,L-malate and ciprofloxacin treatment on the viability of 3-D epithelial cells. In the absence of treatment, *P. aeruginosa* PAO1, AA2, and AA44 infection of 3-D lung epithelial cells caused 90.2, 98.1, and 83.2% cell death, respectively (Fig. 4). The effect of D,L-malate alone on the viability of 3-D lung epithelial cells was minor, and was only significant for *P. aeruginosa* PAO1 ($p = 0.008$) (Fig. 4). Treatment with ciprofloxacin alone did not significantly increase the viability of cells, except for *P. aeruginosa* PAO1 ($p = 0.008$). The combination of 0.25 µg/mL ciprofloxacin and D,L-malate significantly increased the viability of cells infected with *P. aeruginosa* PAO1 ($p = 0.008$) and AA2 ($p = 0.0001$), but not of cells infected with *P.*

aeruginosa AA44 (p = 0.6) compared to the untreated control. The combination of 0.5 μg/mL ciprofloxacin and D,L-malate significantly improved the viability of cells after infection of *P. aeruginosa* AA2 (p = 0.0004) and AA44 (p = 0.002) compared to the untreated control. Compared to treatment with 0.25 μg/mL ciprofloxacin alone, the combination of 0.25 μg/mL ciprofloxacin and D,L-malate significantly increased the viability of cells by 73% (PAO1, p = 0.0002), 39% (AA2, p = 0.008), and 12% (AA44, p = 0.041) (Fig. 4). Similarly, when infected cells were treated with the higher concentration of ciprofloxacin (0.5 μg/mL; not tested with PAO1), the addition of D,L-malate was able to significantly enhance the viability of cells by 57% (AA2, p = 0.007) or 54% (AA44, p = 0.002). These data show that the addition of D,L-malate could increase the viability of infected cells treated with ciprofloxacin and demonstrate that the higher concentration of ciprofloxacin led to significantly higher viability of infected cells when used in combination with D,L-malate.

These results were supported by light microscopy observation (Fig. S7). We observed that the overall integrity of the uninfected 3-D epithelial cells was maintained during the 48 h incubation period, during which a limited amount of cells detached from the microcarrier bead scaffolds. A large amount of the cells detached from the microcarrier bead scaffolds after infection with all three strains of *P. aeruginosa* for 48 h. The addition of D,L-malate, or ciprofloxacin alone did not reduce the detachment of infected cells compared to the untreated control. However, overall we observed higher integrity of 3-D aggregates for *P. aeruginosa*-infected cultures treated with combined treatment ciprofloxacin and D,L-malate compared to either treatment alone. This observation was most pronounced for 3-D cultures treated with the highest concentration of ciprofloxacin used (0.5 μg/ml). Hence, these observations are consistent with the results of the viability assay.

DISCUSSION

The increasing prevalence of infections with antibiotic resistant organisms, together with the limited pipeline of novel antibiotics, is contributing to a severe worldwide public health crisis (55). Consequently, it is crucial and urgent to explore innovative strategies for alternative therapies. Recent studies have shown that the combination of antibiotics with other compounds has the potential to enhance the effectiveness of current antibiotic treatments (21, 23, 24, 26,

27, 56–60). Previously, we found that D,L-malic acid (ciprofloxacin) and sodium acetate (ceftazidime) potentiate antibiotic activity against *P. aeruginosa* biofilms in a synthetic sputum medium (26). Here, we showed that the addition of D,L-malate increased the activity of ciprofloxacin against *P. aeruginosa* in *G. mellonella* larvae and a 3-D lung epithelial cell model, but not in *C. elegans*. Given the potential differences in concentrations between *C. elegans* and *G. mellonella* models (e.g. due to variations in animal size and mode of administration), further research is needed to explore the effect of different concentrations of malate in these infection models.

Our findings show that the model system used can greatly influence the experimental outcomes when evaluating the activity of antibiotic potentiators. In the *C. elegans* nematode model, no potentiating effect of D,L-malate on ciprofloxacin activity was observed, neither on the survival of nematodes nor on bacterial load. Surprisingly, D,L-malate alone was sufficient to significantly increase the survival of infected nematodes. However, results obtained in *G. mellonella* larvae and in the 3-D lung cell epithelial model indicate that D,L-malate could work as a potentiator of ciprofloxacin against *P. aeruginosa* to increase the survival of larvae or cellular viability.

A previous study has shown that antifungal imidazoles econazole and miconazole could potentiate tobramycin activity against *Burkholderia cenocepacia* biofilms formed in 96-well microtiter plates, but not in 3-D lung epithelial cell cultures, *G. mellonella* larvae, or mice models (61). Besides, thioridazine, which belongs to phenothiazines drug class, was shown to potentiate the activity of tobramycin, linezolid and flucloxacillin against *S. aureus* biofilms in 96-well microtiter plates, while this potentiation effect was lost in a chronic wound model of biofilm infection (62). In another study, the outer membrane-acting peptide L6 showed little or no synergistic activity with vancomycin against the tested Gram-negative pathogens *in vitro* (96 well plates), while another outer membrane-acting peptide L8 showed synergistic effect against *Acinetobacter baumannii* and *Klebsiella pneumoniae*. However, in *A. baumannii* infected zebrafish larvae, L6 showed an additive effect on the antimicrobial activity of vancomycin while L8 showed an antagonistic effect (63). The addition of CdTe-2.4, which can produce reactive oxygen species after illumination, potentiated the effect of ciprofloxacin against *Salmonella enterica* serovar Typhimurium in infected HeLa cells grown as 2-D

monolayers and significantly reduced the number of intracellular bacteria compared to ciprofloxacin treatment alone (64). However, this potentiating effect was no longer significant in the *C. elegans* model (64). These examples confirm that model system selection significantly impacts experimental outcomes.

The effect of D,L-malate on the activity of ciprofloxacin in the 3-D lung epithelial model also seems to be antibiotic-concentration dependent. While D,L-malate did not potentiate ciprofloxacin activity at a concentration of 0.25 μg/mL ciprofloxacin, a significant difference was observed when the concentration of ciprofloxacin was increased to 0.5 μg/mL. This suggests that higher concentrations of ciprofloxacin may be more effectively potentiated by D,L-malate.

In conclusion, D,L-malate showed a significant effect on increasing ciprofloxacin activity against *P. aeruginosa* in two out of three models used in the present study. Hence, D,L-malate may be a promising, effective, and easy-to-obtain potentiator of ciprofloxacin to combat *P. aeruginosa*-related infections. In addition, this study highlights the importance of using models that mimic the *in vivo* environment as close as possible as it can affect experimental outcomes.

ACKNOWLEDGEMENTS

This work was supported by the Chinese Scholarship Council (CSC, NO. 201806150021).

REFERENCE

1. Peña C, Suarez C, Tubau F, Dominguez A, Sora M, Pujol M, Gudiol F, Ariza J. 2009. Carbapenem-resistant *Pseudomonas aeruginosa*: factors influencing multidrug-resistant acquisition in non-critically ill patients. Eur J Clin Microbiol Infect Dis 28:519–522.
2. Juan C, Peña C, Oliver A. 2017. Host and Pathogen Biomarkers for Severe *Pseudomonas aeruginosa* Infections. J Infect Dis 215:S44–S51.
3. Kang CI, Kim SH, Kim H Bin, Park SW, Choe YJ, Oh MD, Kim EC, Choe KW. 2003. *Pseudomonas aeruginosa* bacteremia: Risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clin Infect Dis 37:745–751.
4. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. 2006. Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. Antimicrob Agents Chemother 50:43–48.
5. Bassetti M, Vena A, Croxatto A, Righi E, Guery B. 2018. How to manage *Pseudomonas aeruginosa* infections. Drugs Context 7:1–18.
6. Barraud N, Buson A, Jarolimek W, Rice SA. 2013. Mannitol enhances antibiotic sensitivity of persister bacteria in *Pseudomonas aeruginosa* biofilms. PLoS One 8:e84220.
7. Botelho J, Grosso F, Peixe L. 2019. Antibiotic resistance in *Pseudomonas aeruginosa* – Mechanisms, epidemiology and evolution. Drug Resist Updat 44:100640.
8. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. 2011. *Pseudomonas aeruginosa*: All roads lead to resistance. Trends Microbiol 19:419–426.

9. Gellatly SL, Hancock REW. 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis 67:159–173.
10. Rello J, Kalwaje Eshwara V, Lagunes L, Alves J, Wunderink RG, Conway-Morris A, Rojas JN, Alp E, Zhang Z. 2019. A global priority list of the TOp TEn resistant Microorganisms (TOTEM) study at intensive care: a prioritization exercise based on multi-criteria decision analysis. Eur J Clin Microbiol Infect Dis 38:319–323.
11. Balandin B, Ballesteros D, Ruiz de Luna R, López-Vergara L, Pintado V, Sancho-González M, Soriano-Cuesta C, Pérez-Pedrero MJ, Asensio-Martín MJ, Fernández-Simón I, Rodríguez-Serrano D, Silva A, Chicot M, Iranzo R, Martínez-Sagasti F, Royuela A. 2021. Multicenter study of ceftolozane/tazobactam for treatment of *Pseudomonas aeruginosa* infections in critically ill patients. Int J Antimicrob Agents 57:106270.
12. Sindeldecker D, Stoodley P. 2021. The many antibiotic resistance and tolerance strategies of *Pseudomonas aeruginosa*. Biofilm 3:100056.
13. Ciofu O, Tolker-Nielsen T. 2019. Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. Front Microbiol 10:913.
14. Okoliegbe IN, Hijazi K, Cooper K, Ironside C, Gould IM. 2021. Trends of antimicrobial resistance and combination susceptibility testing of multidrug-resistant *Pseudomonas aeruginosa* isolates from cystic fibrosis patients: a 10-year update. Antimicrob Agents Chemother 65:e02483-20.
15. Cianciulli Sesso A, Lilić B, Amman F, Wolfinger MT, Sonnleitner E, Bläsi U. 2021. Gene Expression Profiling of *Pseudomonas aeruginosa* Upon Exposure to Colistin and Tobramycin. Front Microbiol 12:626715.
16. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson M V. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103:8487–8492.
17. Falagas ME, Rafailidis PI. 2009. Nephrotoxicity of Colistin: New Insight into an Old Antibiotic. Clin Infect Dis 48:1729–1731.

18. Allison KR, Brynildsen MP, Collins JJ. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216–220.
19. Kim JS, Cho DH, Heo P, Jung SC, Park M, Oh EJ, Sung J, Kim PJ, Lee SC, Lee DH, Lee S, Lee CH, Shin D, Jin YS, Kweon DH. 2016. Fumarate-mediated persistence of *Escherichia coli* against antibiotics. Antimicrob Agents Chemother 60:2232–2240.
20. Peng B, Su Y Bin, Li H, Han Y, Guo C, Tian YM, Peng XX. 2015. Exogenous Alanine and/or Glucose plus Kanamycin Kills Antibiotic-Resistant Bacteria. Cell Metab 21:249–262.
21. Ye JZ, Su Y Bin, Lin XM, Lai SS, Li WX, Ali F, Zheng J, Peng B. 2018. Alanine enhances aminoglycosides-induced ROS production as revealed by proteomic analysis. Front Microbiol 9:1–14.
22. Farha MA, Verschoor CP, Bowdish D, Brown ED. 2013. Collapsing the proton motive force to identify synergistic combinations against *Staphylococcus aureus*. Chem Biol 20:1168–1178.
23. Su Y bin, Peng B, Li H, Cheng Z xue, Zhang T tuo, Zhu J xin, Li D, Li M yi, Ye J zhou, Du C chao, Zhang S, Zhao X liang, Yang M jun, Peng XX. 2018. Pyruvate cycle increases aminoglycoside efficacy and provides respiratory energy in bacteria. Proc Natl Acad Sci U S A 115:e1578–e1587.
24. Meylan S, Porter CBM, Yang JH, Belenky P, Gutierrez A, Lobritz MA, Park J, Kim SH, Moskowitz SM, Collins JJ. 2017. Carbon Sources Tune Antibiotic Susceptibility in *Pseudomonas aeruginosa* via Tricarboxylic Acid Cycle Control. Cell Chem Biol 24:195–206.
25. Su Y Bin, Peng B, Han Y, Li H, Peng XX. 2015. Fructose restores susceptibility of multidrug-resistant *Edwardsiella tarda* to kanamycin. J Proteome Res 14:1612–1620.
26. Bao X, Bové M, Coenye T. 2021. Organic Acids and Their Salts Potentiate the Activity of Selected Antibiotics against *Pseudomonas aeruginosa* Biofilms Grown in a Synthetic Cystic Fibrosis Sputum Medium. Antimicrob Agents Chemother 66:e0187521.
27. Crabbé A, Ostyn L, Staelens S, Rigauts C, Risseeuw M, Dhaenens M, Daled S, Van Acker H, Deforce D, Van Calenbergh S, Coenye T. 2019. Host metabolites stimulate the bacterial proton motive force to enhance the activity of aminoglycoside antibiotics.

509 PLoS Pathog 15:1–24.
510 28. Bjarnsholt T, Whiteley M, Rumbaugh KP, Stewart PS, Jensen P, Frimodt-Møller N.
511 2022. The importance of understanding the infectious microenvironment. Lancet Infect
512 Dis 22:e88–e92.
513 29. Jensen P, Kolpen M, Kragh KN, Kühl M. 2017. Microenvironmental characteristics and
514 physiology of biofilms in chronic infections of CF patients are strongly affected by the
515 host immune response. Apmis 125:276–288.
516 30. Van den Bossche S, De Broe E, Coenye T, Van Braeckel E, Crabbé A. 2021. The cystic
517 fibrosis lung microenvironment alters antibiotic activity: causes and effects. Eur Respir
518 Rev 30(161):210055.
519 31. Mylonakis E, Ausubel FM, Tang RJ, Calderwood SB. 2003. The art of serendipity:
520 Killing of *Caenorhabditis elegans* by human pathogens as a model of bacterial and
521 fungal pathogenesis. Expert Rev Anti Infect Ther. Taylor & Francis 1(1):167-73.
522 32. Edwards S, Kjellerup B V. 2012. Exploring the applications of invertebrate host-
523 pathogen models for *in vivo* biofilm infections. FEMS Immunol Med Microbiol 65:205–
524 214.
525 33. Vallejo JA, Beceiro A, Rumbo-Feal S, Rodríguez-Palero MJ, Russo TA, Bou G. 2015.
526 Optimisation of the *Caenorhabditis elegans* model for studying the pathogenesis of
527 opportunistic *Acinetobacter baumannii*. Int J Antimicrob Agents 46:1.
528 34. Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM. 1999. *Pseudomonas*
529 *aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence
530 factors. Proc Natl Acad Sci U S A 96:2408–2413.
531 35. Chan SY, Liu SY, Seng Z, Chua SL. 2021. Biofilm matrix disrupts nematode motility
532 and predatory behavior. ISME J 15:260–269.
533 36. Razvi E, Whitfield GB, Reichhardt C, Dreifus JE, Willis AR, Gluscencova OB, Gloag
534 ES, Awad TS, Rich JD, da Silva DP, Bond W, Le Mauff F, Sheppard DC, Hatton BD,
535 Stoodley P, Reinke AW, Boulianne GL, Wozniak DJ, Harrison JJ, Parsek MR, Howell
536 PL. 2023. Glycoside hydrolase processing of the Pel polysaccharide alters biofilm
537 biomechanics and *Pseudomonas aeruginosa* virulence. NPJ Biofilms Microbiomes 9:7.
538 37. De La Fuente-Núñez C, Reffuveille F, Mansour SC, Reckseidler-Zenteno SL,

539 Hernández D, Brackman G, Coenye T, Hancock REW. 2015. D-enantiomeric peptides
540 that eradicate wild-type and multidrug-resistant biofilms and protect against lethal
541 *Pseudomonas aeruginosa* infections. Chem Biol 22:196–205.
542 38. Cutuli MA, Petronio Petronio G, Vergalito F, Magnifico I, Pietrangelo L, Venditti N,
543 Di Marco R. 2019. *Galleria mellonella* as a consolidated *in vivo* model hosts: New
544 developments in antibacterial strategies and novel drug testing. Virulence 10:527–541.
545 39. Benthall G, Touzel RE, Hind CK, Titball RW, Sutton JM, Thomas RJ, Wand ME. 2015.
546 Evaluation of antibiotic efficacy against infections caused by planktonic or biofilm
547 cultures of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in *Galleria mellonella*.
548 Int J Antimicrob Agents 46:538–545.
549 40. Mannala GK, Rupp M, Alagboso F, Kerschbaum M, Pfeifer C, Sommer U,
550 Kampschulte M, Domann E, Alt V. 2021. *Galleria mellonella* as an alternative *in vivo*
551 model to study bacterial biofilms on stainless steel and titanium implants. ALTEX -
552 Altern to Anim Exp 38:245–252.
553 41. Wang W, Bao X, Bové M, Rigole P, Meng X, Su J, Coenye T. 2022. Antibiofilm
554 Activities of Borneol-Citral-Loaded Pickering Emulsions against *Pseudomonas*
555 *aeruginosa* and *Staphylococcus aureus* in Physiologically Relevant Chronic Infection
556 Models. Microbiol Spectr 10(5):e0169622.
557 42. Kaul L, Abdo AI, Coenye T, Krom BP, Hoogenkamp MA, Zannettino ACW, Süss R,
558 Richter K. 2022. The combination of diethyldithiocarbamate and copper ions is active
559 against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms *in vitro* and *in*
560 *vivo*. Front Microbiol 13:999893.
561 43. Wille J, Teirlinck E, Sass A, Van Nieuwerburgh F, Kaever V, Braeckmans K, Coenye
562 T. 2020. Does the mode of dispersion determine the properties of dispersed
563 *Pseudomonas aeruginosa* biofilm cells? Int J Antimicrob Agents 56:106194.
564 44. Barrila J, Radtke AL, Crabbé A, Sarker SF, Herbst-Kralovetz MM, Ott CM, Nickerson
565 CA. 2010. Organotypic 3D cell culture models: using the rotating wall vessel to study
566 host-pathogen interactions. Nat Rev Microbiol 8:791–801.
567 45. Crabbé A, Barrila J, Ott CM, Nickerson CA. 2016. Outpacing infectious disease:
568 Mimicking the host-pathogen microenvironment in three-dimensions. Eff Spacefl

569 Spacefl Analog Cult Hum Microb Cells Nov Insights into Dis Mech 93–119.
570 46. Carterson AJ, Höner Zu Bentrup K, Ott CM, Clarke MS, Pierson DL, Vanderburg CR,
571 Buchanan KL, Nickerson CA, Schurr MJ. 2005. A549 lung epithelial cells grown as
572 three-dimensional aggregates: alternative tissue culture model for *Pseudomonas*
573 *aeruginosa* pathogenesis. Infect Immun 73:1129–1140.
574 47. Crabbé A, Liu Y, Matthijs N, Rigole P, De La Fuente-Nùñez C, Davis R, Ledesma MA,
575 Sarker S, Van Houdt R, Hancock REW, Coenye T, Nickerson CA. 2017. Antimicrobial
576 efficacy against *Pseudomonas aeruginosa* biofilm formation in a three-dimensional
577 lung epithelial model and the influence of fetal bovine serum. Sci Rep 7:43321.
578 48. Cullen L, Weiser R, Olszak T, Maldonado RF, Moreira AS, Slachmuylders L,
579 Brackman G, Paunova-Krasteva TS, Zarnowiec P, Czerwonka G, Reilly J, Drevinek P,
580 Kaca W, Melter O, De Soyza A, Perry A, Winstanley C, Stoitsova SR, Lavigne R,
581 Mahenthiralingam E, Sá-Correia I, Coenye T, Drulis-Kawa Z, Augustyniak D, Valvano
582 MA, McClean S. 2015. Phenotypic characterization of an international *Pseudomonas*
583 *aeruginosa* reference panel: strains of cystic fibrosis (CF) origin show less *in vivo*
584 virulence than non-CF strains. Microbiol (United Kingdom) 161:1961–1977.
585 49. Brackman G, Cos P, Maes L, Nelis HJ, Coenye T. 2011. Quorum sensing inhibitors
586 increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*.
587 Antimicrob Agents Chemother 55:2655–2661.
588 50. Van den Bossche S, Vandeplassche E, Ostyn L, Coenye T, Crabbé A. 2020. Bacterial
589 Interference With Lactate Dehydrogenase Assay Leads to an Underestimation of
590 Cytotoxicity. Front Cell Infect Microbiol 10:494.
591 51. Rigauts C, Aizawa J, Taylor SL, Rogers GB, Govaerts M, Cos P, Ostyn L, Sims S,
592 Vandeplassche E, Sze M, Dondelinger Y, Vereecke L, Van Acker H, Simpson JL, Burr
593 L, Willems A, Tunney MM, Cigana C, Bragonzi A, Coenye T, Crabbé A. 2022. Rothia
594 mucilaginosa is an anti-inflammatory bacterium in the respiratory tract of patients with
595 chronic lung disease. Eur Respir J 59:11.
596 52. Dalhoff A. 2014. Pharmacokinetics and pharmacodynamics of aerosolized antibacterial
597 agents in chronically infected cystic fibrosis patients. Clin Microbiol Rev 27:753–782.
598 53. Stass H, Nagelschmitz J, Willmann S, Delesen H, Gupta A, Baumann S. 2013.

Inhalation of a dry powder ciprofloxacin formulation in healthy subjects: A phase i study. Clin Drug Investig 33:419–427.

54. Edwards CB, Copes N, Brito AG, Canfield J, Bradshaw PC. 2013. Malate and Fumarate Extend Lifespan in *Caenorhabditis elegans*. PLoS One 8:e58345.

55. Murray CJ, Shunji Ikuta K, Sharara F, et. al. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399:629–655.

56. Deng W, Fu T, Zhang Z, Jiang X, Xie J, Sun H, Hu P, Ren H, Zhou P, Liu Q, Long Q. 2020. L-lysine potentiates aminoglycosides against *Acinetobacter baumannii* via regulation of proton motive force and antibiotics uptake. Emerg Microbes Infect 9:639–650.

57. Aedo SJ, Tang J, Brynildsen MP. 2021. Metabolites potentiate nitrofurans in non-growing *Escherichia coli*. Antimicrob Agents Chemother 65:e00858-20.

58. Liu Y, Yang K, Jia Y, Shi J, Tong Z, Wang Z. 2020. Cysteine potentiates bactericidal antibiotics activity against gram-negative bacterial persisters. Infect Drug Resist 13:2593–2599.

59. Liu Y, Yang K, Jia Y, Shi J, Tong Z, Wang Z. 2021. Thymine sensitizes gram-negative pathogens to antibiotic killing. Front Microbiol 12:1–9.

60. Crabbé A, Jensen PØ, Bjarnsholt T, Coenye T. 2019. Antimicrobial tolerance and metabolic adaptations in microbial biofilms. Trends Microbiol 27:850–863.

61. Van Driessche F Den, Vanhoutte B, Brackman G, Crabbé A, Rigole P, Vercruysse J, Verstraete G, Cappoen D, Vervaet C, Cos P, Coenye T. 2017. Evaluation of combination therapy for *Burkholderia cenocepacia* lung infection in different *in vitro* and *in vivo* models. PLoS One 12:e0172723.

62. Van den Driessche F, Brackman G, Swimberghe R, Rigole P, Coenye T. 2017. Screening a repurposing library for potentiators of antibiotics against *Staphylococcus aureus* biofilms. Int J Antimicrob Agents 49:315–320.

63. Schouten GK, Paulussen FM, Kuipers OP, Bitter W, Grossmann TN, van Ulsen P. 2022. Stapling of peptides potentiates the antibiotic treatment of *Acinetobacter baumannii in vivo*. Antibiotics 11:273.

64. Courtney CM, Goodman SM, Nagy TA, Levy M, Bhusal P, Madinger NE, Detweiler

CS, Nagpal P, Chatterjee A. 2017. Potentiating antibiotics in drug-resistant clinical isolates via stimuli-activated superoxide generation. Sci Adv 3:e1701776.

Figure 1. Percentage survival of *C. elegans* nematodes infected with strains PAO1 (A, n=11), AA44 (B, n=7), DK2 (C, n=6) and LESB58 (D, n=5) after 72 h incubation (n represents the number of biological replicates, each biological replicate consisted of three technical replicates). Results are displayed as mean ± standard error; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 2. Kaplan-Meier survival curves of *G. mellonella* larvae infected with *P. aeruginosa* PAO1 and treated with malate, ciprofloxacin, or the combination. Uninfected and untreated *G. mellonella* served as controls. Data shown are average of 3-6 independent experiments. **p < 0.01, ***p = 0.001, ****p < 0.0001, log-rank test with Bonferroni correction for multiple comparisons was applied for significance analysis between different groups.

Figure 3. The number of *P. aeruginosa* cells recovered from 3-D lung epithelial cells. Data are presented as the log value of the number of bacteria (CFU/mL). The results are displayed as mean ± standard error (n = 5 – 6). **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 4. Viability of 3-D lung epithelial cells based on the intracellular LDH assay after incubation with different *P. aeruginosa* strains and subsequent treatment, expressed as a percentage compared to uninfected cells. Cells infected with *P. aeruginosa* without any treatment served as control. The results are displayed as mean ± standard error (n = 5 – 6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.