

Reversion of antibiotic resistance in multidrug-resistant pathogens using non-antibiotic pharmaceutical benzydamine

Yuan Liu ^{1,2,3,4✉}, Ziwen Tong¹, Jingru Shi¹, Yuqian Jia¹, Tian Deng¹ & Zhiqiang Wang ^{1,3,4✉}

Antimicrobial resistance has been a growing concern that gradually undermines our tradition treatment regimens. The fact that few antibacterial drugs with new scaffolds or targets have been approved in the past two decades aggravates this crisis. Repurposing drugs as potent antibiotic adjuvants offers a cost-effective strategy to mitigate the development of resistance and tackle the increasing infections by multidrug-resistant (MDR) bacteria. Herein, we found that benzydamine, a widely used non-steroidal anti-inflammatory drug in clinic, remarkably potentiated broad-spectrum antibiotic-tetracyclines activity against a panel of clinically important pathogens, including MRSA, VRE, MCRPEC and *tet(X)*-positive Gram-negative bacteria. Mechanistic studies showed that benzydamine dissipated membrane potential ($\Delta\Psi$) in both Gram-positive and Gram-negative bacteria, which in turn upregulated the trans-membrane proton gradient (ΔpH) and promoted the uptake of tetracyclines. Additionally, benzydamine exacerbated the oxidative stress by triggering the production of ROS and suppressing GAD system-mediated oxidative defensive. This mode of action explains the great bactericidal activity of the doxycycline-benzydamine combination against different metabolic states of bacteria involve persister cells. As a proof-of-concept, the *in vivo* efficacy of this drug combination was evidenced in multiple animal infection models. These findings indicate that benzydamine is a potential tetracyclines adjuvant to address life-threatening infections by MDR bacteria.

¹College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China. ²Institute of Comparative Medicine, Yangzhou University, Yangzhou 225009, China. ³Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou 225009, China. ⁴Joint International Research Laboratory of Agriculture and Agri-Product Safety, the Ministry of Education of China, Yangzhou University, Yangzhou 225009, China. ✉email: liuyuan2018@yzu.edu.cn; zqwang@yzu.edu.cn

The prevalence of chromosome or plasmid-conferred resistance determinants has severely impaired the efficacy of clinically available antibiotics, rendering the onset of the global antimicrobial resistance crisis¹. Among these pathogenic bacteria, of particular concern are ESKAPE (Enterococcus, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which are responsible for the majority of nosocomial infections worldwide with high morbidity and mortality^{2,3}. With the increasing incidence of drug resistance in ESKAPE clinical isolates, bacterial infection-associated diseases are becoming harder to treat. Notably, carbapenems, colistin, and tigecycline are recognized as extremely crucial antibiotics and last options against these drug-resistant bacteria. However, the emergence of carbapenemase⁴, *mcr-1*-encoded phosphoethanolamine transferase⁵, and *tet(X)*-mediated flavin-dependent monooxygenase^{6,7} in bacteria from animal and human source leaves few choices for clinicians from these traditional pipelines. Meanwhile, few novel antibiotic entities with distinct scaffolds or modes of action have been approved for clinical use during the past decades due to the huge scientific and commercial challenges in the development of new drugs^{8,9}. There is a dire need to identify alternative strategies to address these infections.

Repurposing drugs as potential antibiotic adjuvants to reverse antibiotic resistance and enhance antibiotic activity represents a simple but effective approach to counter this problem^{10–12}. For example, our previous studies have shown that hypoglycemic agent metformin could resensitize *tet(A)*-positive bacteria to tetracyclines through disrupting the functions of efflux pumps¹³. Melatonin, which has been applied for treating sleep disturbances and circadian disorders, potentiated colistin activity against MCR-positive bacteria by enhancing the membrane damage¹⁴. Anti-HIV agent azidothymidine decreased Tet(X3/X4)-mediated bacterial resistance to tigecycline in *Escherichia coli* via specifically inhibiting DNA synthesis and suppressing resistance enzyme activity^{15,16}. In addition, other compounds such as natural products or synthetic drugs have also been demonstrated to enhance the activity of existing antibiotics. The condensed tannins of American cranberry fruit, cranberry proanthocyanidin, increased the activity of a broad range of antibiotic classes against opportunistic pathogens by interfering with intrinsic resistance mechanisms¹⁷. Synthetic tobramycin–lysine conjugates displayed a synergistic effect with minocycline or rifampicin against clinical multidrug-resistant (MDR) *P. aeruginosa* isolates¹⁸. Benzylamine is a locally acting nonsteroidal anti-inflammatory drug with local anesthetic and analgesic properties by selectively binding to prostaglandin synthetase^{19,20}. Recently, benzylamine was found to inhibit osteoclast differentiation and bone resorption via down-regulating the expression of interleukin-1 β ²¹. In addition, benzylamine drastically reduced oral mucositis even at doses >50 Gy in head and neck cancer patients²². However, the adjuvant potential of benzylamine to existing antibiotics is still not fully understood.

In this study, we characterized the synergistic activity of benzylamine with different classes of antibiotics, and found that it drastically potentiated tetracyclines activity against various MDR pathogens. Importantly, benzylamine dissipated membrane potential ($\Delta\Psi$) in both Gram-positive and negative bacteria, which in turn upregulated the transmembrane proton gradient (ΔpH) and promoted the uptake of tetracyclines. Meanwhile, benzylamine synergized with doxycycline on killing a spectrum of bacterial pathogens carrying *mecA*, *bla_{MBL}* and/or *mcr* genes, as well as *tet(X)* by triggering oxidative damage. Notably, benzylamine potentially rescued the activity of doxycycline in multiple animal infection models infected by MDR MRSA T144 or *E. coli* B2. This study reveals the therapeutic potential of benzylamine as an antibiotic adjuvant for the treatment of infection caused by MDR pathogens.

Results

Benzylamine potentiates doxycycline activity in both drug-susceptible and -resistant bacteria. We first evaluated the synergistic activity of benzylamine with eight classes of antibiotics against MDR *E. coli* B2 using checkerboard broth microdilution assays. Out of these drugs, colistin, ciprofloxacin, and doxycycline showed synergistic activity with benzylamine, whereas kanamycin displayed an antagonistic effect with benzylamine (Supplementary Fig. 1 and Supplementary Table 2). Remarkably, the combination of benzylamine and doxycycline possessed the highest synergistic effect (FIC index (FICI) = 0.188), which enabled the MIC value of doxycycline to decrease from 32 to 2 $\mu\text{g}/\text{mL}$ (16-fold). We further tested the potentiation of benzylamine to other tetracyclines, including tetracycline, oxytetracycline, minocycline, and tigecycline. As expected, their antibacterial activity was all substantially improved in the presence of benzylamine (Supplementary Table 2). Subsequently, the checkerboard assays were performed in both drug-susceptible and -resistant bacteria. Interestingly, the combination of benzylamine and doxycycline showed synergistic effect in all test bacteria, including hard-to-treat pathogenic bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) T144 (FICI = 0.188), vancomycin-resistant enterococci (VRE) A4 (FICI = 0.375), *bla_{NDM-5}*-positive *E. coli* G6 (FICI = 0.375), *mcr-1*-carrying *K. pneumoniae* D120 (FICI = 0.375) and *tet(X6)*-positive *A. baumannii* C222 (FICI = 0.5). In detail, the MIC values of doxycycline in drug-susceptible bacteria (including *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. Enteritidis* ATCC 13076, and *E. coli* MG1655) were all lower than 2 $\mu\text{g}/\text{mL}$. And in the presence of 0.25 \times MIC of benzylamine, the MICs of doxycycline were decreased by 4-fold. In contrast, the MICs of doxycycline in drug-resistant pathogens ranged from 16 to 32 $\mu\text{g}/\text{mL}$, which dropped sharply to 1–8 $\mu\text{g}/\text{mL}$ at 0.25 \times MIC of benzylamine, corresponding 4–16-fold potentiating. Overall, this combination displayed a higher synergistic effect on drug-resistant bacteria than -susceptible bacteria, suggesting that its activity is also related to the inhibition of resistance determinants (Fig. 1 and Supplementary Table 3).

Next, we assessed whether the synergistic activity of this combination would result in increased toxicity, including hemolytic activity on mammals RBCs and in vivo toxicity in mice²³. Surprisingly, no detectable toxicity in hemolysis rate, body weight, and blood biochemical analysis was found in the benzylamine–doxycycline combination treatment (Supplementary Figs. 2 and 3). These data suggest that benzylamine is a safe and potent antibiotic adjuvant to tetracyclines.

Benzylamine dissipates the electric potential ($\Delta\Psi$) component of the proton motive force and promotes the uptake of doxycycline.

Our prior results have shown that benzylamine is a universal adjuvant to tetracycline antibiotics in all tested strains, but antagonizes kanamycin activity in *E. coli* B2. We next assessed the interaction of benzylamine and kanamycin in a panel of bacteria. As a consequence, a remarkable antagonism effect was found in all bacteria (FICI > 2.0, Supplementary Fig. 1 and 4). The opposite effect of benzylamine in combination with doxycycline or kanamycin inspired us to speculate that the mechanism of action of benzylamine may be directly related to the destruction of the bacterial proton motive force (PMF)²⁴. In bacteria, the transmembrane transfer of proton H^+ by the respiratory chain results in an electrochemical gradient, named PMF. It consists of two parts, electric potential ($\Delta\Psi$) and transmembrane proton gradient (ΔpH)²⁵. Damage to one will be compensated by increasing another to achieve dynamic balance²⁶. Previous studies have indicated that the uptake of tetracyclines by bacterial cells

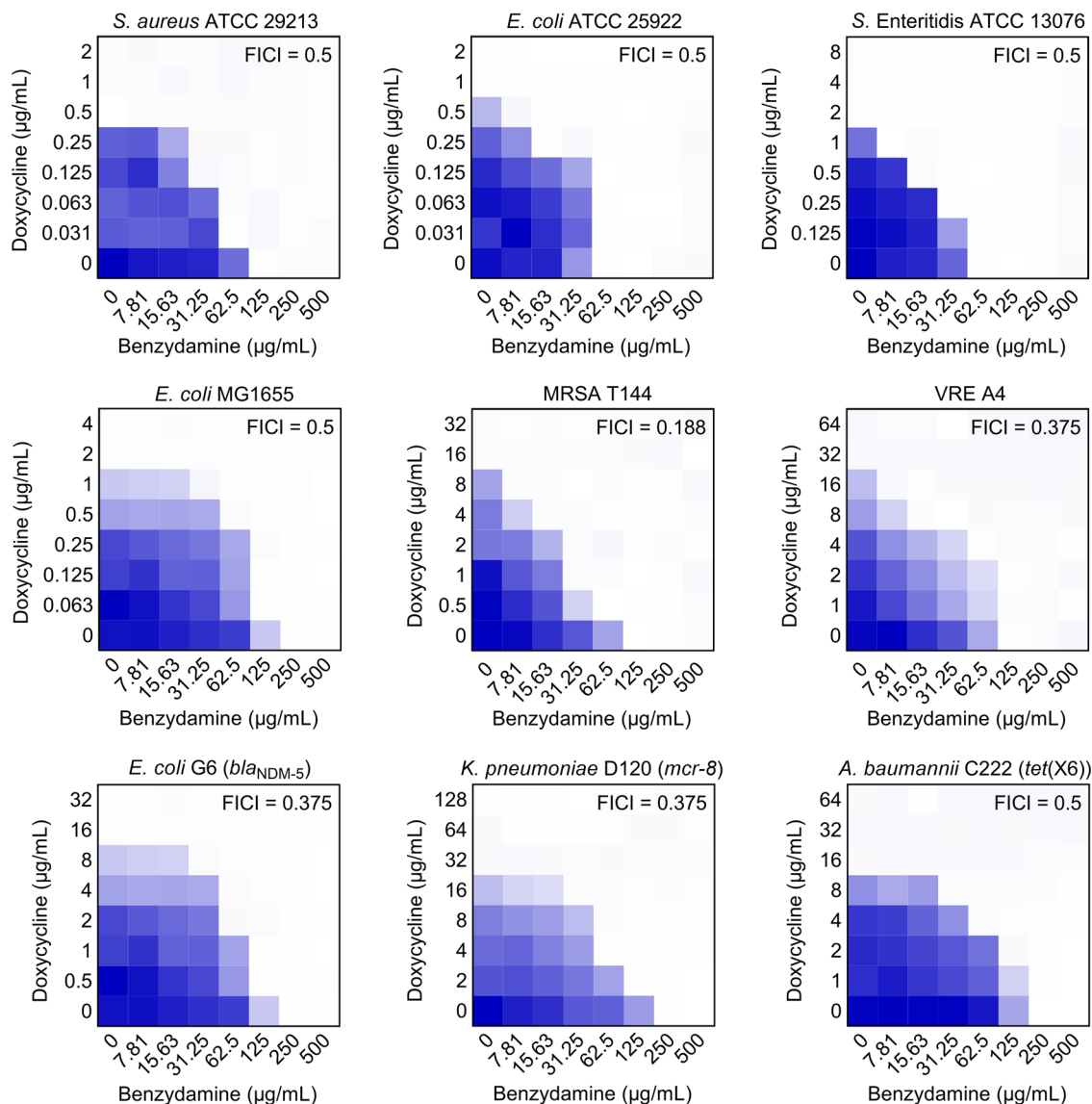


Fig. 1 Synergistic activity between benzylidamine and doxycycline against drug-susceptible and -resistant bacteria by checkerboard assay, related to Supplementary Table 3. Dark blue regions represent higher cell density. Data represent the mean OD₆₀₀ nm of two biological replicates. Synergy is defined with FIC index ≤ 0.5 .

depends on ΔpH , whereas aminoglycosides utilize the $\Delta\Psi$ component for transport, therefore, we are concerned that benzylidamine might target the $\Delta\Psi$ component of bacterial PMF. To test our hypothesis, a fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide (DiSC₃(5))²⁷ was used to assess membrane potential changes induced by doxycycline, benzylidamine alone, or their combination in four representative strains (*S. aureus* ATCC 29213, MRSA T144, *E. coli* ATCC 25922 and *E. coli* B2; two Gram-positive and two Gram-negative bacteria). Consequently, no obvious fluorescence change was observed in the doxycycline treatment group. In contrast, benzylidamine treatment resulted in rapid disruption of electric potential in a dose-dependent manner (Fig. 2a). Next, we measured the fluorescence with one to fourfold MIC of doxycycline or combination with 250 $\mu\text{g}/\text{mL}$ of benzylidamine. Benzylidamine plus doxycycline indeed resulted in increased fluorescence compared with doxycycline alone (Fig. 2b), suggesting that benzylidamine is definitely a potential dissipator of $\Delta\Psi$. The extracellular pH values are also related to the PMF. A previous study demonstrated that the antibacterial activity of the dissipator of $\Delta\Psi$ will be strengthened when the

extracellular pH changed to the alkaline values²⁸. Consistent with the membrane potential results, the MICs of benzylidamine were reduced by four to eightfold when the pH changed from 5.5 to 9.5 in both Gram-negative bacteria and Gram-positive bacteria (Fig. 2c). An intact PMF is required for the bacterial function in flagellar secretion, thus, we next examined the integrity of PMF through swimming motility experiments²⁹. Exposure of four strains to sub-inhibitory concentrations of benzylidamine drastically decreased bacterial motility (Fig. 2d), suggesting the impaired PMF in benzylidamine-treated bacterial cells. These evidence demonstrates that benzylidamine disrupts the PMF by targeting $\Delta\Psi$ component.

After showing that benzylidamine selectively disrupted the $\Delta\Psi$, we set out to test whether ΔpH will be compensatorily upregulated. A membrane-permeable fluorescent probe termed BCECF-AM³⁰ was used to monitor intracellular pH changes in four strains. Interestingly, benzylidamine led to the acidification of the cytoplasm in Gram-positive bacteria, but alkalization of cytoplasm in Gram-negative bacteria (Fig. 3a). Nevertheless, both these actions triggered the upregulation of ΔpH in bacteria. Given

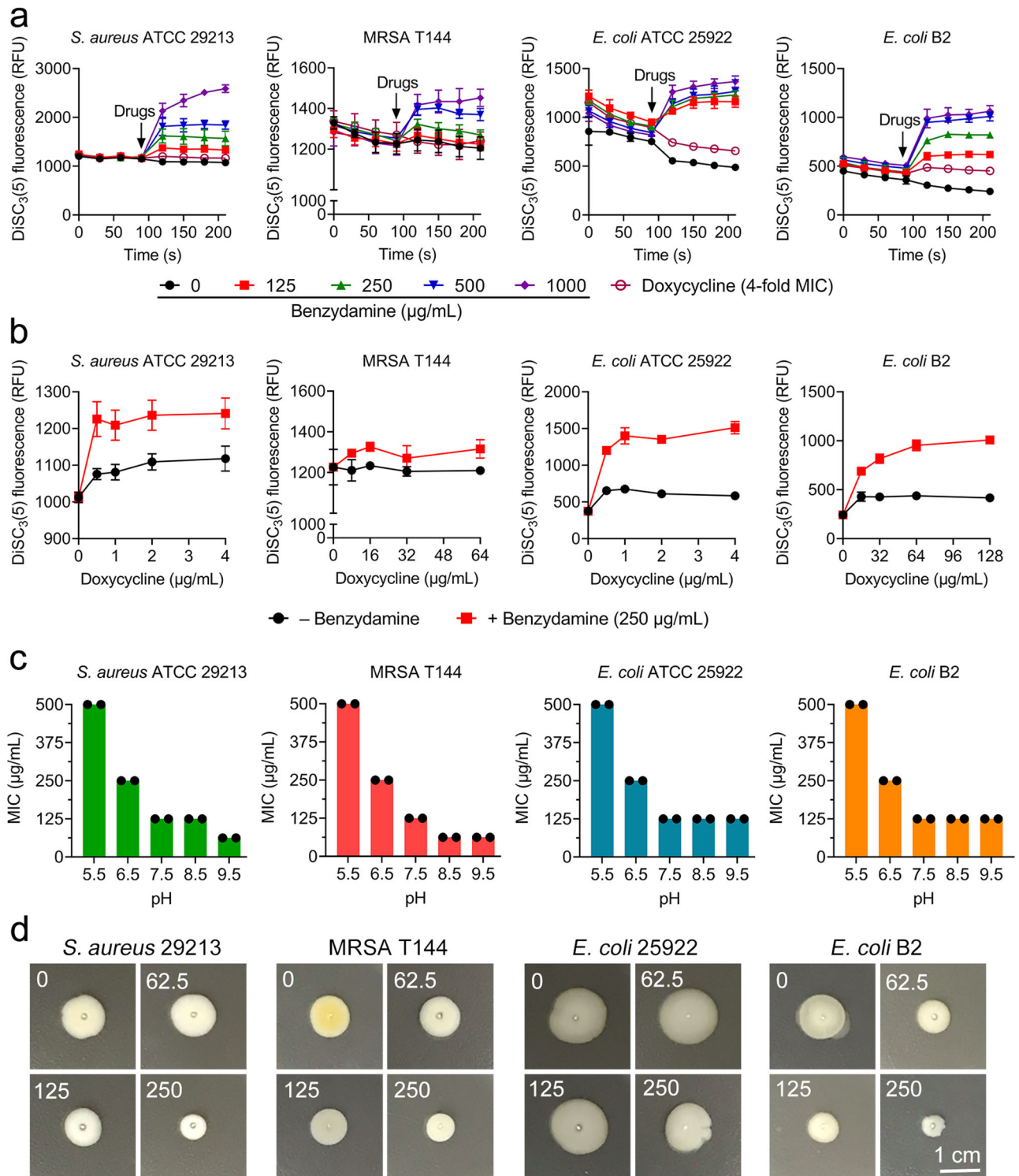


Fig. 2 Benzylamine disrupts proton motive force (PMF) in both Gram-positive and Gram-negative bacteria. **a** Benzylamine dissipates membrane potential in bacteria. Fluorescence intensity of DiSC₃(5) in *S. aureus* ATCC 29213, MRSA T144, *E. coli* ATCC 25922, and *E. coli* B2 after treatment with increasing concentrations of benzylamine and doxycycline (fourfold MIC) was monitored. Drugs were added into DiSC₃(5)-probed cells at 90 s. **b** Combination of doxycycline and benzylamine (250 μg/mL) displays increased disruption on membrane potential compared with doxycycline alone. **c** Decreased MIC values of benzylamine against four strains in an alkaline environment. $\Delta\Psi$ becomes the main component of PMF as the external pH is shifted to an alkaline environment. **d** Benzylamine inhibits the swimming motility of four bacterial strains. Overnight cultures were standardized to OD₆₀₀ nm of 0.5, and inoculated on 0.3% agar plates for 48 h at 37 °C. Scar bar, 1 cm.

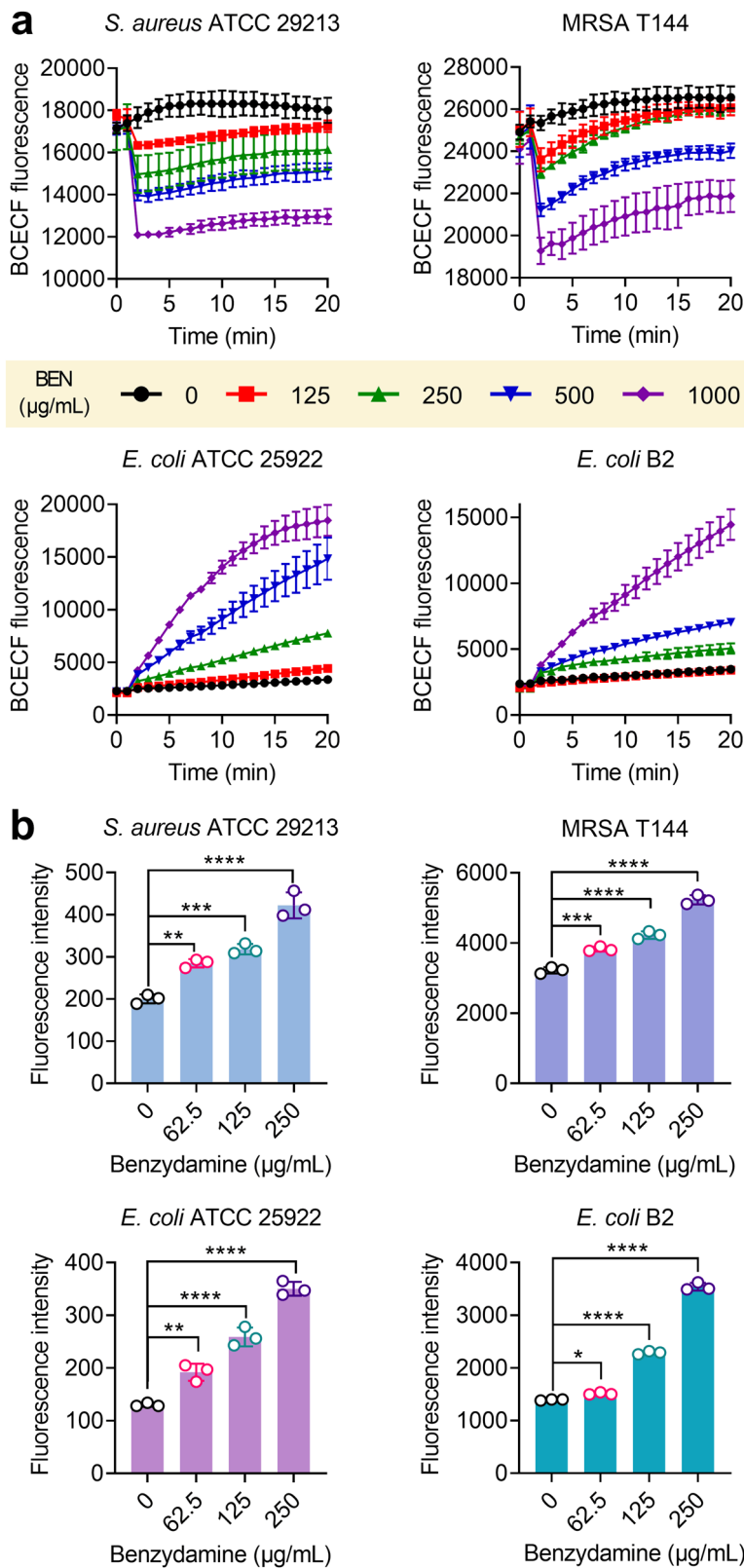


Fig. 3 Benzylamine upregulates Δ pH and promotes the intracellular accumulation of doxycycline. a Upregulation of Δ pH in BCECF-AM-labeled bacterial cells after exposure to varying concentrations of benzylamine. In Gram-positive bacteria, benzylamine decreases fluorescence and the cytoplasmic pH. In contrast, benzylamine increases fluorescence and the cytoplasmic pH in Gram-negative bacteria (*E. coli* ATCC 25922 and *E. coli* B2). **b** Benzylamine supplementation dose-dependently promotes the intracellular accumulation of doxycycline in bacteria. Intracellular antibiotic content was determined by monitoring the fluorescence of doxycycline (excitation wavelength, 405 nm; emission wavelength, 535 nm). All data from three biological replicates were presented as mean \pm SD, and the significance was determined by non-parametric one-way ANOVA (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

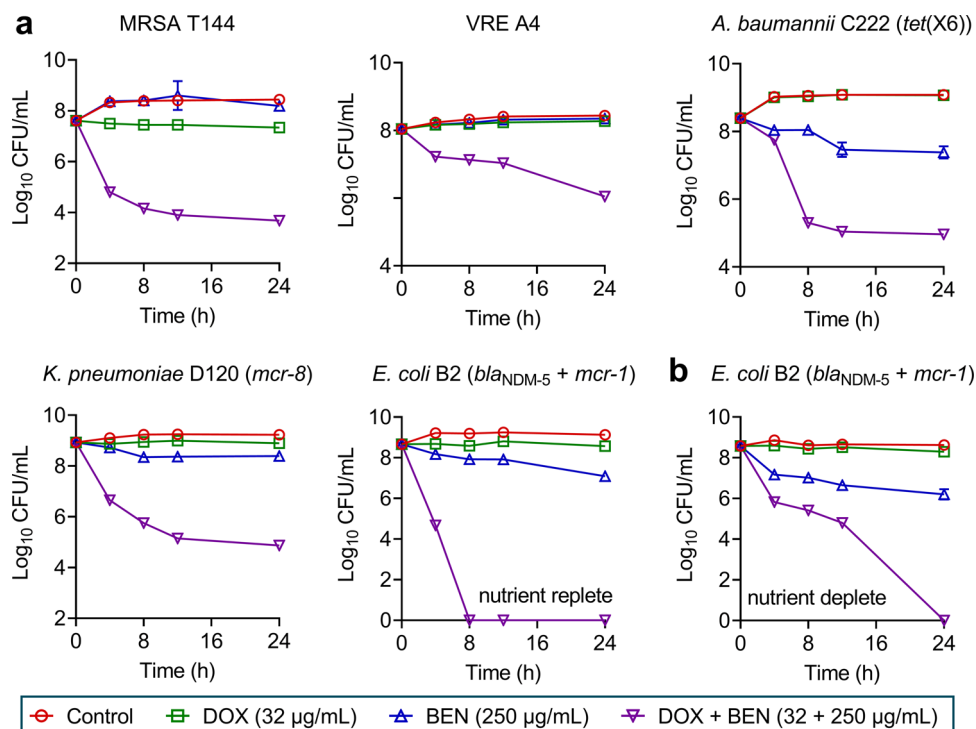


Fig. 4 Combination of doxycycline and benzydamine displays bactericidal activity against various drug-resistant bacteria. **a** Killing activity of doxycycline plus benzydamine in LB media against MDR Gram-positive bacteria (MRSA T144 and VRE A4) and Gram-negative bacteria (*A. baumannii* C222, *K. pneumoniae* D120, and *E. coli* B2). **b** Time-killing curves of doxycycline, benzydamine alone, or their combination in PBS against *E. coli* B2. The initial cell density is about 10^8 CFU/mL. All data were performed from three biological replicates and shown as mean \pm SD.

that the increasing Δ pH may contribute to the uptake of tetracyclines, thus we next determined the intracellular doxycycline accumulation after exposure to varying concentrations of benzydamine³¹. As expected, benzydamine supplementation remarkably enhanced the content of doxycycline in four strains (Fig. 3b). Tetracyclines act by specifically binding to the 30S subunit of the ribosome, thus inhibiting bacterial protein synthesis³². Therefore, the uptake and accumulation of tetracyclines are of importance for their antibacterial activity. Collectively, our results indicate that benzydamine dissipates the Δ Ψ , in turn, upregulates the Δ pH, thereby promoting the uptake of tetracyclines.

Doxycycline plus benzydamine is bactericidal against MDR bacteria and biofilm-producing bacteria. It has been widely acknowledged that tetracyclines belong to bacteriostatic antibiotics. We reasoned whether the benzydamine–doxycycline combination would possess bactericidal activity, which would markedly extend its therapeutic potential. To test this hypothesis, we performed time-killing experiments on various MDR pathogens. Impressively, a direct synergistic bactericidal effect was observed in rich growth conditions (Fig. 4a). Specifically, either 32 μ g/mL doxycycline or 250 μ g/mL benzydamine showed slight bactericidal activities. In comparison, the combination of doxycycline plus benzydamine (32 + 250 μ g/mL) exhibited excellent bactericidal activity, especially for *E. coli* B2. Besides, to determine whether benzydamine has the potency to combat metabolically repressed and non-replicating cells, we tested the bactericidal activity of this drug combination in the nutrient-free buffer. Remarkably, this combination retained potent bactericidal activity against *E. coli* B2 (Fig. 4b).

The formation of antibiotic-tolerant biofilms greatly affects the efficacy of antibiotics^{33,34}. To explore whether benzydamine supplementation can enhance doxycycline activity against the

biofilm-producing bacteria, we performed the formation and eradication of biofilms experiments in the presence or absence of different concentrations of benzydamine. As shown in Supplementary Fig. 5a, b, the combination of benzydamine at 50 μ g/mL with sub-MIC of doxycycline significantly inhibited biofilm formation of MRSA T144 and *E. coli* B2. Notably, in the biofilm inhibition assay, the benzydamine plus doxycycline at concentrations of ≤ 2 μ g/mL did not have bacteriostatic activity against two test strains, indicating that the inhibition of biofilm formation at these concentrations was not owing to the effect on bacterial growth. Besides, in the presence of benzydamine, the eradication effect of doxycycline on mature biofilm was markedly enhanced (Supplementary Fig. 5c, d). Taken together, we unexpectedly found that the combination of doxycycline plus benzydamine displays great bactericidal activity against various MDR pathogens in different metabolic states, including metabolically active cells, antibiotic-tolerant cells, and biofilm-producing bacteria.

Benzydamine aggravates oxidative damage and inhibits the function of MDR efflux pumps. Having shown that the synergistic bactericidal activity of the benzydamin–doxycycline combination, we reasoned that benzydamine may trigger other unknown modes of action except for the promotion of doxycycline uptake. To explore the underlying mechanisms, we performed transcription analysis of *E. coli* B2 under treatment with doxycycline or doxycycline plus benzydamine for 4 h. The comparison of treatment with combination to doxycycline alone revealed an upregulation of 35 differentially expressed genes (DEGs) and downregulation of 14 DEGs (Supplementary Fig. 6a). Go annotation analysis showed that these DEGs were involved in biological processes, cellular components, and molecular functions (Supplementary Fig. 6b). KEGG enrichment analysis displayed that these DEGs with increased expression were involved in ribosome synthesis, and DEGs with repressed expression was

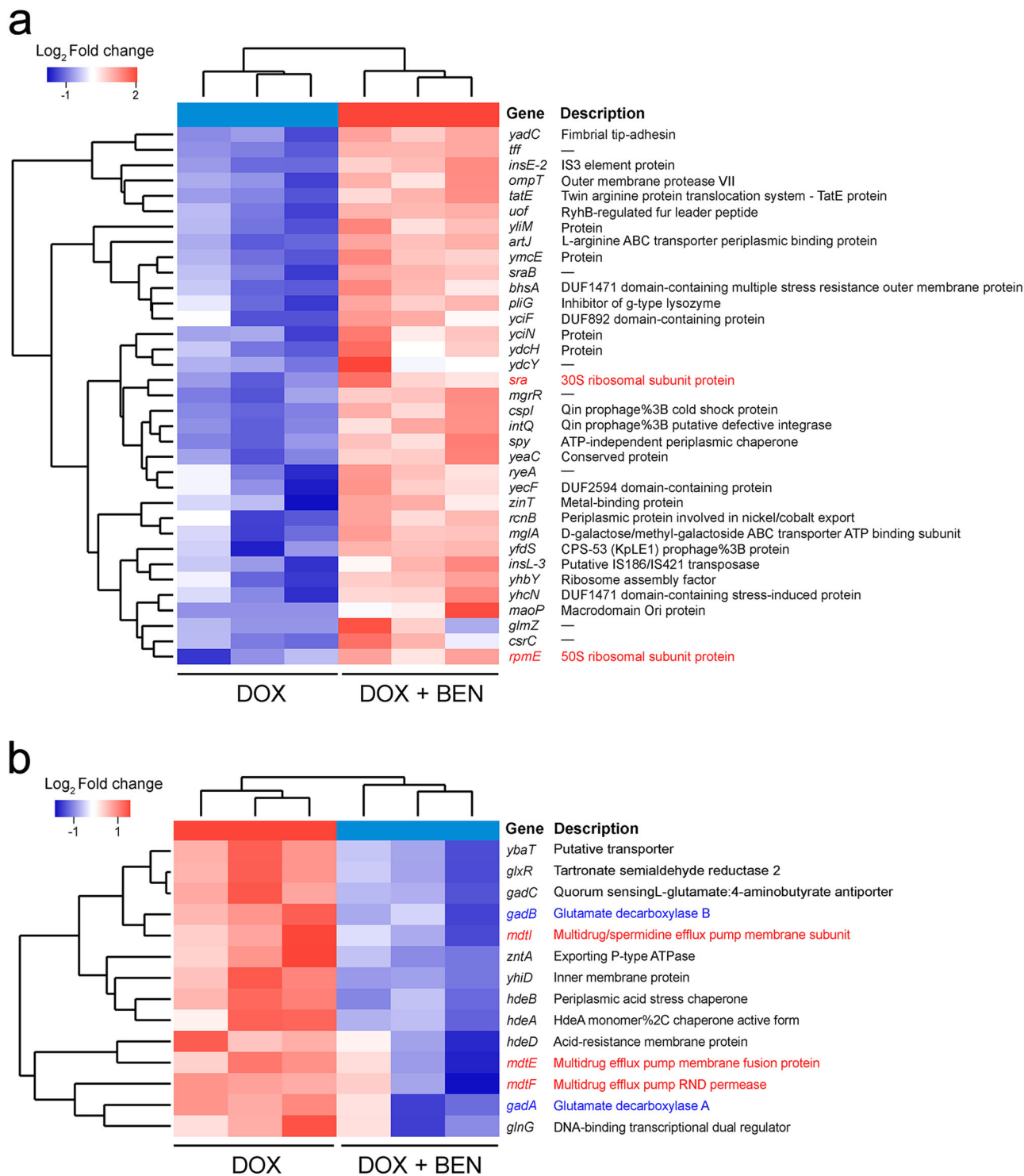


Fig. 5 Differentially expressed genes (DEGs) of *E. coli* B2 after treatment with doxycycline plus benzydamine in comparison to doxycycline alone. Significant upregulated (**a**, $P < 0.05$, $\text{Log}_2\text{Fold change} \geq 1$) and downregulated DEGs (**b**, $P < 0.05$, $\text{Log}_2\text{Fold change} \leq -1$) in combination treatment group compared with doxycycline monotreatment.

associated with glutamate metabolism and GABA shunt (Supplementary Fig. 6c, d). Notably, genes with 30S and 50S subunits were upregulated, which may be caused by increased accumulation of doxycycline that inhibits protein synthesis (Fig. 5a). In contrast, multidrug efflux pumps-related genes, glutamate decarboxylase (GAD) system-associated genes, and acid resistance-related genes were obviously decreased in the combination treatment group (Fig. 5b).

Based on the transcription results, we next performed a series of phenotype experiments to elucidate the other functions of benzydamine. First, we evaluated the activity of drug combinations in the different pH growth environments via time-killing experiments. We found that it had almost no bactericidal effect in the acid media, however, this combination showed enhanced bactericidal activity while a change in pH to alkaline values. After treatment for 4 h, the bacteria were all killed in the MHB broth at

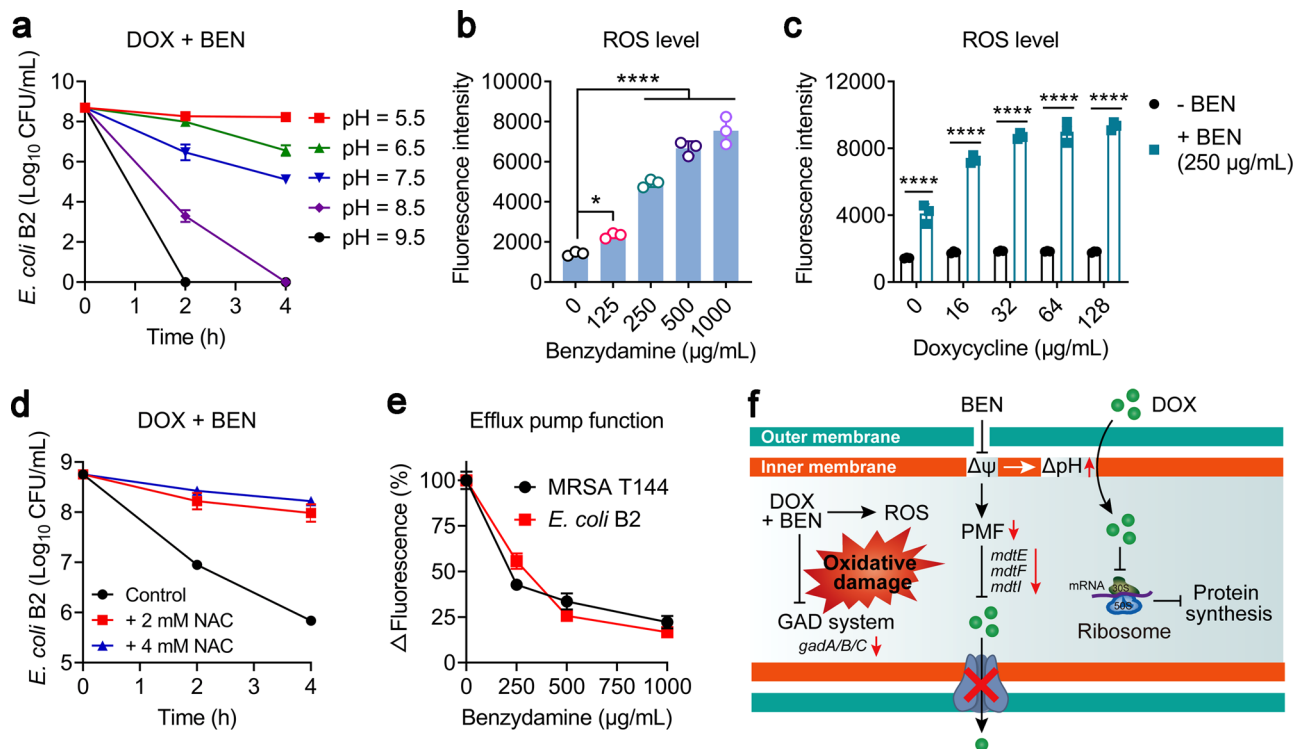


Fig. 6 Benzylamine promotes oxidative damage and inhibits the function of the efflux pump in *E. coli*. **a** Time-killing curves of *E. coli* B2 after treatment with the combination of doxycycline and benzylamine in different pH media from 5.5 to 9.5. **b** Benzylamine promotes the production of ROS in a dose-dependent manner. **c** Doxycycline plus benzylamine (250 µg/mL) shows higher ROS generation compared to doxycycline alone. Fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to monitor the levels of ROS in cells ($\lambda_{\text{excitation}} = 488 \text{ nm}$, $\lambda_{\text{emission}} = 525 \text{ nm}$). All data from three biological replicates were presented as mean \pm SD, and the significance was determined by non-parametric one-way ANOVA ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). **d** The addition of ROS scavenger *N*-acetylcysteine weakens the potentiation of benzylamine to doxycycline. **e** Benzylamine drastically impairs the function of efflux pumps in both MRSA T144 and *E. coli* B2. Rhodamine B ($\lambda_{\text{excitation}} = 540 \text{ nm}$, $\lambda_{\text{emission}} = 625 \text{ nm}$) was used to characterize the activity of efflux pumps in bacteria. **f** Schematic illustrations of the potentiating mechanisms of benzylamine to tetracyclines in the fight against drug-resistant pathogens.

pH 8.5 and 9.5 (Fig. 6a). These data were in agreement with the previous observations that the antibacterial activity of benzylamine was strengthened in the alkaline conditions and the genes associated with acid resistance were downregulated. Recently, several studies reported that GAD systems, which were downregulated in the combination group, play a critical role in protecting bacteria against oxidative stress^{35,36}, we hypothesized that the potentiation of benzylamine to antibiotics may also correlate to enhanced oxidative damage. Thus, we tested the generation of reactive oxygen species (ROS)³⁷ in *E. coli* B2 treated by either benzylamine or in combination with doxycycline. Surprisingly, benzylamine markedly promoted the generation of ROS in a dose-dependent manner (Fig. 6b). Meanwhile, the combination treatment showed higher ROS levels compared to doxycycline monotherapy (Fig. 6c). Accordingly, ROS has been recognized as one of the common mechanisms in the antibiotic-mediated killing of bacteria. The over-production of ROS in the benzylamine–doxycycline combination gives an interpretation of their synergistic bactericidal activity. To further verify it, *N*-acetyl-L-cysteine (NAC), a ROS scavenger, was added in time-killing assays. As shown in Fig. 6d, the potentiation of benzylamine to doxycycline was greatly impaired when incubation with 2 or 4 mM NAC. Finally, we used a fluorescent dye rhodamine B to assay the function of the efflux pump in bacteria under the treatment of benzylamine (Fig. 6e). As a result, it showed that the activity of the efflux pump was dose-dependently suppressed by benzylamine, which further promoted the accumulation of doxycycline in MDR bacteria. Collectively, these data together demonstrate that benzylamine enhances oxidative

damage via triggering the production of ROS and inhibiting the function of MDR efflux pumps (Fig. 6f).

Benzylamine rescues doxycycline efficacy in vivo infection models. In view of the excellent synergy of doxycycline and benzylamine against MDR pathogens *in vitro*, we tested whether they have potent activity *in vivo* using two animal infection models infected by MRSA T144 or *E. coli* B2. First, we used *G. mellonella* larvae infection models to explore their efficacy *in vivo*. As shown in Fig. 7a, the infected larvae with the combination therapy of doxycycline plus benzylamine (50 + 50 mg/kg) resulted in above 80% survival during 5 days, which was higher than the doxycycline monotherapy ($P = 0.0174$ or 0.0397 , corresponding to MRSA T144 and *E. coli* B2, respectively). In addition, the efficacy of this combination therapy in a neutropenic mouse thigh infection model was evaluated (Fig. 7b). Similarly, a single dose of doxycycline plus benzylamine (50 + 10 or 50 + 50 mg/kg) reduced bacterial burden in mice thighs compared with doxycycline alone ($P < 0.0001$). These data demonstrate the benzylamine plus doxycycline has a great synergy effect *in vivo*.

Discussion

The occurrence of MDR phenotype in pathogenic bacteria undermines the clinical efficacy of antibiotics and leaves no available options for the treatment of intractable infections^{38,39}. Despite many ongoing efforts in identifying new classes of antimicrobial agents^{40,41}, few antibiotics have been approved for clinical use in the past 20 years. Accordingly, the average cost of

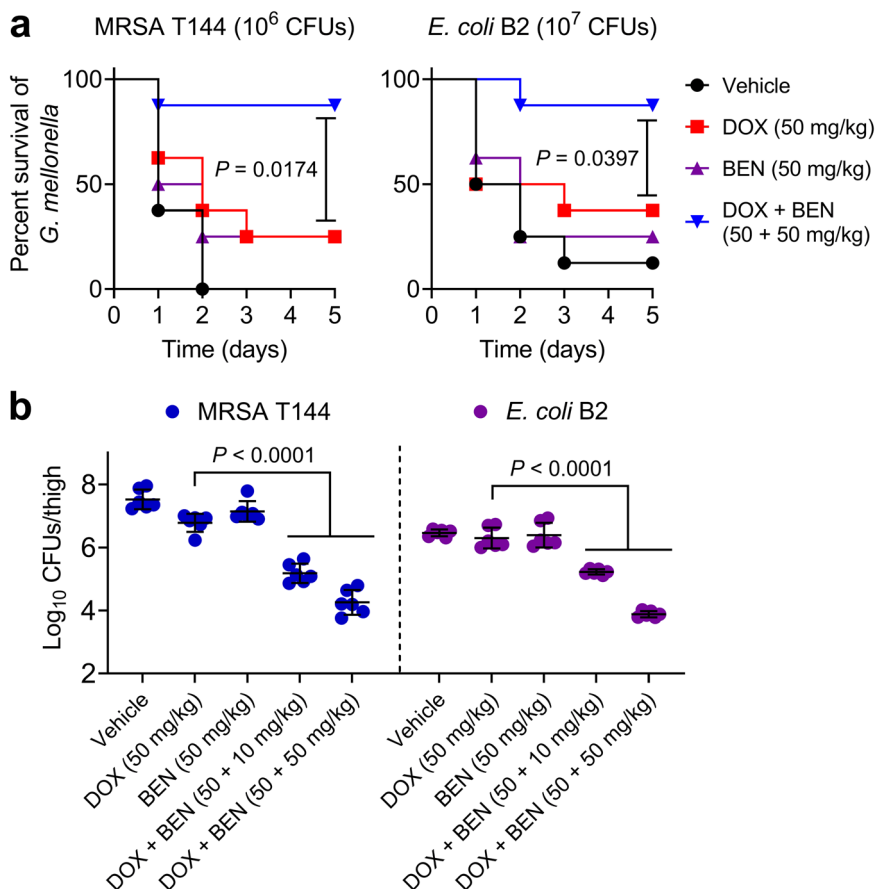


Fig. 7 Combination of doxycycline and bezzydamine is efficacious in various animal infection models. **a** Survival rates of *Galleria mellonella* ($n = 8$ biologically independent animals per group) infected by MRSA T144 or *E. coli* B2 and then treated with doxycycline (50 mg/kg) or bezzydamine (50 mg/kg) alone or a combination of doxycycline plus bezzydamine (50 + 50 mg/kg). P values were determined using the two-sided log-rank (Mantel-Cox) test. **b** Combination of doxycycline (50 mg/kg) and bezzydamine (50 mg/kg) significantly reduces the thigh bacterial loads of mice ($n = 6$ biologically independent animals per group) infected by MRSA T144 or *E. coli* B2 (10^5 CFUs per mouse) compared with doxycycline monotherapy (50 mg/kg). P values were calculated using a two-sided Mann-Whitney U test.

research and development of a new drug from discovery to regulatory approval is US\$2.6 billion, which takes >10 years, and the successful launch is less than one thousandth^{42,43}. As such, alternative strategies are warranted to confront this serious global crisis. Repurposing non-antibacterial drugs as potential antibiotic adjuvants are gaining traction in both the public and private sector⁴⁴. In this study, we revealed the synergistic effect of bezzydamine, a widely used non-steroidal anti-inflammatory drug in the clinic, in combination with three classes of antibiotics against MDR *E. coli* B2. Most importantly, bezzydamine displayed the greatest synergistic activity with tetracyclines, which belong to broad-spectrum antibiotics. For various MDR pathogens, including MRSA, VRE, NDM/MCR/Tet(X)-expressing Gram-negative bacteria, the doxycycline–bezzydamine combination showed unprecedented synergistic activity. Biofilm-producing bacteria are important causes of chronic and recurrent bacterial infection but are commonly overlooked in drug discovery. We found that the doxycycline–bezzydamine combination is able to prevent the formation of *S. aureus* and *E. coli* biofilm, as well as eradicate the established biofilms. These data further support the notion that bezzydamine is a potential antibiotic adjuvant candidate to reverse bacterial resistance. Consistent with our finding, the prior study provided a preliminary observation regarding the antibacterial activity of the bezzydamine–tetracyclines combination, whereas the antibacterial spectrum, mechanisms of action, and in vivo potency are not explored⁴⁵.

Bacterial energy metabolism such as PMF has a critical role in cellular activities including material transport, flagellar motility, and ATP synthesis by the F_1F_0 -ATPase⁴⁶. The disruption of PMF would inhibit the basic functions of bacteria and accelerate its death. Recently, using a deep learning approach, a new broad-spectrum antibiotic termed halicin was identified to selectively destroy the PMF⁴⁷. Molecules I1- I3 and D1-D3, the potential modulators of PMF, showed killing activity against MRSA by preventing electron transport and ATP synthesis²⁸. Generally, PMF is comprised of $\Delta\Psi$ and ΔpH . To maintain the bacterial PMF, dissipation of either component would be compensatory increased by another. In our study, we uncovered that bezzydamine dissipated the $\Delta\Psi$ component of PMF in both Gram-positive and Gram-negative bacteria, in turn, increased the ΔpH , which was critical for the uptake of tetracyclines. These findings were consistent with the previous studies that tetracyclines uptake is driven by ΔpH ⁴⁸, whereas aminoglycosides uptake is highly dependent on $\Delta\Psi$ ⁴⁹. In addition, the successful paradigm of daptomycin depolarizing the cytoplasmic membrane provides a proof-of-concept for PMF-targeted antimicrobial agents⁵⁰. These examples suggest that bacterial PMF is a promising target for the development of novel antimicrobial agents and antibiotic adjuvants. Toxicity concerns are critical factors that limit the clinical trials of new drugs⁵¹. Meaningfully, our experiments tentatively demonstrated that the combination of doxycycline and bezzydamine exhibited negligible toxicity in a murine model.

Nevertheless, considering the potential side effects of benzydamine in clinic such as nausea and vomiting⁵², more preclinical studies are needed for exploring the therapeutic potential of this particular combination. Moreover, we proposed that the structure optimization of benzydamine and the use of functionalized drug delivery system⁵³ may contribute to reducing the toxicity and improving the effectiveness of this drug combination.

Furthermore, the doxycycline–benzydamine combination showed excellent synergistic bactericidal activity for all test MDR isolates, implying that the action of benzydamine is not merely to promote the uptake of tetracyclines. Transcriptomic analysis coupled with phenotype experiments indicated that benzydamine not only triggered the generation of ROS, but also downregulated the GAD system that protects bacteria from oxidative damage. These modes of action resulted in an oxidative burst, which has been proved to be important for antibiotic-mediated killing. In addition, the functions of MDR efflux pumps in bacteria were severely destroyed, partly owing to the dissipation of PMF by benzydamine. It would be interesting to investigate the potential of benzydamine as a new and broad-spectrum inhibitor of MDR efflux pumps.

To conclude, our findings reveal that non-steroidal anti-inflammatory drug benzydamine may serve as a potential antibiotic adjuvant to restore the activity of clinically relevant antibiotics particularly tetracyclines against infections caused by MDR pathogens. In addition, the elucidation of modes of action of benzydamine highlights the remarkable potential of PMF downregulators as a feasible adjuvant therapy to tackle the escalating concern of antibiotic resistance.

Materials and methods

Bacterial strains and reagents. All strains used in this study were listed in Supplementary Table 1. The bacteria were stored in nutrient broth supplemented with 20% (v/v) glycerol at -80°C . For experiments, all strains were grown in Mueller-Hinton Broth (MHB) or on LB agar (LBA) plates. Antibiotics were obtained from China Institute of Veterinary Drug Control and other chemical reagents were purchased from Aladdin (Shanghai, China) or TCI (Shanghai, China).

MIC determinations. The MICs of all antibiotics and benzydamine were determined using the broth dilution method, according to the CLSI 2018 guideline⁵⁴. All drugs were twofold diluted in MHB and equally mixed with bacterial suspensions in a 96-well microtiter plate (Corning, New York, USA). After 16–20 h incubation at 37°C , the MICs were defined as the lowest concentrations of drugs that no bacteria can be detected.

Checkerboard analyses and FIC index determination. The fractional inhibitory concentrations (FIC) indices were measured by the checkerboard analyses⁵⁵. In brief, 100 μL of MHB was added into each well of a 96-well plate with an 8×8 matrix, then the antibiotics and compounds were twofold diluted along the abscissa and ordinate, respectively. After incubated at 37°C for 18 h with bacterial suspension (10^5 CFUs/well), the absorbance of each well at 600 nm was determined. The FIC was calculated as the MIC when the compound is used in combination divided by the MIC when it is used alone. The FICI is the sum of the FICs of two compounds, and synergy is defined with the FIC index ≤ 0.5 .

Hemolysis analysis. Hemolytic activity of doxycycline or in combination with benzydamine was assessed based on a previous study⁵⁶. In brief, 8% of sheep blood cells were equal to volume incubated with 0–256 $\mu\text{g}/\text{mL}$ doxycycline alone or in combination with 250 $\mu\text{g}/\text{mL}$ benzydamine at 37°C for 1 h. Phosphate buffer saline (PBS) and double-distilled water were used as a negative and positive control, respectively. The absorbance of released hemoglobin was determined at 576 nm using an Infinite M200 Microplate reader (Tecan, Männedorf, Switzerland). Hemolysis rate (%) was calculated by the result of absorbance of the sample subtracting the negative control divided by the positive control subtracting the negative control.

In vivo toxicity of benzydamine–doxycycline combination. The in vivo toxicity was evaluated by gavaging a combination of doxycycline plus benzydamine (10 + 10 mg/kg) to female CD-1 mice ($n = 6$ per group). Mice were continuously gavaged for 6 days and body weights were recorded daily. On the seventh day, blood was collected for blood biochemical tests and cell analysis.

Inhibition of biofilm formation. MRSA T144 and *E. coli* B2 suspensions (1×10^7 CFUs per mL) were exposed to doxycycline solutions (final concentrations ranging from 0.125 to 2 $\mu\text{g}/\text{mL}$) in the presence or absence of 50 $\mu\text{g}/\text{mL}$ benzydamine. As a negative control, bacteria were exposed to MHB without drugs. Bacteria were grown for 36 h at 37°C under static conditions, and then 300 μL PBS was used to remove the planktonic bacteria. Then, 200 μL of methanol was added to fix for 15 min, after that, the fixative was aspirated to air dry and 0.1% crystal violet was added for staining for 15 min. The dye solution was removed and stained-biofilm was washed three times with PBS and dried naturally. At last, crystal violet-stained biofilms were solubilized with 33% glacial acetic acid (100 μL) and incubated at 37°C for 30 min. Biofilm mass was determined by monitoring the absorbance of the supernatant at 570 nm⁵⁷.

Biofilm eradication assay. Overnight MRSA T144 and *E. coli* B2 were diluted 1:100 into MHB and incubated at 37°C with shaking at 200 rpm for 6 h. Subsequently, 100 μL bacterial suspensions were mixed with an equal volume of MHB in 96-well microtitre plate. After 36 h incubation at 37°C , the planktonic bacteria were removed. Next, biofilms were treated with 32–256 $\mu\text{g}/\text{mL}$ doxycycline alone or in combination with 50 $\mu\text{g}/\text{mL}$ benzydamine. After 2 h incubation at 37°C , the remaining cells were dispersed via ultrasonic treatment for 20 min. Finally, the mixed liquor was resuspended in sterile PBS and then the dilutions were plated on LBA plates and incubated overnight at 37°C .

Measurement of membrane potential. The membrane potential of *S. aureus* ATCC 29213, MRSA T144, *E. coli* ATCC 25922, and *E. coli* B2 was tested by the fluorescent probe DiSC₃(5) (Aladdin, Shanghai, China). Bacterial cells were grown to the log phase in MHB, then washed with PBS to an OD₆₀₀ of 0.5 and incubated with DiSC₃(5) (0.5×10^{-6} M) for 30 min. Finally, varying concentrations of benzydamine (10 μL) were added into the 190 μL of DiSC₃(5)-loaded cells. For all membrane potential experiments, the fluorescence intensity was measured with the excitation wavelength at 622 nm and emission wavelength at 670 nm using a Microplate reader (Tecan, Männedorf, Switzerland).

Swimming motility experiment. In all, 0.3% (w/v) agar media composed of trypticase peptone (10 g/L), NaCl (10 g/L), and yeast extract (5 g/L) was used to assess bacterial swimming motility³¹. After the medium reached 50°C , the final concentrations of benzydamine at 0, 31.25, 62.5, 125, and 250 $\mu\text{g}/\text{mL}$ were added. A 2 μL volume of *S. aureus* 29213, MRSA T144, *E. coli* 25922, and *E. coli* B2 culture at an OD₆₀₀ of 0.5 was placed in the center of each plate and allowed to stay for 30 min. The plates were placed in a 37°C incubator for 48 h.

Measurement of intracellular pH values. Overnight *S. aureus* ATCC 29213, MRSA T144, *E. coli* ATCC 25922, and *E. coli* B2 were resuspended to OD₆₀₀ of 0.5 with PBS, and the final concentration of pH-sensitive fluorescent probe BCECF-AM³⁰ (2×10^{-6} M for Gram-negative bacteria and 0.5×10^{-6} M for Gram-positive bacteria) was added. After incubation for 30 min, four strains were treated with the final concentration of benzydamine (125–1000 $\mu\text{g}/\text{mL}$). The fluorescence intensity was immediately monitored with the excitation wavelength of 488 nm and emission wavelength of 535 nm.

Uptake of doxycycline. Doxycycline uptake was evaluated by monitoring the fluorescence change of the drug in bacteria³¹. Culture of MRSA T144 and *E. coli* B2 were grown to an OD₆₀₀ of 0.5. Cells were centrifuged at 3500 rpm for 10 min and washed in an equal volume of PBS three times. Subsequently, doxycycline at MIC alone or with a final concentration of benzydamine at 250–1000 $\mu\text{g}/\text{mL}$ was added into the 96-wells plates containing cell suspensions at 100 $\mu\text{L}/\text{well}$. An infinite Microplate reader was used to monitor the fluorescence intensity with the excitation wavelength of 405 nm and emission wavelength of 535 nm.

Measurement of ROS levels. The fluorescence probe 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA, 10 μM)⁵⁸ (Beyotime, Shanghai, China) was used to test the levels of ROS in *E. coli* B2 treated by benzydamine, doxycycline, or their combination. After incubation 1 h, the fluorescence intensity was detected with the excitation and emission wavelength of 488 and 525 nm, respectively.

Efflux pump assay. A fluorescence dye, rhodamine B⁵⁹ has been applied to assay the inhibition of efflux pump of *E. coli* B2 and MRSA T144 treated by benzydamine. Bacterial cells were grown in MHB broth to mid-log phase (OD₆₀₀ = 0.5) at 37°C with shaking 200 rpm, then the cultures were washed and suspended with PBS. Subsequently, a final concentration of rhodamine B (Aladdin, Shanghai, China) (5×10^{-6} M) was added. After incubation at 37°C for 30 min, the cultures were washed and suspended with PBS containing 1% glucose. Next, probe labeled cells were treated with varying concentrations of benzydamine. After incubation at 37°C for 30 min, bacterial cells were centrifuged at 6000 rpm at 4°C for 5 min and the supernatant was collected for fluorescence analysis with the excitation wavelength of 540 nm and emission wavelength of 625 nm.

Time-dependent killing curves. Overnight culture of *E. coli* B2, *K. pneumoniae* D120 (*mcr-8*), *A. baumannii* C222 (*tet(X6)*), MRSA T144, and VRE A4 were diluted 1/1000 in MHB, and incubated for 4 h at 37 °C with shaking at 200 rpm. Bacteria were then treated with either PBS, doxycycline (16 or 32 µg/mL), or benzydamine (250 µg/mL) alone or their combination. At the time points 0, 4, 8, 12, and 24 h, 100 µL aliquots were removed, centrifuged, and resuspended in sterile PBS, the dilutions were plated on LBA plates, and incubated overnight at 37 °C.

Transcriptomic analysis. The final concentration of doxycycline (32 µg/mL) alone or in the combination with benzydamine (250 µg/mL) was added to the early exponential-phase *E. coli* B2. After incubation for 4 h, the total RNA of culture was extracted by an EASYspin Plus kit (Aidlab, Beijing, China) and quantified by using a Nanodrop spectrophotometer (Thermo Scientific, MA, USA). Subsequently, sequenced on HiSeq2000 with Truseq SBS Kit v3-HS (200 cycles) (Illumina) with the read length as 2 × 100 (PE100). Raw sequencing reads were filtered and mapped against the reference genome of *E. coli* K-12. The FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method was used to identify differentially expressed genes with *p* values ≤ 0.05 and fold change (FC) values ≥ 2 (log₂ FC ≥ 1 or log₂ FC ≤ -1). Differences between these two treatments were studied by Cuffdiff program (<http://cufflinks.cbcb.umd.edu/>).

Galleria mellonella infection model. *Galleria mellonella* larvae (Huiyude Biotech Company, Tianjin, China) were divided into four groups (*n* = 8 per group) and infected with MRSA T144 (10⁶ CFU_s) or *E. coli* B2 (10⁷ CFU_s) suspension. After 1 h post infection, group 1 was subjected to PBS treatment, groups 2 and 3 were treated with doxycycline or benzydamine (50 mg/kg) respectively, group 4 was treated with doxycycline plus benzydamine (50 + 50 mg/kg). Survival rates of *Galleria mellonella* larvae were recorded for 5 days.

Neutropenic mouse thigh infection model. 6–8-week-old female CD-1 mice were obtained from the Comparative Medicine Centre of Yangzhou University (Jiangsu, China). Mice studies were performed in accordance with the relevant guidelines of Jiangsu Laboratory Animal Welfare and Ethical of Jiangsu Administrative Committee of Laboratory Animals (permission number, SYXKSU-2007-0005). The laboratory animal usage license number is SCXK-2017-0044, certified by Jiangsu Association for Science and Technology.

Female CD-1 mice (*n* = 6 per group) were treated with cyclophosphamide with 150 mg/kg in the 4 days before infection, and 100 mg/kg in the 1 day before infection. The exponential-phase MRSA T144 or *E. coli* B2 suspension (100 µL, 10⁵ CFU_s per mouse) was injected into the right thighs of mice. After 2 h of infection, a single dose of PBS, doxycycline (50 mg/kg), benzydamine (50 mg/kg), or combinations (50 + 10 mg/kg, 50 + 50 mg/kg) was administered. At 48 h post infection, mice were euthanized due to dislocation of the cervical spine. The right thigh muscle was aseptically resected, homogenized, serially diluted, and plated on LBA to determine bacterial numbers.

Statistics and reproducibility. Statistical analysis was performed using GraphPad Prism version 8.3.0. All data from at least three biological replicates are shown as mean ± SD. Unless otherwise noted, unpaired *t* test between two groups or one-way analysis of variance among multiple groups were used to calculate *P* values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

RNA-sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (PRJNA766533). Source data for the main figures are provided in Supplementary Data 1. All other data are available from the corresponding authors.

Received: 29 June 2021; Accepted: 4 November 2021;

Published online: 25 November 2021

References

- Harrison, E. & Brockhurst, M. A. Plasmid-mediated horizontal gene transfer is a coevolutionary process. *Trends Microbiol.* **20**, 262–267 (2012).
- De Oliveira, D. M. P. et al. Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* **33**, e00181–19 (2020).
- Ma, Y.-X. et al. Considerations and caveats in combating ESKAPE pathogens against nosocomial infections. *Adv. Sci.* **7**, 1901872 (2020).
- Gupta, N., Limbago, B. M., Patel, J. B. & Kallen, A. J. Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clin. Infect. Dis.* **53**, 60–67 (2011).
- Liu, Y.-Y. et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* **16**, 161–168 (2016).
- Sun, J. et al. Plasmid-encoded *tet(X)* genes that confer high-level tetracycline resistance in *Escherichia coli*. *Nat. Microbiol.* **4**, 1457–1464 (2019).
- He, T. et al. Emergence of plasmid-mediated high-level tetracycline resistance genes in animals and humans. *Nat. Microbiol.* **4**, 1450–1456 (2019).
- Liu, Y., Ding, S., Shen, J. & Zhu, K. Nonribosomal antibacterial peptides that target multidrug-resistant bacteria. *Nat. Prod. Rep.* **36**, 573–592 (2019).
- Lewis, K. The science of antibiotic discovery. *Cell* **181**, 29–45 (2020).
- Wright, G. D. Antibiotic adjuvants: rescuing antibiotics from resistance. *Trends Microbiol.* **24**, 862–871 (2016).
- Liu, Y., Li, R., Xiao, X. & Wang, Z. Antibiotic adjuvants: an alternative approach to overcome multi-drug resistant Gram-negative bacteria. *Crit. Rev. Microbiol.* **45**, 301–314 (2019).
- Liu, Y. et al. Drug repurposing for next-generation combination therapies against multidrug-resistant bacteria. *Theranostics* **11**, 4910–4928 (2021).
- Liu, Y. et al. Metformin restores tetracyclines susceptibility against multidrug resistant bacteria. *Adv. Sci.* **7**, 1902227 (2020).
- Liu, Y. et al. Melatonin overcomes MCR-mediated colistin resistance in Gram-negative pathogens. *Theranostics* **10**, 10697–10711 (2020).
- Liu, Y. et al. Anti-HIV agent azidothymidine decreases Tet(X)-mediated bacterial resistance to tetracycline in *Escherichia coli*. *Commun. Biol.* **3**, 162 (2020).
- Zhou, Y.-F. et al. Activity of tetracycline or colistin in combination with zidovudine against harboring *tet(X)* and *mcr-1*. *Antimicrob. Agents Chemother.* **65**, e011172–01120 (2020).
- Maisuria, V. B., Okshevsky, M., Deziel, E. & Tufenkji, N. Proanthocyanidin interferes with intrinsic antibiotic resistance mechanisms of Gram-negative bacteria. *Adv. Sci.* **6**, 1802333 (2019).
- Lyu, Y. F. et al. Amphiphilic tobramycin-lysine conjugates sensitize multidrug resistant Gram-negative bacteria to rifampicin and minocycline. *J. Med. Chem.* **60**, 3684–3702 (2017).
- Avvisati, R. et al. Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action. *Addict. Biol.* **23**, 610–619 (2018).
- Nettis, E., Di Paola, R., Napoli, G., Ferrannini, A. & Tursi, A. Benzydamine: an alternative nonsteroidal anti-inflammatory drug in patients with nimesulide-induced urticaria. *Allergy* **57**, 442–445 (2002).
- Son, H. S. et al. Benzydamine inhibits osteoclast differentiation and bone resorption via down-regulation of interleukin-1β expression. *Acta Pharm. Sin.* **10**, 462–474 (2020).
- Rastogi, M. et al. Role of benzydamine hydrochloride in the prevention of oral mucositis in head and neck cancer patients treated with radiotherapy (>50 Gy) with or without chemotherapy. *Support. Care Cancer* **25**, 1439–1443 (2017).
- Lakshmaiah Narayana, J. et al. Two distinct amphipathic peptide antibiotics with systemic efficacy. *Proc. Natl. Acad. Sci. USA* **117**, 19446–19454 (2020).
- Farha, M. A., French, S., Stokes, J. M. & Brown, E. D. Bicarbonate alters bacterial susceptibility to antibiotics by targeting the proton motive force. *ACS Infect. Dis.* **4**, 382–390 (2018).
- Mitchell, P. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966. *Biochim. Biophys. Acta* **1807**, 1507–1538 (2011).
- Chen, H. et al. Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. *Lett. Appl. Microbiol.* **47**, 180–186 (2008).
- Liu, Y. et al. Potent broad-spectrum antibacterial activity of amphiphilic peptides against multidrug-resistant bacteria. *Microorganisms* **8**, 1398 (2020).
- Farha, M. A., Verschoor, C. P., Bowdish, D. & Brown, E. D. Collapsing the proton motive force to identify synergistic combinations against *Staphylococcus aureus*. *Chem. Biol.* **20**, 1168–1178 (2013).
- Brunelle, B. W., Bearson, B. L., Bearson, S. M. D. & Casey, T. A. Multidrug-resistant *Salmonella enterica* serovar Typhimurium isolates are resistant to antibiotics that influence their swimming and swarming motility. *mSphere* **2**, e00306–e00317 (2017).
- Ozkan, P. & Mutharasan, R. A rapid method for measuring intracellular pH using BCECF-AM. *Biochim. Biophys. Acta* **1572**, 143–148 (2002).
- Ejim, L. et al. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol.* **7**, 348 (2011).
- Chopra, I. & Roberts, M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **65**, 232–260 (2001).
- Hall, C. W. & Mah, T.-F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol. Rev.* **41**, 276–301 (2017).
- Yan, J. & Bassler, B. L. Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms. *Cell Host Microbe* **26**, 15–21 (2019).
- Boura, M., Brensone, D. & Karatzas, K. A. G. A novel role for the glutamate decarboxylase system in *Listeria monocytogenes*; protection against oxidative stress. *Food Microbiol.* **85**, 103284 (2020).

36. Feehily, C. & Karatzas, K. A. G. Role of glutamate metabolism in bacterial responses towards acid and other stresses. *J. Appl. Microbiol.* **114**, 11–24 (2013).
37. Voorhees, A. J. M. A. K. J. Detection of cyclic lipopeptide biomarkers from bacillus species using atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **75**, 1628–1673 (2003).
38. Magiorakos, A. P. et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **18**, 268–281 (2012).
39. Falagas, M. E. & Karageorgopoulos, D. E. Pandrug resistance (PDR), extensive drug resistance (XDR), and multidrug resistance (MDR) among Gram-negative bacilli: need for international harmonization in terminology. *Clin. Infect. Dis.* **46**, 1121–1122 (2008).
40. Luther, A. et al. Chimeric peptidomimetic antibiotics against Gram-negative bacteria. *Nature* **576**, 452–458 (2019).
41. Imai, Y. et al. A new antibiotic selectively kills Gram-negative pathogens. *Nature* **576**, 459–464 (2019).
42. Avorn, J. The \$2.6 billion pill—methodologic and policy considerations. *N. Engl. J. Med.* **372**, 1877–1879 (2015).
43. DiMasi, J. A., Grabowski, H. G. & Hansen, R. W. Innovation in the pharmaceutical industry: new estimates of R&D costs. *J. Health Econ.* **47**, 20–33 (2016).
44. Tyers, M. & Wright, G. D. Drug combinations: a strategy to extend the life of antibiotics in the 21st century. *Nat. Rev. Microbiol.* **17**, 141–155 (2019).
45. Fanaki, N. H. & El-Nakeeb, M. A. Antibacterial activity of benzydamine and antibiotic-benzzydamine combinations against multifold resistant clinical isolates. *Arzneimittelforschung* **46**, 320–323 (1996).
46. Paul, K., Erhardt, M., Hirano, T., Blair, D. F. & Hughes, K. T. Energy source of flagellar type III secretion. *Nature* **451**, 489–492 (2008).
47. Stokes, J. M. et al. A deep learning approach to antibiotic discovery. *Cell* **180**, 688–702 (2020).
48. Yamaguchi, A., Ohmori, H., Kaneko-Ohdera, M., Nomura, T. & Sawai, T. Delta pH-dependent accumulation of tetracycline in *Escherichia coli*. *Antimicrob. Agents Chemother.* **35**, 53–56 (1991).
49. Taber, H. W., Mueller, J. P., Miller, P. F. & Arrow, A. S. Bacterial uptake of aminoglycoside antibiotics. *Microbiol. Rev.* **51**, 439–457 (1987).
50. Hawkey, P. M. Pre-clinical experience with daptomycin. *J. Antimicrob. Chemother.* **62**, iii7–iii14 (2008).
51. Segall, M. D. & Barber, C. Addressing toxicity risk when designing and selecting compounds in early drug discovery. *Drug Discov. Today* **19**, 688–693 (2014).
52. Altinsoy, S., Ütebey, G., Kavak Akelma, F. & Ergil, J. The effects of topical chlorhexidine-benzzydamine spray on laryngeal mask airway application. *Minerva Anesthesiol.* **86**, 277–285 (2020).
53. Pham, T.-N., Loupias, P., Dassonville-Klimpt, A. & Sonnet, P. Drug delivery systems designed to overcome antimicrobial resistance. *Med. Res. Rev.* **39**, 2343–2396 (2019).
54. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. *CLSI* (2018).
55. Song, M. et al. A broad-spectrum antibiotic adjuvant reverses multidrug-resistant Gram-negative pathogens. *Nat. Microbiol.* **5**, 1040–1050 (2020).
56. Liu, Y., Ding, S., Dietrich, R., Märklbauer, E. & Zhu, K. A biosurfactant-inspired heptapeptide with improved specificity to kill MRSA. *Angew. Chem. Int. Ed.* **56**, 1486–1490 (2017).
57. De, A. B. et al. The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms. *Sci. Transl. Med.* **10**, eaan4044 (2018).
58. Aranda, A. et al. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: a quantitative method for oxidative stress assessment of nanoparticle-treated cells. *Toxicol In Vitro* **27**, 954–963 (2013).
59. Forster, S., Thumser, A. E., Hood, S. R. & Plant, N. Characterization of rhodamine-123 as a tracer dye for use in in vitro drug transport assays. *PLoS One* **7**, e33253 (2012).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32172907 and 32002331), National Key Research and Development Program of China (2018YFA0903400), Natural Science Foundation of Jiangsu Province of China (BK20190893), Agricultural Science and Technology Independent Innovation Fund of Jiangsu Province (CX(20)3091 and CX(21)2010), China Postdoctoral Science Foundation funded project (2019M651984 and 2021T140579), A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and Young Elite Scientists Sponsorship Program by CAST (2020QNR001).

Author contributions

Y.L. and Z.W. design and conceived the project. Z.T., J. S., Y.J. and T.D. performed all of the experiments. Z.T. and J.S. performed MIC experiments, checkboard assay, biofilm studies, fluorescence probe-related experiments, and transcriptomic analysis. Y.J. and T.D. performed safety evaluation, swimming motility experiment, and animal studies. Y.L. and Z.T. analyzed the data and wrote/revised the manuscript. All of the authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information


Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s42003-021-02854-z>.

Correspondence and requests for materials should be addressed to Yuan Liu or Zhiqiang Wang.

Peer review information *Communications Biology* thanks the anonymous reviewers for their contribution to the peer review of this work. Primary Handling Editors: Cesar de la Fuente, Anam Akhtar, and Brooke LaFlamme.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021