Small Molecule IITR08367 Potentiates Antibacterial Efficacy of Fosfomycin against *Acinetobacter baumannii* by Efflux Pump Inhibition

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ABSTRACT: Fosfomycin is a broad-spectrum single-dose therapy approved for treating lower urinary tract infections. *Acinetobacter baumannii*, one of the five major UTI-causing pathogens, is intrinsically resistant to fosfomycin. Reduced uptake and active efflux are major reasons for this intrinsic resistance. AbaF, a major facilitator superfamily class of transporter in *A. baumannii*, is responsible for fosfomycin efflux and biofilm formation. This study describes the identification and validation of a novel small-molecule efflux pump inhibitor that potentiates fosfomycin efficacy against *A. baumannii*. An AbaF inhibitor screening was performed against *Escherichia coli* KAM32/pUC18_abaF, using the non-inhibitory concentration of 24 putative efflux pump inhibitors. The inhibitory activity of IITR08367 [bis(4-methylbenzyl) disufide] against fosfomycin/H$^+$ antiport was validated using ethidium bromide efflux, quinacrine-based proton-sensitive fluorescence, and membrane depolarization assays. IITR08367 inhibits fosfomycin/H$^+$ antiport activity by perturbing the transmembrane proton gradient. IITR08367 is a nontoxic molecule that potentiates fosfomycin activity against clinical strains of *A. baumannii* and prevents biofilm formation by inhibiting efflux pump (AbaF). The IITR08367-fosfomycin combination reduced bacterial burden by > 3 log$_{10}$ in kidney and bladder tissue in the murine UTI model. Overall, fosfomycin, in combination with IITR08367, holds the potential to treat urinary tract infections caused by *A. baumannii*.

KEYWORDS: screening, efflux pump inhibitor, urinary tract infection, C. elegans, biofilm inhibition, quinacrine assay
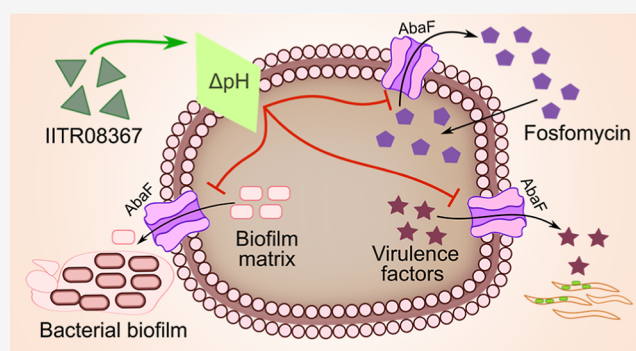
Urinary tract infections (UTIs) are the most prevalent type of infectious disease worldwide. In 2020, about 82.5% of the total bacterial infection cases were attributed to UTIs.[1] Major causative organisms of UTIs are *Escherichia coli*, *Klebsiella* spp., *Enterococcus* spp., *Pseudomonas* spp., and *Acinetobacter baumannii*.[2] The emergence of antimicrobial resistance phenotypes among UTI pathogens is rising rapidly.[3] Additionally, the development of new antibacterials has decreased alarmingly in the last few decades.[4] A dearth of new antibacterial chemical entities and a rise in bacterial antibiotic resistance is leading to therapy failure even in uncomplicated infections. To deal with this current alarming situation, there is an urgent need to discover new antibacterial chemical entities.[5,6] Besides discovering new antibacterials, revival of old antibiotics using adjuvant molecules may serve as an attractive strategy.[7−9]

Due to the increasing incidence of drug resistance, clinicians are exploring older antibiotics. Fosfomycin is one such example, as it has remained active against both Gram-positive and Gram-negative multiple drug resistant (MDR) and extensively drug resistant (XDR) bacteria. Fosfomycin is used for the treatment of uncomplicated UTIs. Single-dose

fosfomycin is one of the most effective treatment options prescribed worldwide.[10] Fosfomycin treatment is very effective against all UTIs-causing pathogens except *A. baumannii*.[11,12]

A. baumannii, a Gram-negative nosocomial bacterium, is one of the five major urinary infection-causing pathogens.[13] Treatment of multidrug-resistant *A. baumannii* infection is shrinking rapidly.[14] Intrinsic resistance of *A. baumannii* against fosfomycin is primarily due to ineffective concentration accumulation of this drug inside the bacterial cells.[15] One of the reasons for low cellular concentration is inefficient uptake, as *A. baumannii* lacks fosfomycin active uptake transporters like GlpT and UhpT.[16] Additionally, *A. baumannii* can also actively efflux out fosfomycin using *A. baumannii*-specific fosfomycin efflux pump, AbaF.[17] AbaF plays a critical role in the intrinsic
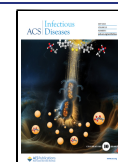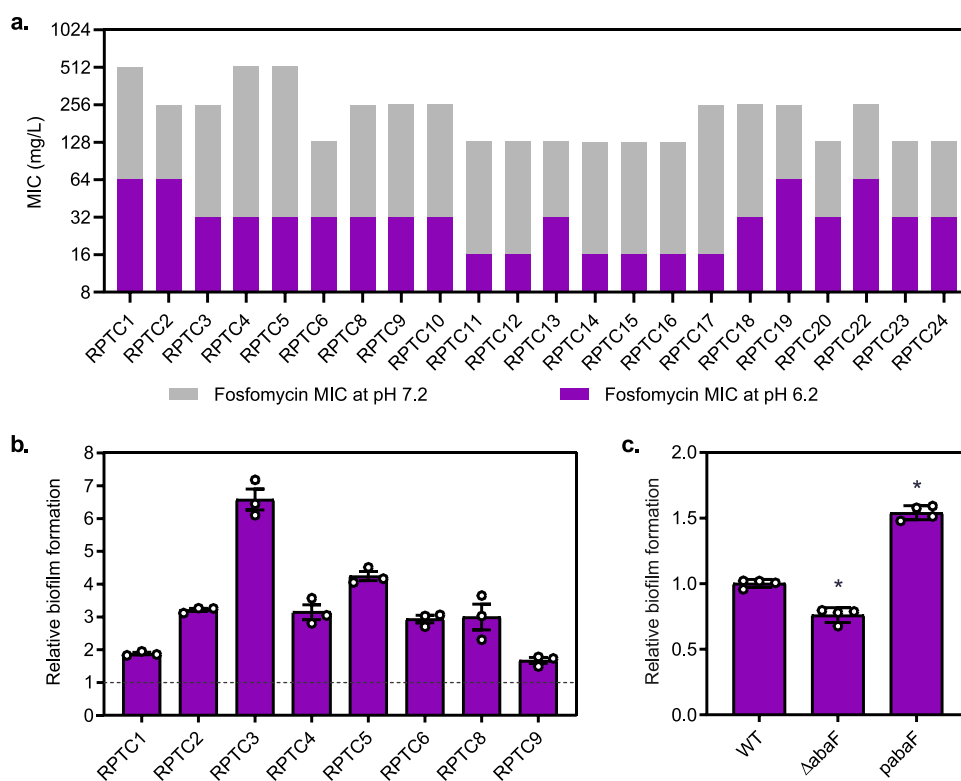
Figure 1. Effect of pH on the susceptibility of fosfomycin against *A. baumannii*. (a) Lowering the system pH by one unit enhances fosfomycin activity up to 16-fold in clinical strains of *A. baumannii*. The experiment was performed with three independent biological replicates. (b) Fosfomycin (128 mg/L) treatment enhances biofilm formation in all tested clinical strains of *A. baumannii*. The dotted line represents the biofilm formation of each strain without any treatment. Each data point represents an independent biological replicate. (c) Relative biofilm formation of *A. baumannii* strains: WT, ΔabaF, and pabaF represent *A. baumannii* ATCC 17978, *A. baumannii* ATCC 17978:Δ*abaF* and *abaF* complimented in *A. baumannii* ATCC 17978:Δ*abaF* respectively. Normalization was performed with respect to *A. baumannii* ATCC 17978. Each data point represents an independent biological replicate. Error bars represent ±s.d. * indicates *p*-value < 0.05 (unpaired student's *t*-test followed by Mann−Whitney posthoc test).

resistance of fosfomycin in *A. baumannii*. Thus, it can serve as a target to revive the fosfomycin efficacy. It is also known that a slightly acidic pH of the system (human body) can facilitate fosfomycin absorption via simple diffusion by keeping the fosfomycin in a partially protonated state.[18,19]

In this study, we identified IITR08367, a potent efflux pump inhibitor against AbaF. We demonstrated inhibition of AbaF activity by IITR08367 using ethidium bromide efflux assay. We also validated efflux inhibition of fosfomycin/H⁺ antiport activity in everted membrane vesicles. Furthermore, IITR08367 showed a reversal of minimum inhibitory concentration (MIC) of fosfomycin against MDR clinical isolates of *A. baumannii* below the clinical breakpoint. Additionally, IITR08367 successfully prevented fosfomycin-induced biofilm formation of *A. baumannii* in clinically relevant conditions. The addition of IITR08367 to fosfomycin treatment in the murine UTI infection model showed a significant reduction of the bacterial burden. Overall, this study identified a bona fide efflux pump inhibitor that potentiates fosfomycin efficacy against a clinically challenging pathogen, *A. baumannii*.

RESULTS

Acidic Environment Enhances Susceptibility of *A. baumannii* against Fosfomycin.
The bacterial uptake of fosfomycin in *E. coli* is facilitated via glucose uptake transporter GlpT and a glucose-6-phosphate inducible hexose uptake transporter HlpT. It is known that *A. baumannii* does not use glucose as a primary energy source due to inefficient uptake.[20] Additionally, glucose uptake transporters GlpT or UhpT or their homologues are not characterized in *A. baumannii*.[16] To ascertain the absence of these transporters in *A. baumannii*, we performed a nucleotide homology search of these genes in the genome of *Acinetobacter* species. We did not find any sequence with more than 5% homology. Thus, it was concluded that the bacterial uptake of the fosfomycin depends solely on the simple diffusion of the partially charged fosfomycin molecules. Fosfomycin has two ionization pHs; the first ionization occurs at pH 1.25, which makes the molecule partially ionized. The second ionization pH of the fosfomycin is 7.82; therefore, more than 90% of fosfomycin molecules remain partially charged at pH ≤ 6.5 (pK_a data obtained from Chemicalize database (https://chemicalize.com/#/calculation) and can be easily absorbed by simple diffusion.[19] The relationship between acidic pH and susceptibility to fosfomycin has been previously explored.[18,19,21] However, there is a lack of information on whether an acidic environment can enhance fosfomycin susceptibility against *A. baumannii*. Surprisingly, our results show that reducing media pH by one unit (7.2 to 6.2) can significantly (up to 16-fold) increase the susceptibility of fosfomycin against clinical strains of *A. baumannii* (Figure 1a). We found increased fosfomycin susceptibility against all tested clinical strains of *A. baumannii* (*n* = 22). Acidification of the bacterial growth medium is an important aspect affecting the efficacy of fosfomycin against *A. baumannii*. However, we
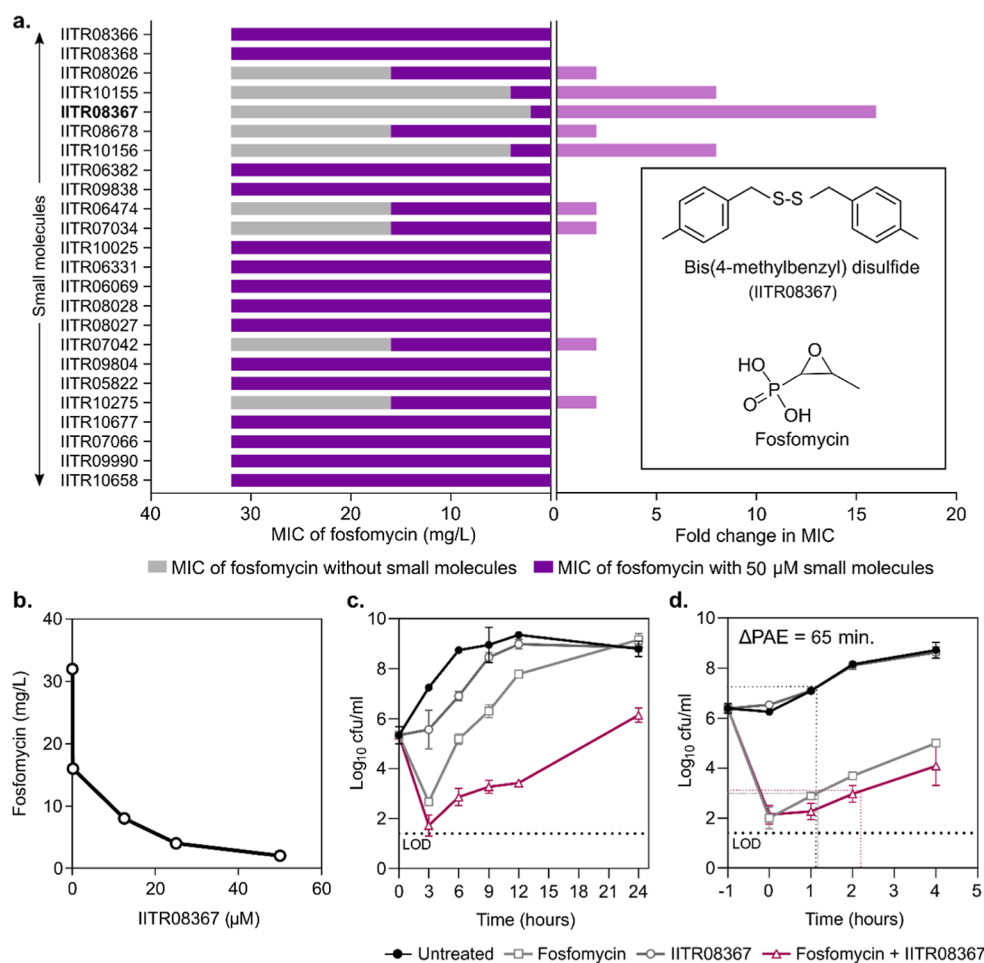
Figure 2. Screening and identification of IITR08367. (a) List of small molecules (n = 24) used as potential efflux pump inhibitors (used at 50 μM) that can potentiate the activity of fosfomycin against AbaF expressing *E. coli* KAM32/pUC18_abaF. IITR08367 (50 μM) decreased the MIC of fosfomycin to 2 mg/L (16-fold reduction). Inset shows the chemical structures of IITR08367 [bis(4-methylbenzyl) disulfide] and fosfomycin. (b) Checkerboard titration assay of fosfomycin with IITR08367 shows the potentiation of fosfomycin activity against AbaF expressing *E. coli* KAM32/pUC18_abaF. (c) Time-kill kinetics assay of fosfomycin (32 mg/L) alone or in combination with 25 μM IITR08367. The limit of detection is 50 cfu/mL. Data points of three independent replicates are shown here. Error bars represent ±s.d. (d) The postantibiotic effect (PAE) of fosfomycin (128 mg/L) alone or in combination with IITR08367 (50 μM). Data points of three independent replicates are shown here. The limit of detection (LOD) represents 50 cfu/mL. Error bars represent ±s.d.

also observed an increase in biofilm formation capacity of these clinical strains (n = 10) after treatment with fosfomycin (Figure 1b). We observed a two- to sixfold increase in biofilm in these tested strains. One of the key factors of *A. baumannii* involved in fosfomycin resistance as well as in biofilm formation is an efflux pump called AbaF. AbaF plays an important role in fosfomycin resistance and extrusion of biofilm matrix out of the cells.[17] We found that deletion of abaF abolished biofilm-forming capacity of *A. baumannii* ATCC 17978, while plasmid-based gene complementation can recover its biofilm formation potential (Figure 1c). This highlighted the fact that targeting AbaF could provide dual benefits in reviving efficacy of fosfomycin against intrinsically resistant *A. baumannii*.

IITR08367 Is a Potent Efflux Inhibitor of AbaF. Recently, our group screened a library of 8000 small molecules and discovered 24 potential efflux pump inhibitors active against AbeM, a proton-driven multidrug and toxic compound extrusion (MATE) family efflux pump of *A. baumannii*.[22] Since the MFS and MATE families use a similar proton-driven efflux mechanism, we hypothesized if our 24 novel efflux inhibitors

could also be efficacious against AbaF. We tested the efficacy of these 24 potential inhibitors against AbaF-expressing strain *E. coli* KAM32/pUC18_abaF, and surprisingly, nine molecules out of 24 restored the efficacy of fosfomycin (Figure 2a). IITR08367 [bis(4-methylbenzyl) disulfide] (Figure 2a inset) displayed the most potent efflux inhibition and potentiates fosfomycin activity up to 16-fold against AbaF expressing strain, *E. coli* KAM32/pUC18_abaF. IITR08367 itself did not kill any tested strains of *E. coli* and *A. baumannii* up to 400 μM (the highest tested concentration). IITR08367 showed a dose-dependent potentiation of fosfomycin activity against AbaF expressing strain, *E. coli* KAM32/pUC18_abaF (Figure 2b). The potentiation of fosfomycin activity in the presence of IITR08367 was further confirmed by a time kill-kinetics assay (Figure 2c).

Treatment of *E. coli* KAM32/pUC18_abaF cells with 32 mg/L fosfomycin showed a 2 $\log_{10}$ cfu decrease within 2 h, which grew back similar to the untreated control within the next 16 h. However, treatment with 32 mg/L fosfomycin in combination with 25 μM IITR08367 demonstrated a significant decrease of 4 $\log_{10}$ cfu within 2 h and slowed

down the regrowth of cells up to 12 h. Overall, $a > 3 \log_{10}$ cfu difference was observed even after 24 h. Postantibiotic effect (PAE), an important clinically relevant pharmacodynamic parameter, helps in designing dosing intervals. PAE indicates the time required by the bacterial population to overcome antibiotic stress after the removal of an antibiotic from the system. The addition of IITR08367 (50 μM) increased the PAE of fosfomycin by 30 min (Figure 2d) with an overall fosfomycin PAE of 1.6 h after 1 h treatment at 4× MIC concentration. Noteworthy, we did not observe the same level of potentiation in time-kill kinetics as well as in PAE experiment with *E. coli* KAM32 harboring only pUC18 plasmid (only plasmid control) (Figure S1a,b).

IITR08367 Is a Bona Fide Efflux Pump Inhibitor That Acts by Disrupting Proton Gradient. Ethidium bromide (EtBr) is a common substrate for many efflux pumps. The fluorescence of EtBr changes proportionally to its concentration inside the cells. EtBr loses its fluorescence when effluxed out due to quenching by water molecules.[23] We observed an enhanced EtBr accumulation in AbaF expressing strain, *E. coli* KAM32/pUC18_abaF, after IITR08367 treatment, thus validating its efflux inhibitory potential (Figure 3a).

To further test the effect of IITR08367 on bacterial membrane potential, an anionic potentiometric bis-oxonol probe DiBAC$_4$(3) [(bis(1,3-dibutylbarbituric acid)trimethine oxonol)] was used. DiBAC$_4$(3) undergoes voltage-dependent partitioning between the aqueous phase and bacterial membrane; membrane depolarization decreases its uptake in the membrane; thus, an increase in fluorescence is observed. Phenylalanine—arginine beta-naphthylamide (PAβN) is a known efflux pump inhibitor that also disrupts membrane potential, and we also observed increased fluorescence in PAβN-treated cells (Figure 3b). Hyperpolarized membrane increases its uptake in the membrane, leading to fluorescence quenching.[24] Bacterial cells treated with IITR08367 showed decreased fluorescence due to hyperpolarization of the cell membrane (Figure 3b). Bacterial cells tend to hyperpolarize when the H$^+$ gradient gets disturbed to maintain overall PMF across the membrane.[25] It is also known that efflux pump inhibitors, which perturb membrane potential, tend to have membrane-damaging effects.[26] Thus, we tested whether IITR08367 has any membrane permeabilizing activity, and we found that it does not cause membrane damage (Figure S2). Overall, IITR08367 disrupts the H$^+$ gradient across the membrane, ultimately inhibiting the H$^+$-gradient-driven efflux pumps.

AbaF belongs to an MFS class of efflux pumps that works as a fosfomycin/H$^+$ antiporter, that is, it effluxes out fosfomycin in exchange for proton ions. To understand the mechanism of the inhibitory effect of IITR08367 on fosfomycin/H$^+$ antiport movement, a quinacrine-based proton ion concentration-sensitive fluorescence assay was performed in everted membrane vesicles. The monoprotonated form of quinacrine quickly equilibrates inside out of vesicles via diffusion and emits fluorescence. The addition of potassium lactate energizes everted membrane vesicles, which leads to fluorescence quenching due to acidification (increased H$^+$ concentration) inside the vesicles. A slight increase in fluorescence after the addition of fosfomycin is attributed to the decrease in intravesicular H$^+$ ion concentration due to AbaF-mediated fosfomycin exchange. The addition of IITR08367 leads to disruption of the H$^+$ gradient, thus increasing the fluorescence
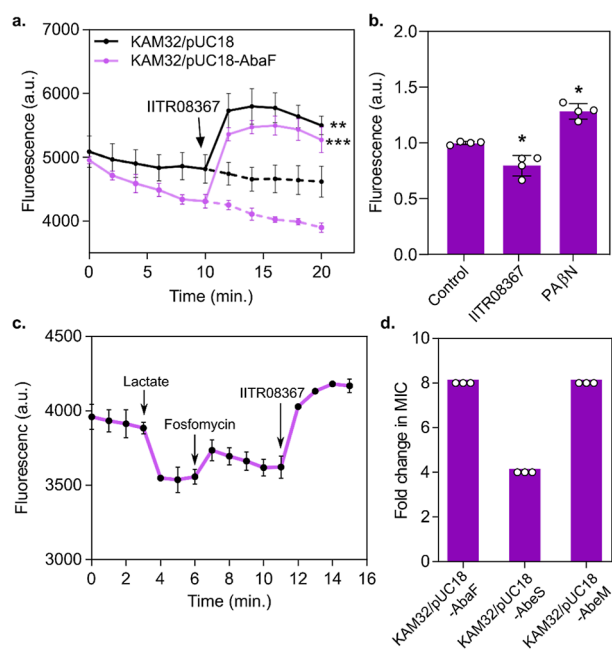

Figure 3. IITR08367 is a bona fide efflux pump inhibitor that acts by affecting the ΔpH component of the cell. (a) Effect of addition of IITR08367 (100 μM, indicated by arrow) on ethidium bromide (EtBr) fluorescence in *E. coli* KAM32/pUC18_AbaF. The dotted line represents fluorescence values without IITR08367 treatment. Data points represent an average of three independent replicates. *E. coli* KAM32/pUC18 (only plasmid) was used as a control. Error bars represent ±s.d. (b) Effect of IITR08367 (100 μM) on cell membrane potential of *E. coli* KAM32/pUC18_AbaF. PAβN (25 μM) was used as a positive control. Each data point represents independent biological replicates. Data was normalized with respective to untreated control. Error bars represent ±s.d. * indicates *p*-value < 0.05 (unpaired student *t*-test followed by Mann—Whitney posthoc test). (c) Effect of IITR08367 on fosfomycin/H$^+$ antiport. The fluorescence signal of quinacrine (420 nm excitation and 500 nm emission) with respect to time in everted membrane vesicles of *E. coli* KAM32/pUC18_AbaF. Potassium-lactate, fosfomycin, and IITR08367 were used at 5 mM, 50 μM, and 25 μM, respectively. Data points represent an average of three independent replicates. Error bars represent ±s.d. (d) Fold change in MIC of fosfomycin against *E. coli* KAM32 expressing different efflux pumps of *A. baumannii* (AbaF—MFS class, AbeS—SMR class, AbeM—MATE class). Data points of three independent replicates are shown here.

output of the system (Figure 3c). This energy decoupling led to the inhibition of the H$^+$-gradient-driven efflux pumps.

Since many bacterial efflux pumps utilize proton gradient as an energy source, we were curious to test whether IITR08367 could also inhibit other efflux pumps that utilize proton gradient. *A. baumannii* harbors multiple types of efflux pumps that utilize proton gradient as an energy source. AbeM (MATE class) and AbeS (SMR) are two validated efflux pumps responsible for providing resistance against fluoroquinolones and other antibiotics in *A. baumannii*.[22] Hence, we also tested the activity of IITR08367 against AbeS and AbeM and found that IITR08367 inhibits AbeS as well as AbeM, which resulted in MIC reversal of ciprofloxacin by four- and eightfold, respectively (Figure 3d). Overall, multiple efflux pumps inhibition by IITR08367 by disrupting the H$^+$ gradient across bacterial membrane may prove beneficial for reviving multiple antibiotics which are actively effluxed out by *A. baumannii*.
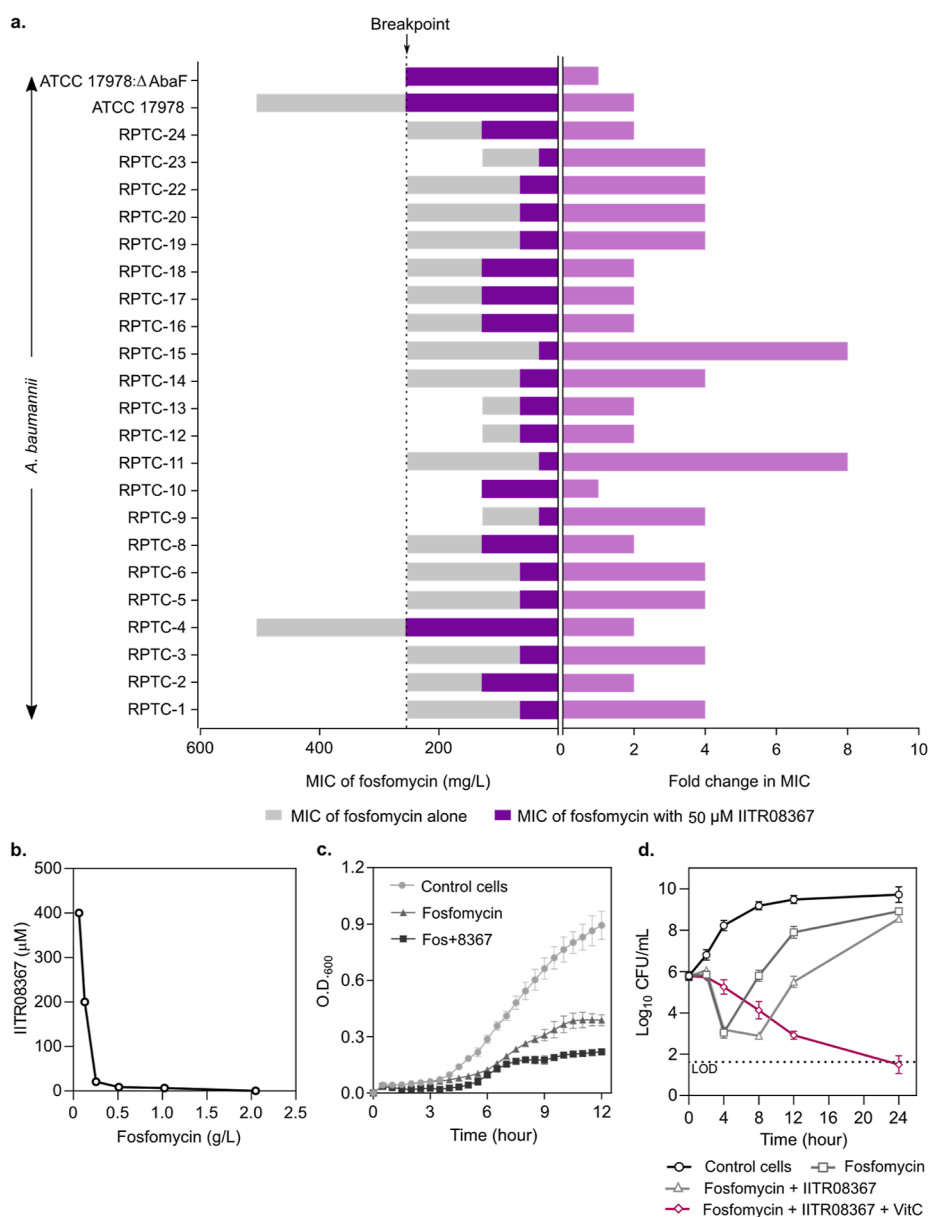
Figure 4. IITR08367 is effective against clinical strains of *A. baumannii*. (a) Reversal of MIC of fosfomycin in the presence of 50 μM IITR08367 against clinical isolates of *A. baumannii*. *A. baumannii* ATCC 17978 and *A. baumannii* ATCC 17978 ΔAbaF were used as positive and negative controls, respectively. Dashed line represents the epidemiological cutoff (breakpoint) of fosfomycin against *P. aeruginosa* according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST); fosfomycin cutoff is not available (December 2023) against *A. baumannii*. (b) Checkerboard titration isobologram represents dose-dependent potentiation of fosfomycin activity in the presence of IITR08367. (c) Growth profile of *A. baumannii* RPTC-15 in human urine (pH ranges 6.5−7) supplemented with 20% (v/v) Cation adjusted Muller Hinton broth. Control cells represent untreated cells. Fosfomycin was used at 64 mg/L (when used alone) or in the presence of 100 μM IITR08367. Data points of a single human urine sample are shown here. (d) Time-kill kinetics assay of fosfomycin (256 mg/L) in combination with IITR08367 (100 μM) in the MH broth acidified (pH 6.9) with the addition of Vitamin C (100 mg/L). The LOD represents 50 cfu/mL. Data points of three independent biological replicates are shown here. Error bars represent ±s.d.

IITR08367 Potentiates Fosfomycin Activity against Clinical Strains of *A. baumannii*. After getting encouraging results of IITR08367 on *E. coli* KAM32/pUC18_AbaF, we tested the efficacy spectrum of IITR08367 against 22 fosfomycin-resistant *A. baumannii* clinical strains harboring AbaF.[17] IITR08367 potentiated the efficacy of fosfomycin in 21 clinical strains as well as ATCC 17978 (Figure 4a). A maximum eightfold reversal of fosfomycin efficacy was observed against *A. baumannii* RPTC-11 and RPTC-15. A fourfold reversal of fosfomycin efficacy was observed against ten clinical isolates, and a twofold reversal was observed against

nine clinical isolates, including *A. baumannii* ATCC 17978. No change in fosfomycin MIC was observed in AbaF knockout and RPTC-10. *A. baumannii* RPTC-15 was selected as a representative strain for further studies. *A. baumannii* RPTC-15 is resistant to multiple clinically important antibiotics. A 2D-checkerboard assay reveals the dose-dependent effect of IITR08367 on fosfomycin activity against *A. baumannii* RPTC-15 (Figure 4b).

As fosfomycin is often prescribed for treating urinary tract infections (UTIs), we were intrigued to test the clinical significance of IITR08367 in a simulated ex vivo urine
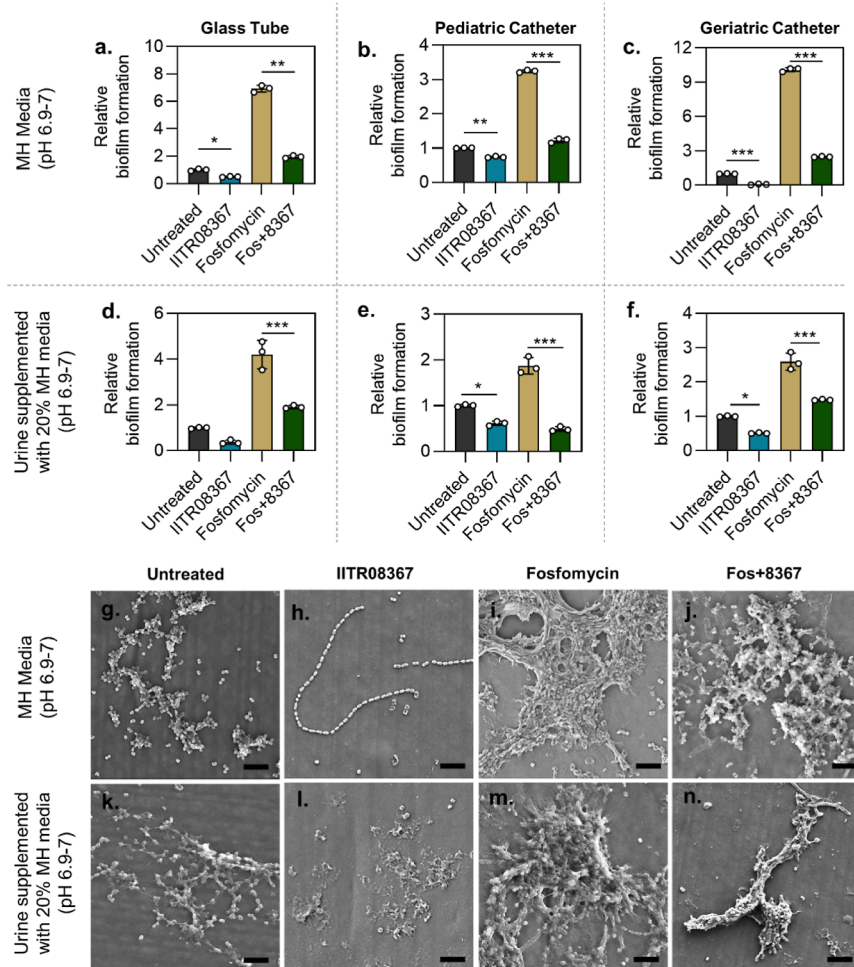
Figure 5. IITR08367−fosfomycin combination is effective against *A. baumannii* biofilm. (a−f) Effect of IITR08367 on biofilm formation of *A. baumannii* RPTC-15 under different clinically relevant simulated conditions. Fosfomycin and IITR08367 were used at 64 mg/L and 100 μM, respectively. Data points of three independent replicates are shown here. Error bars represent ±s.d. *, **, and *** indicate *p*-values of 0.05, 0.01, and 0.001 respectively (one-way ANOVA, with Tukey's multiple comparison test). (g−n) SEM images of *A. baumannii* RPTC-15 biofilm on urinary catheter show increased biofilm after fosfomycin (64 mg/L) treatment. Fosfomycin and IITR08367 were used at 64 mg/L and 100 μM, respectively. Scale bar represents 5 μm.

environment. We tested the growth profile of *A. baumannii* RPTC-15 in the presence of either fosfomycin alone (64−128 mg/L) or in combination with 100 μM IITR08367 in freshly collected filter sterile human urine (n = 21, pH ranges 6.5−7). Our results showed that a combination of fosfomycin (64 mg/L) and IITR08367 (100 μM) could prevent the growth of *A. baumannii* RPTC-15 (an MDR clinical strain), while fosfomycin alone was unable to control growth (Figures 4c and S3).

Considering our results that acidic pH potentiates fosfomycin activity, it would be beneficial to add a pH regulator to ensure the effective uptake of fosfomycin via simple diffusion. Vitamin C is a classical pH regulator widely used as an adjuvant in various clinical therapies.[27] Considering vitamin C itself as a molecule with antibiotic activity,[28] we used 50 times less concentration (100 mg/L) than its minimum effective antimicrobial concentration (5 mg/mL) for the pH regulation. We tested the effect of 100 mg/L vitamin C on the efficacy of 256 mg/L fosfomycin against *A. baumannii* RPTC-15 in a time-kill kinetic assay. Our results confirmed that the tested concentration of vitamin C (100 mg/L) alone did not affect the potency of fosfomycin against *A. baumannii* RPTC-

15 (Figure S4). Our time-kill kinetic assay showed that the addition of vitamin C (100 mg/L) could further enhance the activity of the fosfomycin (256 mg/L)—IITR08367 combination (Figure 4d).

IITR08367 Potentiates Fosfomycin Activity against *A. baumannii* Biofilms. Biofilms are one of the important arsenals that help the survival of *A. baumannii* under different pathophysiological conditions.[13] Previously, it has been established that AbaF helps *A. baumannii* to establish biofilms.[17] We tested whether IITR08367 could hamper biofilm formation by *A. baumannii* via inhibition of AbaF. To replicate hospital-like settings, we performed biofilm inhibition assay on different surfaces such as glass, soft silicone pediatric catheter, and silicone geriatric catheter under two simulated conditions (CAMHB and urine). Our biofilm assay indicated that in addition to the potentiation of fosfomycin's bactericidal effect, adding IITR08367 to fosfomycin could significantly reduce the biofilm formation compared to fosfomycin alone treatment via inhibition of AbaF (Figure 5a−f). We also examined the effect of IITR08367−fosfomycin combination under high-resolution scanning electron microscopy (SEM), and our data show that IITR08367 could abolish biofilm
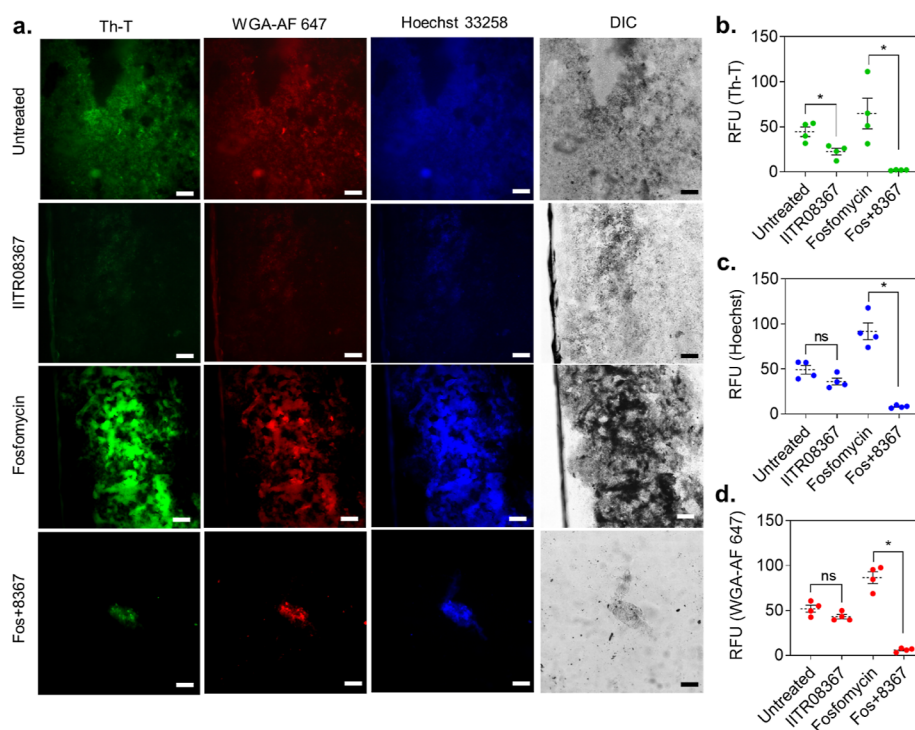
Figure 6. IITR08367−fosfomycin combination reduces biofilm matrix of *A. baumannii*. Fluorescence microscopy imaging and quantitative analysis of biofilm matrix of *A. baumannii* RPTC-15 biofilm on a glass coverslip in the presence of pH-adjusted urine supplemented with 20% MH media. (a) Fluorescence microscopy images of biofilm matrix formed on glass coverslip. Fosfomycin and IITR08367 were used at 64 mg/L and 100 μM, respectively. Thioflavin-T (Th-T), WGA Alexa Fluor 647 (WGA-AF 647), and Hoechst 33258 were used to estimate amyloid proteins, extracellular polysaccharides and extracellular nucleic acid of biofilm, respectively. Scale bar represents 100 μm. Scatter dot plot showing quantitative analysis of fluorescence signal intensity of (b) Th-T (c) Hoechst 33258 and (d) WGA-AF 647. Four independent microscopic images were analyzed for each sample using ImageJ software. Error bar represents ±sem. ns represents nonsignificant; * indicates a *p*-value of <0.05 (unpaired student's *t*-test followed by Mann−Whitney posthoc test).

formation capacity of *A. baumannii* with or without fosfomycin (Figure 5g−n). Additionally, our SEM analysis revealed that *A. baumannii* forms structurally distinct microstructures when supplemented with urine (Figure 5g,k).

Bacterial biofilms are composed of aggregations of bacteria adhered to surfaces and/or to one another, encapsulated within a self-generated matrix. This matrix comprises various substances, including proteins (such as fibrin), polysaccharides (such as alginate), and extracellular DNA (eDNA). Various efflux pumps are also known to be involved in extrusion of different biofilm matrix components.[17,29] Considering the effective biofilm inhibition and efflux pump inhibitory activity of IITR08367, we further investigated the effect of IITR08367 in reducing these matrix components in detail. We employed fluorescence microscopy to measure amyloid proteins, extracellular polysaccharides, and eDNA using their specific fluorescent probes. We found a significant reduction in amyloid proteins of biofilm matrix after treatment with IITR08367 (Figure 6b). This indicates that AbaF may be responsible for the extrusion of proteinaceous components of biofilm matrix. The combination of fosfomycin and IITR08367 significantly reduced all three components of biofilm matrix when compared with fosfomycin alone treatment (Figure 6b−d).

IITR08367 Is a Nonhemolytic and Noncytotoxic Efflux Pump Inhibitor. IITR08367 showed great potential in reviving fosfomycin efficacy against intrinsically resistant *A. baumannii* via inhibiting fosfomycin efflux pump, AbaF. Furthermore, we observed that the efflux pump inhibition

activity of IITR08367 was due to its ability to disrupt the proton gradient across the bacterial membrane. Proton disruptors can be toxic to eukaryotic cells. Hence, we tested the biocompatibility of IITR08367 toward freshly isolated human erythrocytes and peripheral blood mononuclear cells (PBMCs). Our results indicate that the IITR08367 does not show any toxicity to human erythrocytes (Figure S5a). Additionally, IITR08367 did not show much toxicity to human PBMCs at the effective concentration ranges (Figure S5b).

IITR08367 Potentiates Fosfomycin and Reduces In Vivo *A. baumannii* Burden in *C. elegans*. *Caenorhabditis elegans* is a lower eukaryotic model organism used to study the toxicity and efficacy of novel small molecules.[30] Hence, we tested toxicity of IITR08367 and its combination with fosfomycin. Our results indicate that IITR08367 displays only marginal toxicity to *C. elegans* at a very high concentration (400 μM). Similarly, fosfomycin either alone (512 mg/L) or in combination with IITR08367 (fosfomycin at 512 mg/L & IITR08367 at 400 μM) only shows marginal toxicity (Figure 7a). A combination of two different pharmaceutically active compounds can lead to enhanced toxicity in test organisms; however, our results indicate that combination of fosfomycin and IITR08367 does not show any enhanced adverse effect.

C. elegans is a promising model for studying *A. baumannii* pathogenesis.[31] Hence, we evaluated in vivo efficacy of IITR08367 along with fosfomycin in *C. elegans*-*A. baumannii* infection model.[31,32] Our in vivo efficacy results indicate two important evidence. First, in vivo efficacy of fosfomycin is
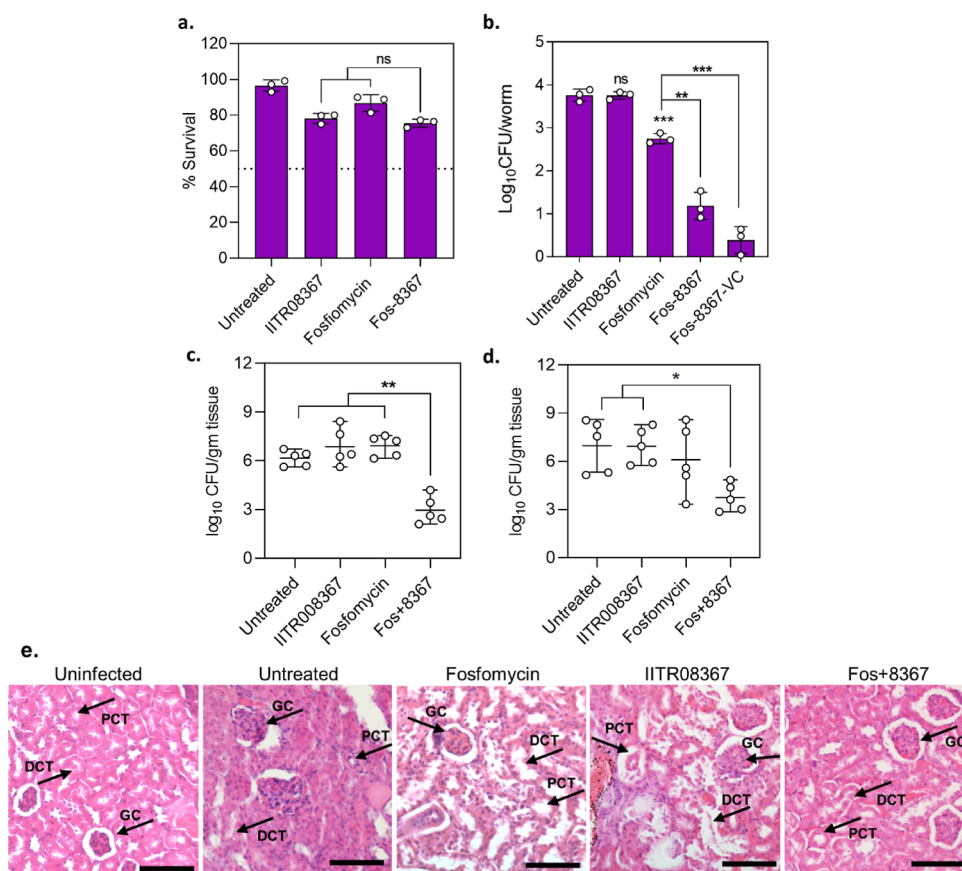
Figure 7. In vivo toxicity and efficacy of fosfomycin-IITR08367 combination (a) Bar graph showing percentage survival of *C. elegans* ($n = 15−20$) under different treatment regimens. Survival was calculated at the end of 72 h. Error bars represent ±s.d. ns represents nonsignificant (one-way ANOVA, with Tukey's multiple comparison test) (b) Bar graph showing *A. baumannii* RPTC-15 (a pathogenic clinical strain) reduction in *C. elegans* ($n = 15−20$) under different treatment regimens. Data points of three independent replicates are shown here. Error bars represent ±s.d. ns represents nonsignificant; *, **, and *** indicate *p*-values of 0.05, 0.01, and 0.001, respectively (one-way ANOVA, with Tukey's multiple comparison test). (c) *A. baumannii* RPTC-15 load in the kidney of mice after UTI infection. The LOD corresponds to 30 cfu/mL. Data points of five individual mice are shown here. Error bars represent ±s.d. ** indicates a *p*-value of <0.01 (nonparametric one-way ANOVA, with Kruskal−Wallis multiple comparison test). (d) *A. baumannii* RPTC-15 load in bladder of mice after UTI infection. Data points of five individual mice are shown here. Error bars represent ±s.d. * indicates a *p*-value of <0.05 (nonparametric one-way ANOVA, with Kruskal−Wallis multiple comparison test). (e) Histopathology of mice kidney under different treatment regimens. GC, PCT, and DCT are indicated by black arrow. Scale bar represents 100 μm.

better than what is in an in vitro assay. Our test pathogen, *A. baumannii* RPTC-15, shows in vitro susceptibility at 256 mg/L. Surprisingly, fosfomycin (at 256 mg/L) shows ≈1 $\log_{10}$ cfu reduction in in vivo conditions, even after it has to go under ADME (absorption, distribution, metabolism, and excretion) pathway. Second, IITR08367 shows potentiation of fosfomycin activity in the *C. elegans* model, as the combination of fosfomycin and IITR08367 displayed ≈3 $\log_{10}$ cfu reduction ($p < 0.001$). Addition of a noninhibitory concentration of vitamin C (100 mg/L) to the fosfomycin-IITR08367 combination further reduces the bacterial load in the *C. elegans* infection model (Figure 7b). Additionally, we also tested the effect of IITR09367-fosfomycin combination on the pathogenicity of *A. baumannii* in *C. elegans* infection model. We found better survivability of *C. elegans* under fosfomycin-IITR08367 treatment regimen. Notably, 80% of the *C. elegans* survived the virulent *A. baumannii* infection even when treated with subinhibitory concentration of fosfomycin-IITR08367 combination, while only 40% of the population survived under only fosfomycin treatment regimen (Figure S6).

IITR08367 Potentiates Fosfomycin and Reduces In Vivo *A. baumannii* Burden in Murine UTI Model. Motivated by our in vivo efficacy results in *C. elegans*, we extended the efficacy study in the murine UTI model. We selected *A. baumannii* RPTC-15 (MDR clinical isolate) for murine UTI infection and found that it can successfully colonize the mice bladder. Our efficacy data show that fosfomycin-IITR08367 combination successfully decreased the *A. baumannii* RPTC-15 burden by ~3 $\log_{10}$, both in the kidney and in the bladder (Figure 7c,d). On the other hand, fosfomycin alone only decreased *A. baumannii* RPTC-15 burden in the bladder by ~1 $\log_{10}$; moreover, it did not reduce any bacterial load in the kidney. Next, we performed histopathological examinations of kidney. We found that *A. baumannii* RPTC-15 caused extensive damage to functional units of kidney, that is, glomerular capsule (GC), proximal convoluted tubules (PCT), and distal convoluted tubules (DCT) in untreated mice (Figure 7e). Additionally, there was very high infiltration of neutrophils, leading to tissue inflammation. Mice treated with either only fosfomycin or IITR08367 showed marked damage in PCT, DCT linings, and

minor damage was observed in GC. However, mice treated with a combination of fosfomycin and IITR08367 showed significant improvement, and tissue ultrastructures were similar to uninfected mice (Figure 7e). In conclusion, IITR08367, along with fosfomycin, can play a major role in the fight against *A. baumannii* infections.

■ DISCUSSION

Bacteria possess multiple arsenal to tackle antibiotics, such as target site protection and modification and enzymatic inactivation of antibiotics.[33] However, efflux pumps are one of the most important arsenals for a resistant bacterial pathogen.[34] Efflux pumps play a critical role in the pathophysiology of many human pathogens.[34−36] What makes bacterial efflux pumps so special is broad substrate specificity, that is, a single type of efflux pump can expel multiple antibiotics. Active expression of a single efflux pump can cause treatment failure against multiple antibiotics.[35,37] Furthermore, efflux pumps also play an important role in biofilm formation by transporting various biofilm matrix components.[29]

Fosfomycin shows broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens.[38] Its usage has been endorsed by the Infectious Diseases Society of America (IDSA) in USA.[39] Despite the very high level of antibiotic resistance among *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *E. faecalis*, fosfomycin has retained its susceptibility in different countries against these human pathogens.[40] Additionally, it shows activity against some unusual and difficult-to-treat pathogens like *Klebsiella oxytoca*, *Serratia marcescens*, and *Proteus mirabilis*. The most noteworthy fact regarding the efficacy of fosfomycin is its retained activity against *Enterobacteriaceae* producing extended-spectrum β-lactamase (ESBL), New Delhi metallo-β-lactamase-1 (NDM-1), and KPC-2 enzymes.[40] In contrast, fosfomycin appears to be ineffective against notorious *A. baumannii*.[13,15] Antibiotic resistance in *A. baumannii* is increasing rapidly.[3,41] *A. baumannii* is intrinsically resistant to fosfomycin due to several factors. First is the lack or modification in the transport systems for fosfomycin uptake, such as glycerol-3-phosphate transporter system (GlpT) and the hexose phosphate transporter (UhpT). Second is the presence of fosfomycin-inactivating enzymes such as FosX. Third is a mutation in UDP-*N*-acetylglucosamine enolpyruvyl transferase gene (*murA*). Fourth is an active efflux of fosfomycin by the AbaF (Acinetobacter baumannii Fosfomycin efflux) system.[15,17] AbaF is a type of MFS transporter found in clinical strains of *A. baumannii* and is primarily responsible for high-level fosfomycin resistance in clinical settings.[15,17] Hence, targeting AbaF would be an excellent choice for rejuvenating fosfomycin against *A. baumannii*.

Fosfomycin is primarily available as oral preparation for the treatment of uncomplicated UTIs and other infections.[42] However, there is an increase in the usage of intravenous fosfomycin along with other antibiotics for severe infections as a combinatorial therapy.[38,43,44] Fosfomycin has excellent oral bioavailability and tissue dissemination at different body sites such as lungs, kidneys, liver, and cerebrospinal fluids.[45,46] What makes fosfomycin unique for the treatment of UTIs is its bioavailability at the bladder wall and in urine. Active concentration of fosfomycin in urine can reach 2500 mg/L within 2 h after a single oral dose of 50 mg/kg.[45,46] Additionally, a very high urinary concentration (up to 700

mg/L) can be maintained for 24 h with a single oral dose of 50 mg/kg.[45] These pharmacokinetic properties of fosfomycin make it the first choice for the treatment of uncomplicated UTIs. There is an increasing appreciation of efficacy of fosfomycin for the treatment of critically ill patients.

Several physiological factors, such as plasma-binding capacity, renal clearance, pH of the system, and so on, control efficacy of a given antibiotic. Fluoroquinolones, aminoglycosides, and trimethoprim are among the antibiotics that exhibit greater effectiveness in alkaline environments, contrary to antibiotics whose activity is favored in acidic media are fosfomycin, tetracycline, nitrofurantoin.[47] Strategically, the system's pH can be controlled by providing systemic alkalizers or mild acidifiers. Vitamin C is one such acidifier used in clinics.[27] Surprisingly, potency of fosfomycin is enhanced under acidic pH, and we observed up to 16-fold susceptibility enhancement against clinical strains of *A. baumannii* in our study. However, only pH does not solve the inherent problem of reduced susceptibility of fosfomycin against *A. baumannii*, because this bacterium tries to evade fosfomycin by making robust biofilm which protects them against fosfomycin.[29] Moreover, *A. baumannii* has fosfomycin specific efflux pump, AbaF.[17] Taking into account these factors, we developed a double-edged sword strategy to overcome this problem by identifying IITR08367, a potent efflux inhibitor that targets AbaF and thus decreases biofilm matrix as well as fosfomycin efflux. We also uncover that IITR08367 is a safe and nonspecific inhibitor of multiple efflux pumps of *A. baumannii*, which may be advantageous for clinical settings.

In order to further broaden the efficacy of fosfomycin against clinically challenging pathogens like *A. baumannii*, we screened, identified, and validated a novel efflux pump inhibitor of AbaF in *A. baumannii*. IITR08367 potentiates the antibacterial efficacy of fosfomycin against *A. baumannii*. IITR08367 inhibits efflux pumps by disrupting the proton gradient across the bacterial membrane. Relatively high activity/toxicity index of IITR08367 offers it as a potent adjuvant for fosfomycin treatment against *A. baumannii*.

■ MATERIAL AND METHODS

Ethical Statement. All animal experiments were approved by the Institutional Animals Ethics Committee of the Indian Institute of Technology Roorkee (Protocol no. BT/IAEC/2014/08/REV). All the animal experiments were conducted as per Institutional Animals Ethics Committee (IAEC) guidelines (CPCSEA registration number 563/GO/Re/S/02/CPCSEA). The collection of human urine and blood samples from healthy volunteers was approved by the Institutional Human Ethics Committee of the Indian Institute of Technology Roorkee (Protocol no. BT/IHEC-IITR/2019/7300).

Chemicals. All small molecules used as efflux pump inhibitors in this work were purchased from Maybridge (Now Thermo Fisher Scientific, USA). DiBAC$_4$(3), PAβN, Thioflavin-T (Th-T), Hoescht 33258, and wheat germ agglutinin- Alexa Fluor 647 (WGA-AF 647) were purchased from Thermo Fisher Scientific (USA). All other chemical probes and antibiotics were purchased from Sigma-Aldrich (USA).

Bacterial Strains and Media. All the bacterial strains used in this study are listed in Table S1. All strains were maintained on Cation-adjusted Muller Hilton (CAMH) Agar (Himedia, India). During all experiments, cells were grown in Cation-

adjusted Muller Hilton Broth (CAMHB) (Himedia, India) until specified.

Determination of MIC. MICs of various antibacterial and small molecules were determined in 96-well clear bottom plates with an initial inoculum of 10^5 cfu/mL as per Clinical and Laboratory Standards Institute (CLSI) guidelines in CAMH broth.[48] Details are described in the Supporting Information methods (Section S1).

Biofilm Inhibition Assay. A biofilm inhibition experiment was performed with various clinical strains of *A. baumannii*, ATCC 17978 (WT), *A. baumannii* Δ*abaF* (Δ*abaF*), and *abaF* complimented in *A. baumannii* Δ*abaF* (*pabaF*) according to previously described method with minor modification.[49] Details are described in the Supporting Information methods (Section S2).

Screening of AbaF Inhibitors Using a Chemical Genetic Approach. Screening of AbaF inhibitor was performed against *E. coli* KAM32 harboring cloned copy of *abaF* in pUC18 plasmid (overexpressor strain of AbaF).[17,50] *E. coli* KAM32 is deficient of important efflux pumps (YdhE, AcrAB), making it an ideal host to test putative efflux pump or efflux pump inhibitors.[50] *E. coli* KAM32 containing only pUC18 was used as the control strain for this assay. A fold change reversal in MIC of fosfomycin was tested with 24 putative efflux pump inhibitors. All putative efflux pump inhibitors were used at a concentration of 50 μM, which is noninhibitory for test strain. Molecules showing more than a fourfold reduction in MIC of fosfomycin were considered potential inhibitors of AbaF.

Evaluation of Synergistic Drug Interactions of Fosfomycin with Potential Inhibitors of AbaF Using Checkerboard Assays. A 2D checkerboard broth micro-dilution assay was performed using twofold serially diluted concentrations of fosfomycin with IITR08367 against *E. coli* KAM32/pUC18_abaF as previously described.[32] Cell growth was monitored by measuring optical density at 600 nm (OD_{600nm}) after 18 h of incubation at 37 °C. The fractional inhibitory concentration (FIC) index was calculated by the following formula

$$\text{FIC index} = \left\{ \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} \right\} + \left\{ \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}} \right\} \quad (1)$$

Synergy was defined as an FIC index value of ≤0.5, and additivity or indifference was defined as an FICI value of ≥0.5 to <4, whereas antagonism was defined as an FICI value of ≥4.[51]

Time-Kill Kinetics Assay. The time-kill experiments were performed in CAMHB (Himedia, India) according to the previously described method with a slight modification.[32] Briefly, *E. coli* KAM32/pUC18_abaF cells (approximately ~10^5 cfu/mL) were treated either with fosfomycin alone (32 mg/L) or in combination with IITR08367 (25 μM) at 37 °C with aeration for 24 h. For *A. baumannii* RPTC-15, fosfomycin was used at 256 mg/L when used alone or with 100 μM of IITR08367. 100 μL of culture was removed at different time intervals (2, 4, 8, 12, and 24 h) and plated on CAMH agar after 10-fold serial dilution. Viable colonies were counted after 24 h of incubation and presented at a $\log_{10}$ scale over time.

In Vitro Postantibiotic Effect. The PAE of IITR08367 was studied according to a previously published protocol with minor modifications.[52,53] Briefly, the culture of *E. coli* KAM32/pUC18_abaF (approximately 10^7 cfu/mL) was treated with fosfomycin alone (128 mg/L) or in combination with IITR08367 (50 μM) for 1 h at 37 °C with aeration (180 rpm). Cells were washed thrice with fresh MH broth to remove test compounds. Cells were further incubated in fresh MH broth at 37 °C with aeration. Viable bacterial counts were determined at regular time intervals (0, 2, 4, 6, and 8 h). The PAE was estimated by the formula PAE = $T^n - T^c$ where T^n is the time required for a unit $\log_{10}$ bacterial growth (cfu/mL) increase in a drug-treated sample, while T^c is the time required for a unit $\log_{10}$ growth in an untreated sample.[54]

Ethidium Bromide Efflux Assay. Freshly harvested mid-log phase cells of *E. coli* KAM32/pUC18_abaF and *E. coli* KAM32/pUC18 were washed and resuspended in 1x PBS to an $OD_{600} \sim 0.3$. EtBr (10 μg/mL) was added to each cell suspension, followed by 30 min incubation at 37 °C with intermittent mixing. Cell suspension (200 μL) of each sample was added to a UV opaque flat bottom black 96-well microtiter plate (BRAND, Germany). 0.4% (w/v) glucose was added to each well to initiate the efflux of EtBr, and fluorescence was measured at 480 nm excitation and 610 nm emission at the 1 min interval using the spectrofluorometer (SynergyH1, BioTek, USA).[55] After 10 min, 100 μM IITR08367 was added, and fluorescence measurement was continued for the next 10 min. Cells without IITR08367 treatment were used as a control for this experiment.

Assessment of IITR08367 Effect on Membrane Potential. Logarithmic-phase *E. coli* KAM32/pUC18_abaF cells were harvested, washed, and resuspended in 1× PBS, containing 0.4% glucose (w/v) to an $OD_{600} \sim 0.2$. The cell suspension was treated with 100 μM IITR08367 for 2 h at 37 °C. After 1 h, 1 μM DiBAC$_4$(3) (Invitrogen, USA) was added and incubated for 15 min. The fluorescence of DiBAC$_4$(3) was measured at an excitation of 490 nm and emission at 520 nm using the spectrofluorometer (SynergyH1, BioTek, USA). Fluorescence was normalized by OD_{600} to calculate relative fluorescence values (RFU).

Preparation of Everted Membrane Vesicles for In Vitro Fosfomycin/H$^+$ Antiport Assay. Everted membrane vesicles of *E. coli* KAM32/pUC18_abaF cells were prepared according to the previously described method with slight modifications.[56,57] For fosfomycin/H$^+$ antiport assay, quinacrine fluorescence quenching was estimated in a mixture of membrane vesicles (0.2 mg/mL of protein) and quinacrine (1 μM). Details are described in the Supporting Information methods (Sections S3 and S4).

Drug Susceptibility Assay/MIC Determination. The MICs of different antibiotics (alone as well as in the presence of 50 μM IITR08367) were determined against *E. coli* KAM32/pUC18_abaF,[17] *E. coli* KAM32/pUC18_abeS,[22] *E. coli* KAM32/pUC18_abeM,[22] and clinical strains of *A. baumannii* (Table S1). The MIC was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[48]

Assessment of IITR08367 Effect on Bacterial Cell Membrane Integrity. Membrane damage assay was performed as previously described.[58] Briefly, logarithmic phase bacterial cells were harvested, washed, and resuspended in 1× PBS, containing 0.4% w/v glucose up to an OD_{600} of 0.2. The cell suspension was treated with 100 μM IITR08367 and

4 mg/L polymyxin B (positive control). After 2 h of incubation at 37 °C, one mg/L propidium iodide (Sigma-Aldrich, USA) was added and incubated for 15 min. Optical density at 600 nm and fluorescence at 535 nm (excitation) and 617 nm (emission) wavelengths were measured by using the spectrofluorometer (SynergyH1, BioTek, USA). Fluorescence was normalized by OD_{600} to calculate RFU.

Ex Vivo Growth Kinetics Study of *A. baumannii* in Human Urine. A growth kinetic was put forward in freshly collected, pH-adjusted (6.5−7), and filtered sterile urine samples supplemented with 20% (v/v) CAMH broth. Each sample was inoculated with 10^5 cells of *A. baumannii* clinical isolate RPTC-15. Each replicate was treated with fosfomycin (64 and 128 mg/L) alone as well as in the presence of IITR08367 (100 μM). Culture was incubated for 12 h at 37 °C with shaking, and optical density was measured at 600 nm after 30 min interval using the spectrofluorometer (SynergyH1, BioTek, USA). This experiment was performed with urine obtained from 21 healthy volunteers.

Scanning Electron Microscopy of Biofilm on Urinary Catheters. Biofilm of *A. baumannii* RPTC-15 was grown on urinary catheters were prepared as previously described with some minor modifications.[59] Briefly, a small section fixed biofilm-formed catheters were dehydrated with an ethanol gradient and visualized under the scanning electron microscope (Zeiss, Germany). Details are described in the Supporting Information (Section S5).

Fluorescence Microscopy and Quantitative Analysis of Biofilm Matrix. Samples were prepared as previously described with some modification.[60] Briefly, biofilm of *A. baumannii* RPTC-15 on glass coverslips were treated with Th-T, WGA-AF 647, and Hoechst 33258. Fluorescence imaging was performed and the fluorescence intensity of each image was measured using ImageJ software.[61] Details are described in the Supporting Information (Section S6).

Erythrocytes (RBCs) Hemolysis and Cytotoxicity Assay. Human RBC hemolysis and toxicity against freshly isolated PBMCs was performed as previously described with minor modifications.[62] Briefly, erythrocytes and PBMCs were treated with different concentrations of IITR08367. Details are described in Supporting Information methods (Section S7).

Toxicity and Efficacy Studies of IITR08367 in *Caenorhabditis elegans* Model. Toxicity and efficacy studies of IITR08367 in *C. elegans* were performed according to the previously described protocol with minor modifications.[63] Infected or uninfected L2-stage worms were used. The number of live and dead worms after 72 h for toxicity, and bacterial load per worm was calculated for efficacy. Details are described in supplementary methods (Section S8).

***C. elegans* Survival Assay.** Worms of *C. elegans* strain AU37 were exposed to *A. baumannii* RPTC-15 in the presence of a subinhibitory concentration of fosfomycin alone as well as in combination with IITR08367. The number of live and dead worms per well was recorded every 24 h for up to 7 days. Details are described in supplementary methods (Section S9).

In Vivo Murine UTI Model. In vivo efficacy of fosfomycin-IITR08367 combination in murine UTI model was performed in 6-week-old BALB/c female mice (*n* = 5). Mice were procured at the age of 5 weeks and housed for 7 days with free access to food and water. RandoMice v1.1.7 software was used to randomly distribute mice among four groups on the basis of urinary pH and body weight. Mice were provided with a 12 h day−night light cycle. Mice were immunocompromised on day −4 and day −1 by injecting cyclophosphamide (150 mg/kg body wt.; I.P.) (TCI Chemical, Japan). On day 0, mice were anesthetized with a 1:1 dose of ketamine (75 mg/kg body wt.; I.P.) (Themis Medicare, Ltd.) and xylazine (16 mg/kg body wt.; I.P.) (Indian Immunologicals, Ltd., India).[64] Mice were infected with *A. baumannii* RPTC-15 (10^5 cfu) by slowly injecting 10 μL culture into the urinary bladder using a neonatal I.V. cannula (26G, IVWP-26; Prime Healthcare Products Pvt. Ltd., India).[65] Treatment was started after 6 h of infection. Group 1 was given normal saline and served as vehicle control (untreated); groups 2, 3, and 4 were treated with fosfomycin (10 mg/kg body wt.), IITR08367 (30 mg/kg body wt.), and an equivalent amount of fosfomycin and IITR08367, respectively. Four doses of treatment were applied every 12 h starting from T0 (6 h postinfection). All mice were sacrificed after 54 h by giving an overdose of anesthesia; the kidney and urinary bladder were excised, and the weight of tissue was measured. One kidney from each mouse was fixed in 4% formalin solution and used for tissue histology (hematoxylin and eosin staining). One kidney and bladder from each mouse were homogenized with a tissue homogenizer (Genetix, India). 100 μL of each sample was 10-fold serially diluted and plated on MH agar plates. CFU counts were enumerated after incubating the plates for 24 h. The results were analyzed with nonparametric one-way ANOVA, with Kruskal−Wallis multiple comparison test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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R.P. and M.S. contributed to the conceptualization; M.S., A.G. and A.H. contributed to the methodology; R.P. contributed to the resources:; M.S. and A.G. contributed to formal analysis; R.P. contributed to funding acquisition; R.P. contributed to supervision; R.P., M.S., A.G., and, A.H. contributed to Writing—review and editing.

Notes

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REFERENCES

(1) World Health Organization. *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report: 2021; 978-92-4-000558-7,* 2021; p 76.

(2) NCDC. *National Antimicrobial Resistance Surveillance Network AMR Annual Report-2021,* 2021; p 46.

(3) Manesh, A.; Varghese, G. M. Rising antimicrobial resistance: an evolving epidemic in a pandemic. *Lancet Microbe* 2021, 2 (9), e419−e420.

(4) Shore, C. K.; Coukell, A. Roadmap for antibiotic discovery. *Nat. Microbiol.* 2016, 1 (6), 16083.

(5) Lewis, K. The Science of Antibiotic Discovery. *Cell* 2020, 181 (1), 29−45.

(6) Laxminarayan, R.; Duse, A.; Wattal, C.; Zaidi, A. K. M.; Wertheim, H. F. L.; Sumpradit, N.; Vlieghe, E.; Hara, G. L.; Gould, I. M.; Goossens, H.; Greko, C.; So, A. D.; Bigdeli, M.; Tomson, G.; Woodhouse, W.; Ombaka, E.; Peralta, A. Q.; Qamar, F. N.; Mir, F.; Kariuki, S.; Bhutta, Z. A.; Coates, A.; Bergstrom, R.; Wright, G. D.; Brown, E. D.; Cars, O. Antibiotic resistance—the need for global solutions. *Lancet Infect. Dis.* 2013, 13 (12), 1057−1098.

(7) Venter, H. Reversing resistance to counter antimicrobial resistance in the World Health Organisation's critical priority of most dangerous pathogens. *Biosci. Rep.* 2019, 39 (4), BSR20180474.

(8) Si, Z.; Lim, H. W.; Tay, M. Y. F.; Du, Y.; Ruan, L.; Qiu, H.; Zamudio-Vazquez, R.; Reghu, S.; Chen, Y.; Tiong, W. S.; Marimuthu, K.; De, P. P.; Ng, O. T.; Zhu, Y.; Gan, Y. H.; Chi, Y. R.; Duan, H.; Bazan, G. C.; Greenberg, E. P.; Chan-Park, M. B.; Pethe, K. A Glycosylated Cationic Block Poly(*β*-peptide) Reverses Intrinsic Antibiotic Resistance in All ESKAPE Gram-Negative Bacteria. *Angew. Chem., Int. Ed. Engl.* 2020, 59 (17), 6819−6826.

(9) Ejim, L.; Farha, M. A.; Falconer, S. B.; Wildenhain, J.; Coombes, B. K.; Tyers, M.; Brown, E. D.; Wright, G. D. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol.* 2011, 7 (6), 348−350.

(10) Huttner, A.; Kowalczyk, A.; Turjeman, A.; Babich, T.; Brossier, C.; Eliakim-Raz, N.; Kosiek, K.; Martinez de Tejada, B.; Roux, X.; Shiber, S.; Theuretzbacher, U.; von Dach, E.; Yahav, D.; Leibovici, L.; Godycki-Cwirko, M.; Mouton, J. W.; Harbarth, S. Effect of 5-Day Nitrofurantoin vs Single-Dose Fosfomycin on Clinical Resolution of Uncomplicated Lower Urinary Tract Infection in Women: A Randomized Clinical Trial. *JAMA* 2018, 319 (17), 1781−1789.

(11) Gardiner, B. J.; Stewardson, A. J.; Abbott, I. J.; Peleg, A. Y. Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. *Aust. Prescr.* 2019, 42 (1), 14−19.

(12) Reffert, J. L.; Smith, W. J. Fosfomycin for the treatment of resistant gram-negative bacterial infections. Insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 2014, 34 (8), 845−857.

(13) Peleg, A. Y.; Seifert, H.; Paterson, D. L. Acinetobacter baumannii: emergence of a successful pathogen. *Clin. Microbiol. Rev.* 2008, 21 (3), 538−582.

(14) Gould, I. M. Coping with antibiotic resistance: the impending crisis. *Int. J. Antimicrob. Agents* 2010, 36, S1−S2.

(15) Leite, G. C.; Perdigão-Neto, L. V.; Ruedas Martins, R. C.; Rizek, C.; Levin, A. S.; Costa, S. F. Genetic factors involved in fosfomycin resistance of multidrug-resistant Acinetobacter baumannii. *Infect., Genet. Evol.* 2021, 93, 104943.

(16) Singkham-In, U.; Chatsuwan, T. Synergism of imipenem with fosfomycin associated with the active cell wall recycling and heteroresistance in Acinetobacter calcoaceticus-baumannii complex. *Sci. Rep.* 2022, 12 (1), 230.

(17) Sharma, A.; Sharma, R.; Bhattacharyya, T.; Bhando, T.; Pathania, R. Fosfomycin resistance in Acinetobacter baumannii is mediated by efflux through a major facilitator superfamily (MFS) transporter-AbaF. *J. Antimicrob. Chemother.* 2017, 72 (1), 68−74.

(18) Pourbaix, A.; Guérin, F.; Burdet, C.; Massias, L.; Chau, F.; Cattoir, V.; Fantin, B. Unexpected Activity of Oral Fosfomycin against Resistant Strains of Escherichia coli in Murine Pyelonephritis. *Antimicrob. Agents Chemother.* 2019, 63 (8), No. e00903-19.

(19) Fedrigo, N. H.; Mazucheli, J.; Albiero, J.; Shinohara, D. R.; Lodi, F. G.; Machado, A.; Sy, S. K. B.; Tognim, M. C. B. Pharmacodynamic Evaluation of Fosfomycin against Escherichia coli and Klebsiella spp. from Urinary Tract Infections and the Influence of pH on Fosfomycin Activities. *Antimicrob. Agents Chemother.* 2017, 61 (8), No. e02498-16.

(20) Ren, X.; Palmer, L. D. Acinetobacter Metabolism in Infection and Antimicrobial Resistance. *Infect. Immun.* 2023, 91 (6), No. e0043322.

(21) Martín-Gutiérrez, G.; Docobo-Pérez, F.; Rodriguez-Beltrán, J.; Rodríguez-Martínez, J. M.; Aznar, J.; Pascual, A.; Blázquez, J. Urinary Tract Conditions Affect Fosfomycin Activity against Escherichia coli Strains Harboring Chromosomal Mutations Involved in Fosfomycin Uptake. *Antimicrob. Agents Chemother.* 2018, 62 (1), No. e01899-17.

(22) Bhattacharyya, T.; Sharma, A.; Akhter, J.; Pathania, R. The small molecule IITR08027 restores the antibacterial activity of fluoroquinolones against multidrug-resistant Acinetobacter baumannii by efflux inhibition. *Int. J. Antimicrob. Agents* 2017, 50 (2), 219−226.

(23) Paixão, L.; Rodrigues, L.; Couto, I.; Martins, M.; Fernandes, P.; de Carvalho, C. C.; Monteiro, G. A.; Sansonetty, F.; Amaral, L.; Viveiros, M. Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. *J. Biol. Eng.* 2009, 3, 18.

(24) Invitrogen by Thermo Fisher Scientific. *Molecular Probes Handbook—A Guide to Fluorescent Probes and Labeling Technologies,* 11th ed.; Thermo Fisher Scientific, 2010.

(25) 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.* 2013, 20 (9), 1168−1178.

(26) Lamers, R. P.; Cavallari, J. F.; Burrows, L. L. The Efflux Inhibitor Phenylalanine-Arginine Beta-Naphthylamide (PA*β*N) Permeabilizes the Outer Membrane of Gram-Negative Bacteria. *PLoS One* 2013, 8 (3), No. e60666.

(27) Hickling, D. R.; Nitti, V. W. Management of recurrent urinary tract infections in healthy adult women. *Rev. Urol.* 2013, 15 (2), 41−48.

(28) Ghosh, T.; Srivastava, S. K.; Gaurav, A.; Kumar, A.; Kumar, P.; Yadav, A. S.; Pathania, R.; Navani, N. K. A Combination of Linalool, Vitamin C, and Copper Synergistically Triggers Reactive Oxygen Species and DNA Damage and Inhibits Salmonella enterica subsp. enterica Serovar Typhi and Vibrio fluvialis. *Appl. Environ. Microbiol.* 2019, 85 (4), No. e02487-18.

(29) Upmanyu, K.; Haq, Q. M. R.; Singh, R. Factors mediating Acinetobacter baumannii biofilm formation: Opportunities for developing therapeutics. *Curr. Res. Microb. Sci.* 2022, 3, 100131.

(30) Dengg, M.; van Meel, J. C. Caenorhabditis elegans as model system for rapid toxicity assessment of pharmaceutical compounds. *J. Pharmacol. Toxicol. Methods* 2004, 50 (3), 209−214.

(31) Vallejo, J. A.; Beceiro, A.; Rumbo-Feal, S.; Rodríguez-Palero, M.; Russo, T. A.; Bou, G. Optimisation of the Caenorhabditis elegans model for studying the pathogenesis of opportunistic Acinetobacter baumannii. *Int. J. Antimicrob. Agents* 2015.

(32) Gaurav, A.; Gupta, V.; Shrivastava, S. K.; Pathania, R. Mechanistic insights into synergy between nalidixic acid and tetracycline against clinical isolates of Acinetobacter baumannii and Escherichia coli. *Commun. Biol.* **2021**, *4* (1), 542.

(33) Blair, J. M.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **2015**, *13* (1), 42−51.

(34) Piddock, L. J. Multidrug-resistance efflux pumps - not just for resistance. *Nat. Rev. Microbiol.* **2006**, *4* (8), 629−636.

(35) Piddock, L. J. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* **2006**, *19* (2), 382−402.

(36) Gaurav, A.; Bakht, P.; Saini, M.; Pandey, S.; Pathania, R. Role of bacterial efflux pumps in antibiotic resistance, virulence, and strategies to discover novel efflux pump inhibitors. *Microbiology* **2023**, *169* (5), 001333.

(37) Blair, J. M.; Richmond, G. E.; Piddock, L. J. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future Microbiol.* **2014**, *9* (10), 1165−1177.

(38) Falagas, M. E.; Giannopoulou, K. P.; Kokolakis, G. N.; Rafailidis, P. I. Fosfomycin: use beyond urinary tract and gastro-intestinal infections. *Clin. Infect. Dis.* **2008**, *46* (7), 1069−1077.

(39) Gupta, K.; Hooton, T. M.; Naber, K. G.; Wullt, B.; Colgan, R.; Miller, L. G.; Moran, G. J.; Nicolle, L. E.; Raz, R.; Schaeffer, A. J.; Soper, D. E. International Clinical Practice Guidelines for the Treatment of Acute Uncomplicated Cystitis and Pyelonephritis in Women: A 2010 Update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. *Clin. Infect. Dis.* **2011**, *52* (5), e103−e120.

(40) Aghamali, M.; Sedighi, M.; Zahedi Bialvaei, A.; Mohammadzadeh, N.; Abbasian, S.; Ghafouri, Z.; Kouhsari, E. Fosfomycin: mechanisms and the increasing prevalence of resistance. *J. Med. Microbiol.* **2019**, *68* (1), 11−25.

(41) Murray, C. J. L.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Robles Aguilar, G.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; Johnson, S. C.; Browne, A. J.; Chipeta, M. G.; Fell, F.; Hackett, S.; Haines-Woodhouse, G.; Kashef Hamadani, B. H.; Kumaran, E. A. P.; McManigal, B.; Achalapong, S.; Agarwal, R.; Akech, S.; Albertson, S.; Amuasi, J.; Andrews, J.; Aravkin, A.; Ashley, E.; Babin, F. X.; Bailey, F.; Baker, S.; Basnyat, B.; Bekker, A.; Bender, R.; Berkley, J. A.; Bethou, A.; Bielicki, J.; Boonkasidecha, S.; Bukosia, J.; Carvalheiro, C.; Castañeda-Orjuela, C.; Chansamouth, V.; Chaurasia, S.; Chiurchiù, S.; Chowdhury, F.; Clotaire Donatien, R.; Cook, A. J.; Cooper, B.; Cressey, T. R.; Criollo-Mora, E.; Cunningham, M.; Darboe, S.; Day, N. P. J.; De Luca, M.; Dokova, K.; Dramowski, A.; Dunachie, S. J.; Duong Bich, T.; Eckmanns, T.; Eibach, D.; Emami, A.; Feasey, N.; Fisher-Pearson, N.; Forrest, K.; Garcia, C.; Garrett, D.; Gastmeier, P.; Giref, A. Z.; Greer, R. C.; Gupta, V.; Haller, S.; Haselbeck, A.; Hay, S. I.; Holm, M.; Hopkins, S.; Hsia, Y.; Iregbu, K. C.; Jacobs, J.; Jarovsky, D.; Javanmardi, F.; Jenney, A. W. J.; Khorana, M.; Khusuwan, S.; Kissoon, N.; Kobeissi, E.; Kostyanev, T.; Krapp, F.; Krumkamp, R.; Kumar, A.; Kyu, H. H.; Lim, C.; Lim, K.; Limmathurotsakul, D.; Loftus, M. J.; Lunn, M.; Ma, J.; Manoharan, A.; Marks, F.; May, J.; Mayxay, M.; Mturi, N.; Munera-Huertas, T.; Musicha, P.; Musila, L. A.; Mussi-Pinhata, M. M.; Naidu, R. N.; Nakamura, T.; Nanavati, R.; Nangia, S.; Newton, P.; Ngoun, C.; Novotney, A.; Nwakanma, D.; Obiero, C. W.; Ochoa, T. J.; Olivas-Martinez, A.; Olliaro, P.; Ooko, E.; Ortiz-Brizuela, E.; Ounchanum, P.; Pak, G. D.; Paredes, J. L.; Peleg, A. Y.; Perrone, C.; Phe, T.; Phommasone, K.; Plakkal, N.; Ponce-de-Leon, A.; Raad, M.; Ramdin, T.; Rattanavong, S.; Riddell, A.; Roberts, T.; Robotham, J. V.; Roca, A.; Rosenthal, V. D.; Rudd, K. E.; Russell, N.; Sader, H. S.; Saengchan, W.; Schnall, J.; et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **2022**, *399* (10325), 629−655.

(42) Falagas, M. E.; Vouloumanou, E. K.; Samonis, G.; Vardakas, K. Z. Fosfomycin. *Clin. Microbiol. Rev.* **2016**, *29* (2), 321−347.

(43) Grabein, B.; Graninger, W.; Rodríguez Baño, J.; Dinh, A.; Liesenfeld, D. B. Intravenous fosfomycin-back to the future.

(44) Sirijatuphat, R.; Thamlikitkul, V. Preliminary study of colistin versus colistin plus fosfomycin for treatment of carbapenem-resistant Acinetobacter baumannii infections. *Antimicrob. Agents Chemother.* **2014**, *58* (9), 5598−5601.

(45) Bergogne-Bérézin, E.; Muller-Serieys, C.; Joly-Guillou, M. L.; Dronne, N. Trometamol-Fosfomycin (Monuril) Bioavailability and Food-Drug Interaction. *Eur. Urol.* **1987**, *13*, 64−68.

(46) Roussos, N.; Karageorgopoulos, D. E.; Samonis, G.; Falagas, M. E. Clinical significance of the pharmacokinetic and pharmacodynamic characteristics of fosfomycin for the treatment of patients with systemic infections. *Int. J. Antimicrob. Agents* **2009**, *34* (6), 506−515.

(47) Ordaz, G.; Dagà, U.; Budia, A.; Pérez-Lanzac, A.; Fernández, J.; Jordán, C. Urinary pH and antibiotics, choose carefully. A systematic review. *Actas Urol. Esp.* **2023**, *47* (7), 408−415.

(48) CLSI. *Performance Standards for Antimicrobial Susceptibility Testing 13th ed. CLSI Standard M02*; Clinical and Laboratory Standards Institute: Wayne, PA, 2018.

(49) Pour, N. K.; Dusane, D. H.; Dhakephalkar, P. K.; Zamin, F. R.; Zinjarde, S. S.; Chopade, B. A. Biofilm formation by Acinetobacter baumannii strains isolated from urinary tract infection and urinary catheters. *FEMS Immunol. Med. Microbiol.* **2011**, *62* (3), 328−338.

(50) Chen, J.; Morita, Y.; Huda, M. N.; Kuroda, T.; Mizushima, T.; Tsuchiya, T. VmrA, a Member of a Novel Class of Na+ Coupled Multidrug Efflux Pumps from Vibrio parahaemolyticus. *J. Bacteriol.* **2002**, *184* (2), 572−576.

(51) Odds, F. C. Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* **2003**, *52* (1), 1.

(52) Cunha, B. A.; Domenico, P.; Cunha, C. B. Pharmacodynamics of doxycycline. *Clin. Microbiol. Infect.* **2000**, *6* (5), 270−273.

(53) Gaurav, A.; Kothari, A.; Omar, B. J.; Pathania, R. Assessment of polymyxin B-doxycycline in combination against Pseudomonas aeruginosa in vitro and in a mouse model of acute pneumonia. *Int. J. Antimicrob. Agents* **2020**, *56* (1), 106022.

(54) Korakianitis, I.; Mirtsou, V.; Gougoudi, E.; Raftogiannis, M.; Giamarellos-Bourboulis, E. J. Post-antibiotic effect (PAE) of moxifloxacin in multidrug-resistant Stenotrophomonas maltophilia. *Int. J. Antimicrob. Agents* **2010**, *36* (4), 387−389.

(55) Blair, J. M.; Piddock, L. J. How to measure export via bacterial multidrug resistance efflux pumps. *mBio* **2016**, *7* (4), No. e00840-16.

(56) Verkhovskaya, M. Preparation of Everted Membrane Vesicles from Escherichia coli Cells. *Bio-Protoc.* **2017**, *7* (9), No. e2254.

(57) Kodama, K.; Hashimoto, A.; Morita, Y.; Tomochika, K.-i.; Tsuchiya, T. Preparation and characterization of everted membrane vesicles from cells of Staphylococcus aureus. *Biol. Pharm. Bull.* **1998**, *21* (1), 5−9.

(58) Patel, M. B.; Garrad, E.; Meisel, J. W.; Negin, S.; Gokel, M. R.; Gokel, G. W. Synthetic ionophores as non-resistant antibiotic adjuvants. *RSC Adv.* **2019**, *9* (4), 2217−2230.

(59) Djeribi, R.; Bouchloukh, W.; Jouenne, T.; Menaa, B. Characterization of bacterial biofilms formed on urinary catheters. *Am. J. Infect. Control* **2012**, *40* (9), 854−859.

(60) Guillonneau, R.; Baraquet, C.; Bazire, A.; Molmeret, M. Multispecies Biofilm Development of Marine Bacteria Implies Complex Relationships Through Competition and Synergy and Modification of Matrix Components. *Front. Microbiol.* **2018**, *9*, 1960.

(61) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **2012**, *9* (7), 676−682.

(62) Bhando, T.; Bhattacharyya, T.; Gaurav, A.; Akhter, J.; Saini, M.; Gupta, V. K.; Srivastava, S. K.; Sen, H.; Navani, N. K.; Gupta, V.; Biswas, D.; Chaudhry, R.; Pathania, R. Antibacterial properties and in vivo efficacy of a novel nitrofuran, IITR06144, against MDR pathogens. *J. Antimicrob. Chemother.* **2020**, *75* (2), 418−428.

(63) Jayamani, E.; Rajamuthiah, R.; Larkins-Ford, J.; Fuchs, B. B.; Conery, A. L.; Vilcinskas, A.; Ausubel, F. M.; Mylonakis, E. Insect-derived cecropins display activity against Acinetobacter baumannii in

Systematic review and meta-analysis of the clinical literature. *Clin. Microbiol. Infect.* **2017**, *23* (6), 363−372.

a whole-animal high-throughput Caenorhabditis elegans model. *Antimicrob. Agents Chemother.* **2015**, *59* (3), 1728−1737.

(64) Musterman, M. *Guidelines of Rat and Mouse Anasthesia and Analgesia Formulary and General Drug Information*, 2016; pp 1−14.

(65) Hung, C.-S.; Dodson, K. W.; Hultgren, S. J. A murine model of urinary tract infection. *Nat. Protoc.* **2009**, *4* (8), 1230−1243.