pH-Mediated Potentiation of Aminoglycosides Kills Bacterial Persisters and Eradicates In Vivo Biofilms

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Background. Limitations in treatment of biofilm-associated bacterial infections are often due to subpopulation of persistent bacteria (persisters) tolerant to high concentrations of antibiotics. Based on the increased aminoglycoside efficiency under alkaline conditions, we studied the combination of gentamicin and the clinically compatible basic amino acid L-arginine against planktonic and biofilm bacteria both in vitro and in vivo.

Methods. Using Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli bioluminescent strains, we studied the combination of L-arginine and gentamicin against planktonic persisters through time-kill curves of late stationary-phase cultures. In vitro biofilm tolerance towards gentamicin was assessed using PVC 96 well-plates assays. Efficacy of gentamicin as antibiotic lock treatment (ALT) at 5 mg/mL at different pH was evaluated in vivo using a model of totally implantable venous access port (TIVAP) surgically implanted in rats.

Results. We demonstrated that a combination of gentamicin and the clinically compatible basic amino acid L-arginine increases in vitro planktonic and biofilm susceptibility to gentamicin, with 99% mortality amongst clinically relevant pathogens, i.e. S. aureus, E. coli and P. aeruginosa persistent bacteria. Moreover, although gentamicin local treatment alone showed poor efficacy in a clinically relevant in vivo model of catheter-related infection, gentamicin supplemented with L-arginine led to complete, long-lasting eradication of S. aureus and E. coli biofilms, when used locally.

Conclusion. Given that intravenous administration of L-arginine to human patients is well tolerated, combined use of aminoglycoside and the non-toxic adjuvant L-arginine as catheter lock solution could constitute a new option for the eradication of pathogenic biofilms.

Keywords. biofilm; persister; aminoglycoside; proton motive force; catheter-related infections.

Biofilm bacterial communities that develop on the surface of indwelling devices and mucosa play a key role in healthcare-associated infections due to their characteristic high tolerance to antibiotics [1]. Biofilm formation therefore impairs treatment efficacy and requires frequent therapeutic removal of colonized devices, leading to increased morbidity and medical costs [1–3]. Most treatment difficulties are related to biofilm tolerance, defined as the ability of a subset of biofilm cells to survive in the presence of high concentrations of bactericidal antibiotics [3]. Although biofilm tolerance is multifactorial, it is believed to originate mainly from a subpopulation of nongrowing persistent bacteria characterized by phenotypically high, but reversible, tolerance to otherwise lethal concentrations of antibiotics [3]. These persisters are not resistant mutants and may resume growth and repopulate biofilm after partially effective antibiotic treatment, thereby leading to infection recurrence [3]. Several studies have identified promising antibiofilm strategies that inhibit bacterial adhesion or induce dispersal [4, 5]. However, such approaches were associated with risk of massive release of pathogenic bacteria from the infected site into the patient’s bloodstream.
[6]. Hence, in addition to identification of nonbiocidal antibiotic compounds, antipersister approaches that are able to kill planktonic and biofilm bacteria are currently being investigated [7, 8].

For instance, use of adjuvant molecules such as mannitol or fructose was demonstrated to increase aminoglycoside uptake, thereby enhancing their in vitro and in vivo efficacy against persisters [9]. More recently, it has been shown that corrupting a target in dormant cells through the activation of a bacterial protease could also increase antibiotic-induced persister cell death [8].

It has been shown that sensitivity of planktonic bacteria exposed to aminoglycosides is increased in alkaline media and, inversely, reduced under acidic conditions [10–12]. Those results, along with several other reports, raised the possibility that local, physiologically compatible alkalization could constitute an alternative approach to improving antibiotic efficiency and eradicating biofilm persisters [10, 13].

Here, we show that raising pH through the use of harmless, clinically compatible basic amino acid L-arginine leads to an increase in the killing efficiency of gentamicin toward persisters. We demonstrate that L-arginine and pH-mediated potentiation of aminoglycosides are effective against both in vitro and in vivo biofilms produced by gram-positive and gram-negative nosocomial pathogens. These results demonstrate that pH-mediated stimulation of the effects of aminoglycosides could constitute a new curative approach to eradicating localized vascular catheter-associated biofilm infections.

MATERIAL AND METHODS

Bacterial Strains and Growth Media

Luminescent variants of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were purchased (*S. aureus* Xen36 from Caliper, Hopkinton, MA) or constructed (*E. coli* EAEC 55989 transformed with stable plasmid pAT881 and *P. aeruginosa* PAKlux, a bioluminescent derivative of the PAK clinical strain) [14–16]. *Staphylococcus aureus* was grown in tryptic soy broth (TSB) supplemented with 0.25% glucose (TSB glucose). Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were grown in lysogeny broth (LB). *Escherichia coli* TGIΔargA was obtained by P1vir transduction from MG1655ΔargA::Tn10 to TGI. Unless specified, all chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Basic amino acids were resuspended in LB, TSB glucose, or 1× phosphate-buffered saline (PBS) and then diluted. L-arginine at 0.4% was buffered using MOPS (3-(N-morpholino)propanesulfonic acid) at 0.05 M. Media were alkalized using adjusted 0.1 M AMPSO (N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid) buffer. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was purchased from Interchim (Montluçon, France). CCCP stock solutions were prepared in dimethyl sulfoxide at 5 mM and stored at 4°C.

Growth Inhibition Assays of Planktonic Bacteria

Growth curves were performed in TPP 96-well polystyrene microtiter plates (Sigma-Aldrich) using a Tecan Sunrise microplate reader (Tecan Group, Männedorf, Switzerland). Each condition was replicated in 3 wells. Minimal inhibitory concentrations (MICs) were determined by broth microdilution. To determine whether the basic amino acid effect was pH mediated, we first looked for a pH-independent effect of L-arginine buffered with MOPS at 0.05 M in order to reach the pH of growth media alone (buffered L-arginine). It is noteworthy that by using TG1ΔargA, which is an *E. coli* derivative auxotrophic toward L-arginine, we were able to confirm that the buffering process with MOPS did not alter L-arginine since bacterial growth was possible with 5–10 µg/mL of L-arginine ± MOPS. Next, 0.1 M–adjusted AMPSO was used as an alkaline buffer so as to reach the same pH as L-arginine 0.4%.

Effects on Stationary-Phase Bacteria and Persister Cells

Since stationary-phase cultures of *E. coli* routinely reach pH 9 and were therefore already alkalinized, late stationary-phase cultures of *S. aureus*, *E. coli*, or *P. aeruginosa* were washed twice (6000 rpm, 10 minutes), resuspended in 1× PBS, and treated with different concentrations of gentamicin supplemented or not with L-arginine or buffered L-arginine under continuous agitation. This strategy allowed standardization of initial pH while maintaining nutrient limitations and thus impeding persisters’ resuscitation. At different time points, a 0.5-mL sample was removed and washed twice in 1× PBS to remove excess antibiotic and surviving colony-forming units (CFUs) were then quantified. In order to eradicate all nonpersistent bacteria, we first sought to determine the gentamicin concentration and time of exposure that led to a surviving CFU plateau (Supplementary Figure 1A, 1C, and 1E). Use of 100-fold of the gentamicin MIC for 6 hours (*S. aureus* and *E. coli*) or 500-fold the MIC for 6 hours (*P. aeruginosa*) led to maximum bacterial mortality. To confirm that this population was hypertolerant to bactericidal antibiotics, this 6-hour–treated culture was washed twice and resuspended in 1× PBS and then treated with gentamicin or ofloxacin at 10-, 100-, or 500-fold the MIC (Supplementary Figure 1B, 1D, and 1F). For *E. coli*, L-arginine was used at 0.2%, as higher concentrations led to significant bacterial mortality even without gentamicin. Buffered L-arginine with MOPS at 0.05 M was used as control, as described above.

Effect on Exponential-Phase Bacteria and Disruption of the Proton Motive Force

Exponential-phase cultures of *E. coli* 55989 (Optical Density 0.3–0.6) were washed twice (6000 rpm, 10 minutes), resuspended in 1× PBS, and treated with 10× MIC gentamicin (80 µg/mL) supplemented or not with L-arginine (0.4%) under continuous agitation. To study the consequences of proton motive force (PMF) disruption on the effect of L-arginine, we used
CCCP, a proton ionophore that abolishes both $\Delta \psi$ and $\Delta \text{pH}$ [17]. CCCP was added at a final concentration of 20 µM. After 30 minutes, a 15-µL sample was removed and serially diluted for quantification of surviving CFUs.

**In Vitro Biofilm Formation and Treatment**

In vitro biofilms were grown in triplicate for 24 hours (S. aureus and E. coli) or 48 hours (P. aeruginosa) on polyvinyl chloride 96-well plates sterilized with ultraviolet light (Thermo Scientific, Rochester, NY). Planktonic bacteria were removed by 1 × PBS washing, and biofilms were treated for 24 hours with increasing concentrations of antibiotic (gentamicin or amikacin) ± L-arginine at 0.4% in TSB glucose or LB. For amikacin, L-arginine at 0.8% was chosen because of better reproducibility compared with L-arginine 0.4%. Buffered L-arginine with MOPS at 0.05 M and pH-modified media with adjusted AMPSO at 0.1 M were used as controls as described above, except for L-arginine 0.8% that required MOPS at 0.1 M in order to be buffered. Then, each well was washed twice with 1 × PBS to remove planktonic bacteria and excess antibiotics; surviving CFUs were then quantified.

**In Vivo Model and Ethics Statement**

Animals were housed in the Institut Pasteur animal facilities, accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 06th February, 2013). The protocols used in this study for the animal model, catheter placement, in vivo biofilm formation, and in vivo study of inflammatory responses were approved by the ethics committee Paris Centre et Sud N°59 under reference 2012–0045. We used a previously described rat model of biofilm-related infection totally implantable venous access port (TIVAP) [18]. Pediatric TIVAP (cat # 2105ISP, Polysite Micro, Titanium 2000 series, Perouse Medical, Ivry le temple, France) were inserted in CD/SD (IGS:Crl) rats (Charles River) and then left to recover for 4 days [18]. One hundred microliters of 1 × PBS containing $10^6$ cells (for S. aureus or P. aeruginosa) or $10^4$ cells (for E. coli) were injected inside the port through the silicone septum using a Huber needle (Perouse Medical) in order to contaminate TIVAP. Bioluminescence was monitored every day using an IVIS-100 imaging system with a charge-coupled device camera (Xenogen Corporation, Alameda, CA). After confirmation of complete catheter colonization (around day 3), rats were treated with systemic antibiotics (daily subcutaneous injection of vancomycin at 50 mg/kg for S. aureus or gentamicin at 30 mg/kg for gram-negative bacteria) and antibiotic lock therapy (ALT) [19, 20]. Vancomycin and gentamicin were chosen as they have been extensively used in animal models with a limited number of injections each day, allowing for reduction of the number of animal manipulations and thus an increase in animal comfort. Day 0 corresponds to the beginning of treatment. ALT consisted of sterile PBS (control), gentamicin or amikacin (5 mg/mL), L-arginine (0.4%), gentamicin (or amikacin) + L-arginine. For amikacin locks, L-arginine was used at 0.8%. All ALT were prepared in sterile water. As controls, we used buffered L-arginine with MOPS or adjusted 0.1 M AMPSO, as described above. Treatment was carried out for 5 days by replacing the old lock with a new one each day. Seven

**Figure 1.** Basic amino acids increase Staphylococcus aureus susceptibility to gentamicin under planktonic conditions. A, Gentamicin minimal inhibitory concentrations of S. aureus Xen36 in tryptic soy broth (TSB) glucose medium supplemented with different basic amino acids. Data represented are the means ± standard error of the mean of at least 3 independent experiments. B, Staphylococcus aureus Xen36 growth curves in TSB glucose without antibiotics with increasing concentrations of L-arginine. L-arginine was buffered using 3-(N-morpholino)propanesulfonic acid at 0.05 M. Abbreviations: AMPSO, N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; L-arg, L-arginine; L-lys, L-lysine; MIC, minimal inhibitory concentration; TSBg, TSB + glucose at 0.25%.
days after the end of treatment, rats were sacrificed and TIVAP were removed for bioluminescence imaging and CFU count, as described [18].

Statistical Analysis
Each experiment was performed at least 3 times. The Wilcoxon Mann–Whitney test (included in Graphpad Prism version 5.04) was used to compare continuous variables. Treatment groups were considered statistically different if $P < .05$.

RESULTS

Basic Amino Acids Increase the Susceptibility of Planktonic Bacteria to Gentamicin
Previous reports showed that medium alkalinization enhanced the effects of aminoglycosides on planktonic bacteria [10]. Consistently, we observed that medium alkalinization with 0.1 M AMPSO buffer adjusted to pH approximately 8.5 decreased the gentamicin MIC of gram-positive (S. aureus) and gram-negative (E. coli and P. aeruginosa) bacteria (Figure 1A and Supplementary Figure 2) [10]. To increase pH using physiologically tolerated compounds, we supplemented TSB and LB broth with the nonbuffered basic amino acids L-arginine, L-histidine, and L-lysine. By increasing nonbuffered L-arginine or L-lysine, it was possible to attain a wide range of pH values in supplemented TSB or LB media (Supplementary Figure 2) and enhanced bacterial susceptibility to gentamicin (Figure 1A and Supplementary Figure 2). Moreover, growth curves performed with increasing concentrations of L-arginine without antibiotic showed no significant inhibition at concentrations that would otherwise have led to increased susceptibility to gentamicin for S. aureus, E. coli, and P. aeruginosa (Figure 1B and Supplementary Figure 2). To confirm that the effect of L-arginine on gentamicin susceptibility was pH dependent, we demonstrated that gentamicin MIC was not affected by buffered L-arginine (Figure 1A and Supplementary Figure 2).

Nonbuffered L-arginine Reduces Planktonic Persister Tolerance to Gentamicin
Stationary-phase bacterial cultures display higher antibiotic tolerance due to an increased level of persistent bacteria compared with exponential-phase cultures [21]. To test whether combining basic amino acid L-arginine and gentamicin could reduce this tolerance, we treated overnight PBS-washed S. aureus, E. coli, and P. aeruginosa cultures for 6 hours with high concentrations of gentamicin in order to kill all nonpersistent bacteria. Indeed, we showed that bacteria that survived this 6-hour gentamicin

Figure 2. L-arginine decreases the tolerance of Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa stationary-phase cultures toward gentamicin. A, Survival curve of S. aureus Xen36; B, enterohemorrhagic E. coli 55989; and C, P. aeruginosa PAKlux overnight cultures treated in 1× phosphate-buffered saline with 100× or 500× minimal inhibitory concentration (MIC) of gentamicin supplemented with or without L-arginine or buffered L-arginine. Data represented are the means ± standard error of the mean of at least 3 independent experiments. *$P < .05$. Values for gentamicin + L-arginine or gentamicin + buffered L-arginine were compared with the group treated with gentamicin alone at 100× (S. aureus and E. coli) or 500× MIC (P. aeruginosa; dark squares). Dotted line represents the limit of detection. Abbreviations: CFU, colony-forming unit; L-arg, L-arginine; ON, overnight.
treatment displayed high tolerance to bactericidal antibiotics characteristically described for persisters (Supplementary Figure 1). Supplementation with L-arginine 0.4% led to a 100-fold reduction in the tolerance of *S. aureus* stationary-phase cultures to gentamicin, while use of buffered L-arginine had no significant effect (Figure 2A). Similar results were obtained with *E. coli* (Figure 2B) and *P. aeruginosa* (Figure 2C), with surviving bacteria below the limit of detection in the latter case when gentamicin was supplemented with nonbuffered L-arginine.

**L-arginine Increases the Efficiency of Gentamicin by Modifying the Membrane pH Gradient**

Gentamicin efficacy was previously shown to depend on the PMF composed of both membrane electrical potential (Δψ) and the transmembrane difference in the H⁺ concentration (ΔpH) [11, 22]. We investigated the consequences of PMF disruption on the effect of L-arginine using CCCP, a proton ionophore that abolishes both Δψ and ΔpH [17]. As stationary-phase bacteria displayed high mortality when exposed to CCCP associated with L-arginine, we used exponential-phase culture bacteria. We first established that L-arginine also mediates the potentiation of the effect of gentamicin against *E. coli* exponential-phase cultures, as demonstrated by a significantly higher bacterial mortality (Figure 3A). Next, we showed that the addition of CCCP abolished gentamicin antibacterial activity, confirming that gentamicin efficacy is dependent on the PMF. Strikingly, the addition of L-arginine in the presence of CCCP partially restored gentamicin activity (Figure 3A). This suggests that the pH effect mediated by nonbuffered L-arginine generates a CCCP-insensitive pH gradient, consistent with similar results obtained using alkaline buffer instead of L-arginine (Figure 3B).

**L-arginine and Alkaline pH Reduce In Vitro Biofilm Tolerance to Aminoglycosides**

Although persisters are produced under both planktonic and biofilm culture conditions [21, 23], they are considered to be the major determinants of biofilm-associated antibiotic tolerance [3]. The enhanced gentamicin efficacy in bacteria subjected to an L-arginine-mediated pH increase prompted us to determine whether this effect is also found in biofilm bacteria. We first observed that, as expected for biofilm bacteria, *S. aureus, E. coli,* and *P. aeruginosa* biofilms displayed between 0.1% and 2% survival at gentamicin concentrations as high as 200×MIC (Figure 4). Strikingly, while L-arginine alone had no effect on biofilm bacteria, nonbuffered L-arginine increased the effect of gentamicin by 2 to 3 logs (Figure 4). Interestingly, although buffered L-arginine combined with gentamicin had no significant effect on *E. coli* biofilms (Figure 4B), it led to a 2-log decrease in the bacterial viability of *S. aureus* and *P. aeruginosa* biofilms, suggesting that L-arginine might also have a pH-independent effect on these bacteria (Figure 4A and 4C). Similar results were obtained for

**P. aeruginosa** with amikacin, another clinically relevant aminoglycoside (Supplementary Figure 3A and 3B).

**Gentamicin Combined With L-Arginine Leads to In Vivo Biofilm Eradication**

Our in vitro results suggest that nonbuffered L-arginine constitutes a new antibiofilm adjuvant for use in combination with...
aminoglycosides in case of local biofilm-associated infections. We tested this hypothesis using an in vivo rat model of a TIVAP amenable to ALT [18, 24]. ALT corresponds to instillation of a high concentration of antibiotics dwelling in the lumen of a catheter, designed to cure resilient biofilms on a central venous catheter [25]. We performed controlled inoculation of a rat-implanted TIVAP using bioluminescent *S. aureus*, *E. coli*, or *P. aeruginosa* strains and monitored the evolution of biofilm formation in the device, which was fully colonized after 3 days (day 0 on Figure 5).

While ALT solutions corresponding to PBS (control), L-arginine, or gentamicin (625 × MIC) alone had little or no effect on biofilm formed in TIVAP (Figure 5A–C), combined use of gentamicin with nonbuffered L-arginine led to complete, long-lasting eradication of *S. aureus* and *E. coli* biofilms (Figure 5D and Figure 6A and 6B). This was demonstrated by the absence of bioluminescence and the disappearance of surviving CFUs, even at 7 days after the end of combined L-arginine-gentamicin ALT (Figure 5G and 5H and Figure 6A and 6B). In contrast, gentamicin supplemented with nonbuffered L-arginine reduced but failed to eradicate in vivo *P. aeruginosa* biofilms (Figure 6C and 6D).

Interestingly, although gentamicin combined with alkaline buffer did not eradicate biofilms in any of the tested rats, use of gentamicin and buffered L-arginine eradicated *S. aureus* biofilms in 4 of 5 rats (Figure 5E to 5H). Similarly, use of gentamicin in an alkaline buffer led to eradication of *E. coli* biofilm in 2 of the 3 tested rats (Figure 6B).

Taken together, these results demonstrate that gentamicin supplemented with L-arginine enables eradication of in vivo biofilm formed by *S. aureus* and *E. coli* via both a pH-mediated and a pH-independent effect.

**DISCUSSION**

The existence of subpopulations of persistent bacteria that are highly tolerant to antibiotics constitutes a major cause of therapeutic inefficacy against biofilms that colonize indwelling devices in medical settings [3, 26]. Here, we show that use of nonbuffered L-arginine as a nontoxic basic compound leads to enhancement of aminoglycoside effectiveness and eradication of persisters formed by several nosocomial pathogens in both in vitro and in vivo biofilms.

The effect of gentamicin is dependent on the PMF, which is composed of Δψ (the electrical potential across the membrane) and ΔpH (the transmembrane difference in the H⁺ concentration) [11, 22, 27]. While use of mannitol or fructose leads to Nicotinamide Adenine Dinucleotide – Hydrogen (NADH) production that stimulates PMF through an increase in membrane electrical potential [9], our results demonstrate that the pH-mediated L-arginine effect probably relies on an effect on the ΔpH-dependent transmembrane difference in the H⁺ ion.

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**Figure 4.** L-arginine reduces in vitro biofilm tolerance of gram-positive and gram-negative bacteria toward gentamicin. *A. Staphylococcus aureus Xen36; B. enteric aggregative Escherichia coli 55989; and C. Pseudomonas aeruginosa PAK/lux* in vitro biofilms were treated with increasing concentrations of gentamicin ± L-arginine, buffered L-arginine with 3-(N-morpholino)propanesulfonic acid 0.05 M, or media buffered with adjusted N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid at 0.1 M. Data represented are means ± standard error of the mean of at least 3 independent experiments. *P < .05, **P < .001, and ***P < .0001. Values were compared with the control (dark bar) at the same antibiotic concentration. Abbreviations: AMPSO, N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; CFU, colony-forming unit; glu, glucose; LB, lysogeny broth; MIC, minimal inhibitory concentration; TSB, tryptic soy broth.
concentration rather than on membrane electrical potential. The mortality of stationary-phase bacteria exposed to CCCP and L-arginine was not expected and was not observed using exponential-phase cultures. We hypothesized that stationary-phase cultures are less able to cope with high pH when CCCP is added. In stationary-phase culture, PMF is probably more important for survival at high pH.

Although L-arginine-mediated effects were found to be exclusively pH-mediated against S. aureus, E. coli, and P. aeruginosa planktonic bacteria and against E. coli biofilm, we observed that medium supplementation with buffered L-arginine significantly reduced S. aureus and P. aeruginosa in vitro biofilm tolerance toward aminoglycosides. Although mechanistic aspects of this effect are currently under investigation, this pH-independent activity could be due to known metabolic stimulation of S. aureus and P. aeruginosa biofilm bacteria exposed to L-arginine [28, 29]. Indeed, supplementation of another aminoglycoside (tobramycin) with 0.4% L-arginine was shown to reduce P. aeruginosa biofilm antibiotic tolerance in vitro, possibly via stimulation of anaerobic metabolism in deep layers of P. aeruginosa biofilm [30]; however, it was not mentioned whether or not this L-arginine solution was buffered. In their study, the effect was more potent in anaerobic conditions than in aerobic conditions. It has been shown

Figure 5. In vivo eradication of Staphylococcus aureus biofilm with gentamicin supplemented with L-arginine. Antibiotic lock therapy (ALT) was instilled in TIVAP (day 0) and was associated with systemic vancomycin to treat 3-day-old S. aureus Xen36 biofilm (number of rats used for each treatment, n = 5). ALT was renewed every 24 hours for 5 days, and its efficacy was monitored as photon emissions. A, 1× phosphate-buffered saline ALT (control); B, L-arginine ALT (0.4%); C, gentamicin ALT (5 mg/mL); D, combined gentamicin (5 mg/mL) and L-arginine (0.4%) ALT; E, combined gentamicin (5 mg/mL) and buffered L-arginine (0.4%) with 3-(N-morpholino)propanesulfonic acid 0.05 M; and F, gentamicin (5 mg/mL) in adjusted N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid 0.1 M. A–F, Results from a representative animal. G, Seven days after administration of the last ALT, rats were sacrificed; TIVAP was harvested and monitored for photon emissions. H, Bacterial cells from TIVAP were harvested, serially diluted, and plated on tryptic soy broth agar for colony-forming unit (CFU) counting. Results for each rat are represented for each treatment group. Mean CFU and standard deviation are also presented as horizontal lines and whiskers. P < .05 was considered significant; *P < .05; **P < .001. Abbreviations: AMPSO, N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; L-arg, L-arginine; ns, not significant; PBS, phosphate-buffered saline.
that anaerobic conditions could prevail in deep layers of biofilms [31]. Thus, we believe that some observations made in anaerobic conditions [30] can be translated into aerobic conditions.

While clinical use of alkaline buffers may be potentially toxic, our choice of L-arginine as an in vivo alkalization adjuvant to aminoglycoside-based therapies is justified by numerous reports of the lack of toxicity of intravenous administration of L-arginine to patients in various clinical situations [32, 33]. We tested the clinical relevance of our results using an appropriate rat model of catheter-associated biofilm infection amenable to controlled ALT. We showed that increasing the pH of highly concentrated yet partially ineffective gentamicin solutions in the lumen of a TIVAP eradicated the representative gram-positive and gram-negative bacterial pathogens that are frequently responsible for catheter-related infections [34, 35].

Since pH-mediated stimulation of antibiotic activity is likely to be effective against a wide range of bacteria, our results suggest that the observed rapid (1 day for S. aureus and E. coli) and long-lasting eradication of in vivo bacterial biofilms could constitute a clinically relevant therapeutic alternative to the current standard catheter-removal approach used in common biofilm infections [25, 36, 37]. Moreover, while the L-arginine effect is not restricted to gentamicin aminoglycoside and also increases

Figure 6. In vivo eradication of gram-negative bacteria biofilm using L-arginine as an adjuvant to gentamicin. Antibiotic lock therapy (ALT) was instilled in TIVAP and associated with systemic gentamicin to treat 3-day-old Escherichia coli 55989 (A and B) or Pseudomonas aeruginosa PAKlux (C and D) biofilms (number of rats used for each treatment, n = 3). Seven days after the administration of the last ALT, rats were sacrificed; TIVAP was harvested and monitored for photon emissions (A and C). Bacterial cells from TIVAP were harvested, serially diluted, and plated on lysogeny broth agar for colony-forming unit (CFU) counting (B and D). Results for each rat are represented for each treatment group. Mean CFU and standard deviation are also presented as horizontal lines and whiskers. L-arginine was buffered using 3-(N-morpholino)propanesulfonic acid at 0.05 M. Abbreviations: AMPSO, N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; L-arg, L-arginine; PBS, phosphate-buffered saline.
amikacin activity in bacterial biofilms, the existence of both pH-dependent and pH-independent stimulation by L-arginine of aminoglycoside effects on biofilm bacteria suggests that L-arginine might be a good adjuvant candidate for reducing biofilm persistor tolerance toward gentamicin in clinical situations in which pH might be locally modified at the site of biofilm-associated infections. In addition, other classes of antibiotics may have a pH-dependent modified spectrum of action, such as fluoroquinolones, the effects of which could be increased under alkaline conditions in clinical settings such as that of urinary tract infections [38, 39]. Last, as some amino acids can be inhaled, the association of L-arginine and aminoglycosides could be studied in the treatment of P. aeruginosa biofilm-associated pneumonia in cystic fibrosis patients [40].

In conclusion, these results point to an original and alternative antibiofilm approach to potentiating the effects of aminoglycosides, eliminating persisters, and achieving biofilm eradication of different types of clinical and industrial biofilm contamination.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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