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Efficacy of Artilysin® Art-175 against resistant and persistent *Acinetobacter baumannii*

Art-175 against *Acinetobacter baumannii*

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Abstract

Bacteriophage-encoded endolysins have shown promise as a novel class of antibacterials with a unique mode of action, i.e. peptidoglycan degradation. However, Gram-negative pathogens are generally not susceptible due to their protective outer membrane. The concept of Artilysin®s overcomes this barrier. Artilysin®s are optimized, engineered fusions of selected endolysins with specific outer membrane destabilizing peptides. Artilysin® Art-175 comprises a modified variant of endolysin KZ144 with an N-terminal fusion to SMAP-29. Previously, we have shown the high susceptibility of *P. aeruginosa* for Art-175. Here, we report that Art-175 is highly bactericidal against stationary phase cells of multidrug-resistant *Acinetobacter baumannii*, even resulting in a complete elimination of large inocula (≥ 10^8 CFU/ml). Besides actively-dividing cells, also persisters are killed. Instantaneous killing of *A. baumannii* upon contact with Art-175 could be visualized after immobilization of the bacteria in a microfluidic flow cell. Effective killing of a cell takes place through osmotic lysis after peptidoglycan degradation. The killing rate is enhanced by the addition of 0.5 mM EDTA. No resistance development against Art-175 under selection pressure or no cross-resistance with existing resistance mechanisms could be observed. In conclusion, Art-175 represents a highly active Artilysin® against both *A. baumannii* and *P. aeruginosa*, two of the most life-threatening pathogens of the order of the *Pseudomonadales*.

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Introduction

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen which has become one of the most important pathogens responsible for hospital-acquired infections including urinary tract infections, secondary meningitis, (burn) wound infections, and ventilator-associated pneumonia. Particularly, patients admitted to the intensive care units are affected. Treatment options are becoming increasingly limited due to the ongoing spread of antibiotic resistance mechanisms. Combined with a high intrinsic resistance, this has resulted in the emergence of multidrug-resistant clinical isolates, leaving colistin as the only therapeutic option. However, an outbreak of pandrug-resistant (including colistin) strains has been recently reported in Spain (1–3). The presence of a protective outer membrane limits not only the passage or diffusion of many currently available therapeutic antibiotics, most novel antibiotics in the development pipeline are too large to pass this barrier (4). *A. baumannii* infections often have a chronic nature, which can be attributed to the formation of persister cells, a small fraction of phenotypic variants highly tolerant to different classes of antibiotics (5, 6). For many antibiotics, persisters remain unaffected because of their dormant, non-dividing and metabolically quiescent state. They are, however, also tolerant to antibiotics targeting non-dividing cells and evidence is accumulating that dormancy is not the only reason for the observed tolerance. The role of active mechanisms, such as those related with the suppression of oxidative stress, is currently gaining more support (7–9). When antibiotic treatment ceases, persisters may resuscitate and initiate a new infection. Eradication of persisters would thus lead to an improved long-term recovery (6, 10).

We have recently reported the development of a novel class of enzyme-based antibacterials, coined Artilysin®s (11). Their mode of action relies on the fast enzymatic degradation of peptidoglycan by

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endolysins, resulting in osmotic lysis. Endolysins are produced by bacterial viruses (or bacteriophages). At the end of the infection cycle, they pass the cytoplasmic membrane through holes formed by small membrane proteins (holins) in a precisely timed manner, causing a sudden lysis of the infected host cell to disperse the newly formed viral particles (12). Exogenous addition of recombinantly produced endolysin molecules are successfully being used to kill Gram-positive pathogens, but they are too large to pass the outer membrane of Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii*. Artilysin®s consist of optimized fusions of a selected endolysin and a specific lipopolysaccharide (LPS) destabilizing peptide that effectively facilitates the outer membrane translocation of the endolysin moiety, eventually resulting in a physical cell lysis (13).

In this study, the antibacterial effect of Artilysin® Art-175 is analyzed against highly prevalent epidemiological clones of multidrug-resistant *A. baumannii* and persisters thereof. Art-175 combines a mutated variant of endolysin KZ144, encoded by the giant *P. aeruginosa* bacteriophage ϕKZ, and the sheep myeloid antibacterial peptide 29 (SMAP-29). Art-175 has previously been shown to be highly bactericidal against multidrug-resistant *P. aeruginosa* with no cross-resistance with existing resistance mechanisms of 21 different antibiotics. In addition, resistant strains could not be selected after serial exposure to sub-inhibitory doses of Art-175 (14). In this study, we found that Art-175 represents a highly active antibacterial capable of killing large inocula of multidrug-resistant stationary phase cells of *A. baumannii*, including their persisters, with a high killing rate.

Material and methods

Bacterial strains and culture conditions

A. baumannii strains used in this study are presented in Table S1. Eighteen strains were received from the Ruhr-universität Bochum (Nationales Referenzzentrum für gramnegative Krankenhauserreger), one *A. baumannii* and one *A. lwoffiii* strain were received from the Universitätsklinikum Regensburg and seven strains from the Queen Astrid Military Hospital, Brussels (15). All strains were grown in 1:20 Tryptic Soy Broth (TSB), unless mentioned otherwise, while shaking at 30°C. For solidified medium, 1.5% agar was added to TSB.

MIC determination

Minimum inhibitory concentrations (MIC) for Art-175 (0.25-20 µg/ml), ciprofloxacin (Sigma-Aldrich) (0.01-17 µg/ml) and tobramycin (0.125-64 µg/ml) were determined by the microdilution method in 96-well microtiter plates, according to the CLSI guidelines. The respective overnight bacterial cultures were adjusted to 0.5 McFarland standard, corresponding to 1 x 10^8 CFU/ml (OD_{625} ~ 0.08-0.1). Dilutions (100x) were made in 2x Mueller Hinton (MH) medium with or without 0.5 mM EDTA-Na_2 to obtain a start inoculum of 1 x 10^6 CFU/ml. Bacterial cultures were distributed over the required number of wells and 50 µl volumes of cells were incubated for 24 h in the presence of 50 µl of a 2-fold dilution series of Art-175 or antibiotic, prepared in 20 mM HEPES-NaOH 100 mM NaCl pH 7.4. 2x MH medium (0.5 mM EDTA-Na_2) with cells and 2x MH medium (0.5 mM EDTA-Na_2) with 50 µl 20 mM HEPES-NaOH 100 mM NaCl pH 7.4 were included as positive and negative control, respectively. MIC

values were determined as the minimum concentration where no bacterial growth was detected. MIC values for colistin were determined with the Etest® strips for colistin of bioMerieux SA (Marcy l'Etoile, France) according to the manufacturer's instructions.

Antibacterial assay

A. baumannii overnight cultures were diluted 100x in 50 ml 1:20 Tryptic Soy Broth (TSB) and grown overnight (18 h) to achieve stationary phase cultures. RUH134 was selected as a reference strain and the MIC of ciprofloxacin, tobramycin and Art-175 in the presence of 0.5 mM EDTA were determined as 0.5 µg/ml, 2 µg/ml and 2 µg/ml, respectively. Stationary phase cultures of RUH875, LUH5875, RUH134 and NCTC 13423 were treated with 5x to 30x MIC_{RUH134} concentrations of ciprofloxacin, tobramycin, Art-175 or the mixture of Art-175 with these antibiotics in total volumes of 200 µl, both in the presence and the absence of 0.5 mM EDTA-Na_2. After 20', 40',1 h, 2 h or 24 h for RUH134 and 1 h or 24 h for RUH875, LUH5875 and NCTC 13423, respectively, treated cultures were washed in 20 mM HEPES-NaOH pH 7.4 and appropriate dilutions were plated on TSB agar plates. Colonies were counted after 48 h of growth at 37°C. Each experiment was repeated independently at least three times.

Persister killing

Persister cells were isolated from a stationary phase culture as described previously, with minor modifications (16). An overnight culture of *A. baumannii* RUH134 was inoculated in 50 ml 1:20 TSB. After 18 h of growth at 37°C, the culture was treated with 60x MIC concentrations of tobramycin for 5

h at 37°C, while shaking at 200 rpm. Persister cells surviving antibiotic treatment were isolated by centrifugation (5250 x g, 15 min, 4°C) and the cell pellet was washed twice with 20 mM HEPES-NaOH pH 7.4. Cells were resuspended in 1:10 the initial volume of 20 mM HEPES-NaOH pH 7.4 after which a killing assay was performed as described previously (17), with minor modifications. A volume of 100 µl of the isolated persister fraction was mixed with ciprofloxacin, Art-175 (final concentrations of 30x MIC) or an equimolar amount of KZ144, in the absence or presence of 0.5 mM EDTA-Na_2 (final concentration). Control treatments with Art-175 storage buffer (20 mM HEPES-NaOH 0.5 M NaCl pH 7.4), 0.5 mM EDTA and tobramycin (30x MIC) were performed in parallel. The mixtures were shaken (200 rpm) for 1 h at 37°C. Treated persister cells were washed twice with 20 mM HEPES-NaOH pH 7.4, appropriate dilutions were plated on TSB agar plates and incubated at 37°C. The number of surviving persister cells was determined after 72 h incubation to allow complete resuscitation of all surviving persisters. Each experiment was independently repeated at least three times.

Time-lapse microscopy

A. baumannii RUH134 cells were grown to mid-exponential phase (OD_{600} = 0.6) in lysogeny broth (LB) at 37°C, washed three times with 20 mM HEPES-NaOH pH 7.4 (for cells with high turgor pressure) or 20 mM HEPES-NaOH 0.5 M NaCl pH 7.4 (for cells without turgor pressure), and finally concentrated five times by resuspension in the corresponding wash buffer, supplemented with 0.5 mM EDTA-Na_2. Cells were kept on ice until use. Bacterial cells (with high or low turgor pressure) were loaded into a ONIX B04A-03 Microfluidic Plate (EMD Millipore Corporation, Hayward, CA, USA), placed under the microscope and subsequently exposed to Art-175 to monitor cell inactivation and lysis. Art-175 (0.4 mg/ml) was dialyzed before use against 20 mM HEPES-NaOH pH 7.4 (for cells with high turgor

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pressure) or 20 mM HEPES-NaOH 0.5 M NaCl pH 7.4 (for cells with a lower turgor pressure) and was supplemented with 50 mM EDTA-Na_2 to a final concentration of 0.5 mM EDTA-Na_2. Visualization of cells with high turgor pressure was done by immobilization of the cells in the inlet channel of Art-175 to ensure high Art-175 concentrations. The cells with lower turgor pressure, however, were visualized in the mid of the flow cell where due to diffusion effects the concentration of Art-175 is lower. Time-lapse microscopy experiments were performed in a temperature controlled (Okolab, Ottaviano, Italy) Eclipse Ti-E inverted microscope (Nikon, Champigny-sur-Marne, France), equipped with a Ti-CT-E motorized condenser and a CoolSNAP HQ2 FireWire CCD camera (18). Images were acquired using NIS-Elements (Nikon), and the resulting pictures were further handled with open-source software, ImageJ (http://rsbweb.nih.gov/ij/). The CellASIC® ONIX Microfluidic System was operated using the CellASIC® ONIX FG Software, provided by the supplier (EMD Millipore Corporation).

Resistance development

The MIC of Art-175 and ciprofloxacin was determined against *A. baumannii* RUH134, RUH875 and LUH5875 as described above. Briefly, cultures were grown in Mueller Hinton broth and adjusted to 5 x 10^5 CFU/ml. Two-fold dilution series of Art-175 and ciprofloxacin were prepared in 20 mM HEPES-NaOH 100 mM NaCl pH 7.4 in a round bottom 96-well microtiter plate, in the presence of the bacterial cells and a final concentration of 250 µM EDTA. After 24 h, the MICs were recorded as the minimal concentration that completely inhibited growth. A new cycle was initiated for each strain/compound combination using a 100x dilution of cell suspensions at the highest concentration with full growth. This was repeated for 20 cycles.

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Hemolytic assay

A standardized hemolytic assay was performed by Pharmacelsus® GmbH to assess the level of erythrocyte damage caused by Art-175. Human erythrocytes were exposed to increasing concentrations of Art-175 for 0.5 – 4 hours on an orbital shaker. Positive controls consisted of 2% Tween 80 and 2% Triton-X100; 20 mM HEPES-NaOH 0.5 M NaCl pH 7.4 was used as negative control. The hemolytic effect of Art-175 was determined as the fraction of hemolysis relative to that of 2% Triton-X100.

Results

Art-175 is an effective antibacterial against relevant A. baumannii strains

Growth inhibition by Art-175 was tested against a panel of thirty-three *A. baumannii* strains and one *A. lwoffii* strain from different sources, containing both lab strains and clinical (multidrug-resistant) isolates (Table S1). MICs of Art-175 range between 4 µg/ml and 20 µg/ml, showing the broad inhibitory activity of Art-175 against different (multidrug-resistant) *A. baumannii* strains. Addition of 200 µM of EDTA reduced the MIC value of Art-175 and improved its antibacterial effect for all strains tested (ranging from ≤4 to 10 µg/ml), providing a potentially interesting feature for topical applications. Colistin resistance (>2-4 µg/ml) arises through addition of phosphoethanolamine or 4-amino-4-arabinose to the phosphate groups of lipid A moiety of the lipopolysaccharides (19, 20) and may thus directly affect the susceptibility for Art-175. However, no correlation was found between

187 the MIC values for colistin and Art-175, respectively (n=28; R^2=0.0043), indicating the lack of cross-
188 resistance (Table S2).

189 The increased number of outbreaks of *A. baumannii* infections in clinical settings, which often share a
190 genetic background, is believed to be linked to the global spread of a number of highly successful
191 clones (21, 22). The strains tested in this study include the reference strains of EU clones I (RUH875), II
192 (RUH134) and III (LUH5875) and the NCTC 13423 strain with MIC values of 8 µg/mL, 10 µg/mL, 12
193 µg/mL and 6 µg/mL, respectively, all multidrug-resistant strains showing resistance against
194 aminoglycosides, quinolones and carbapenems (15). The NCTC 13423 or T strain was first isolated in
195 2003 from a casualty returning from the Iraqi war, deeming it the nickname 'Iraqibacter'. Despite not
196 having greatly contributed to an increase in *A. baumannii* infections, it possesses all characteristics of
197 a highly successful outbreak strain and has become a major cause of concern in conflict zones (23,
198 24). In sum, Art-175 shows a good antibacterial activity against all tested *A. baumannii* strains,
199 independent of the presence of multidrug-resistance mechanisms.

200

201 *Art-175 outcompetes conventional antibiotics in bactericidal activity and killing rate against A.*
202 *baumannii*

203 Time-kill experiments were done for Art-175 and compared to those of ciprofloxacin and tobramycin,
204 two first-line antibiotics against *A. baumannii* infections (Figure 1A-B). Stationary phase cultures of
205 RUH134, RUH875, LUH5875 and NCTC 13423 (~10^7 – 10^8 CFU/ml) were treated for 1 and 24 h with
206 30x MIC_{RUH134} concentrations of Art-175, ciprofloxacin or tobramycin. A single dose of Art-175
207 eradicated all strains completely within 24 h (approximately 8 – 9 log reduction) with a strain-

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dependent killing rate. RUH875 was already completely eliminated after 1 h, while RUH134 and NCTC 13423 still showed a minute surviving fraction (approximately 7 – 8 log reduction), which was completely eliminated after 24 h. Art-175 had the slowest antibacterial effect against LUH5875, with a 2.5 log reduction after 1 h. The bactericidal effect of an Art-175 treatment significantly exceeded the effect of either ciprofloxacin or tobramycin, both in extent and rate. Ciprofloxacin and tobramycin killed between 1.0 and 4.5 log in 24 hours, with the exception of RUH134 and NCTC 13423. Both strains were completely eliminated by tobramycin after 24 hours, but still at a slower rate than in the case of Art-175 (Figure 1A-B).

Subsequently, the effect of the combination of a conventional and experimental antibacterial was evaluated to assess the usefulness of Art-175 as a supplement to currently used antibiotics and to exclude possible antagonistic effects (Figure 1A-B). The same four strains were exposed to either a mixture of Art-175 and tobramycin or Art-175 and ciprofloxacin (each 30x MIC). The antibacterial effect of the mixtures was significantly better ($P<0.005$) than the respective conventional antibiotics alone. No significant differences ($P>0.05$) were observed compared to Art-175 treatment alone. However, one exception is observed; strain LUH5875, which was affected by Art-175 at a slower rate compared to the three other strains, was killed faster by a mixture of ciprofloxacin and Art-175 (4 log reduction after 1 h) than by Art-175 alone (2.5 log reduction after 1 h). After 24 h of treatment with both combinations, the surviving fractions of all bacterial strains tested were reduced to well below the detection limit. In conclusion, the efficacy of a mixture of Art-175 and ciprofloxacin/tobramycin is comparable to Art-175 alone. No antagonistic effects are observed, and it is likely that most cells are already killed by Art-175 before ciprofloxacin and tobramycin can exert their action.

To gain insight in the high killing rate of Art-175, additional time-kill curves were determined for stationary phase cells of RUH134 in a shorter time frame (after 20 min, 40 min, 1 h and 2 h) (Figure 1C). While the killing rate for ciprofloxacin is low, all other treatments lead to a fast killing, which is most pronounced for Art-175 and the combinations of Art-175 with either ciprofloxacin or tobramycin. Within 20 minutes, reductions between 4.5 and 6.5 log are observed and complete killing is effectively reached within two hours. Again, no significant differences are observed between Art-175 alone and combinations of ciprofloxacin or tobramycin with Art-175. These findings confirm the rapid killing rate of Art-175 and show that, even when large inocula of stationary phase cells are used, complete eradication can be achieved in a short time (2 h).

To evaluate the dose-dependence of the antibacterial effect exerted by Art-175 in the absence of 0.5 mM EDTA, RUH134 was exposed to increasing concentrations of Art-175 for 20 min, 40 min, 1 h and 2 h (Figure 1D). A clear dose- and time-dependent effect is observed with significant killing starting from 10x MIC Art-175. After 2 h of treatment reductions of 0.26 ± 0.08 log, 1.55 ± 0.23 log, 6.38 ± 0.28 log and 7.83 ± 0.67 log are obtained for concentrations of 5x MIC, 10x MIC, 20x MIC and 30x MIC, respectively. Comparison of the effect of 30x MIC Art-175 in the presence (Figure 1C) and absence (Figure 1D) of 0.5 mM EDTA shows that EDTA mainly has an accelerating effect and the extent of killing after 2 h is similar. Also stationary phase cells of RUH875 and NCTC 13423 can be completely eradicated within 24 h with Art-175 (20x MIC) in the absence of EDTA, whereas LUH5875 is slightly less affected with a decrease in surviving cell number of 6.93 ± 0.49 log (data not shown).

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Real-time time lapse microscopy shows bactericidal effect upon contact

The rapid bactericidal effect of Art-175 previously hindered real-time monitoring of the mode-of-action, unless Art-175 was sufficiently diluted (14). Therefore, a flow cell was used to immobilize *A. baumannii* RUH134 cells in a microfluidic channel and the immobilized cells were monitored in real-time when a flow of Art-175 (0.4 mg/ml) made contact (Figure 2A; Movie S1A). All cells lysed immediately in the first frame (3 sec) and the cell contents were dispersed in the flow of Art-175 solution. This clearly demonstrates that Art-175 can act upon contact. Cells were resuspended in a buffer with low ionic strength (20 mM HEPES-NaOH pH 7.4), resulting in a high internal osmotic pressure and thus likely contributing to a drastic lysis phenomenon. In a similar experiment but with cells resuspended in a buffer with high ionic strength (20 mM HEPES-NaOH pH 7.4 supplemented with 0.5 M NaCl) and a lowered Art-175 concentration, different observations were made (Figure 2B; Movie S1B). Lysis took place again, but was slower (<1 h) and the cells first adopted a spherical shape before becoming unstable and lysing. Under these conditions, the contribution of two separate effects caused by Art-175 could be visualized, i.e. peptidoglycan degradation resulting in the adoption of a spherical shape and osmotic lysis due to the lack of a protective cell wall.

Art-175 is a highly efficient anti-persister compound

Persister cells constitute a small sub fraction of the bacterial population transiently tolerant to treatment with high doses of antibiotics (6). Art-175 is capable of completely eradicating the persister fraction of *P. aeruginosa* (14). This effect can be attributed to the mode of action of Artilysin®s that physically cleave the peptidoglycan, instead of inhibiting an essential step in the metabolism.

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Consequently, there is no need for an active metabolism, making them very suitable for the elimination of persister cells (14). To investigate whether the anti-persister effect of Art-175 is broadly conserved, *A. baumannii* RUH134 persister cells were isolated through 5 h treatment with high doses of tobramycin. After isolation, the remaining fraction was treated for 1 h with a concentration corresponding to 30x MIC of ciprofloxacin or Art-175, respectively, in the absence or presence of 0.5 mM EDTA. In addition, equimolar amounts of the endolysin KZ144 alone were tested. As controls, treatments with 20 mM HEPES-NaOH pH 7.4 (storage buffer), 0.5 mM EDTA and 30xMIC tobramycin were included.

The isolated persister cells could not be killed by a new tobramycin exposure, confirming that the actual persister subfraction was isolated. Controls such as the storage buffer, EDTA, and ciprofloxacin cause a small but significant reduction of the persister fraction, which may be best explained by the occasional waking up of persister cells during sequential washing steps. Treatment of the persister cells with 30x MIC Art-175, in the absence or presence of 0.5 mM EDTA, significantly reduced the remaining persister fraction with 2.35 ± 0.42 log and 2.66 ± 0.45 log, respectively. Under these conditions, EDTA did not significantly enhance the anti-persister effect. KZ144, on the other hand, caused reductions of only 1.24 ± 0.43 log and 1.93 ± 0.49 log in the absence and presence of 0.5 mM EDTA, respectively (Figure 3).

In conclusion, Art-175 reduces the surviving persister fraction most compared to the controls, demonstrating a potent anti-persister effect. Although the occurrence of persister cells has been described in *A. baumannii* (5, 25), Art-175 and KZ144 are, to our knowledge, the first antibacterial compounds described to exert an anti-persister effect against *A. baumannii*.

Art-175 shows no hemolytic activity

The antibacterial effect of SMAP-29 is explained by its membrane pore formation (26). Membrane pore formation requires oligomerization, and this effect is also correlated with the hemolytic activity of SMAP-29 (7.9% hemolysis at 50 µM or 0.16 ng/ml) (26). Although it is expected that SMAP-29 oligomerization cannot take place in a fusion with the endolysin KZ144 due to steric hindrance, we have evaluated the hemolytic potential of Art-175 by assessing the damage of erythrocytes after exposure to Art-175. No hemolysis was observed for doses up to 200 µg/ml (100x MIC with 200 µM EDTA; 10-50x MIC without EDTA) in contrast to the positive controls Tween80 and Triton X-100 (data not shown).

Resistance of A. baumannii against Art-175 cannot be provoked in vitro

The rate of resistance development is an important parameter to evaluate the potential of a new antibacterial compound. *A. baumannii* is acknowledged as a multi-drug resistant organism, capable of developing resistance rapidly compared to other clinically relevant pathogens (27, 28). To analyze the resistance development of *A. baumannii* against Art-175, the epidemiological European reference strains (RUH134, RUH875 and LUH5875) were serially exposed to sub-inhibitory doses of Art-175. Ciprofloxacin was included as a control antibiotic. A maximal two-fold increase of the MIC of Art-175 was observed for all strains after 20 cycles (Figure 4B). In contrast, sub-inhibitory doses of ciprofloxacin resulted in the recovery of highly resistant strains (according to the breakpoints defined by EUCAST) of both RUH134 and RUH875 after 8 and 3 cycles, showing a 256- and 512-fold increase in

MIC value, respectively (Figure 4A). The MIC of RUH134 further developed to a 512-fold increase in cycle 14. For LUH5875 a four-fold increase in MIC was observed after 20 cycles. However, this strain already showed a relatively high initial resistance against ciprofloxacin, i.e., 85 µg/mL versus 1.3 µg/mL (RUH134 and RUH875), corresponding to a 64-fold higher MIC. Resistance against Art-175 does not appear to be provoked through genetic mutations that are typically selected upon exposure to sub-inhibitory doses.

Discussion

A. baumannii causes a wide range of infections and is becoming an important risk in health care settings. Besides its great intrinsic resistance, extensive antibiotic use has fostered the selection for resistance against all known antimicrobials (28). Despite its increasing clinical relevance, few or no compounds are currently found in late stages of clinical development for the treatment of multidrug-resistant *A. baumannii* infections (3). Increasing multidrug resistance in most bacterial pathogens and lack of new antibiotics have increased the need for alternative treatment options to combat these infections.

The ability of purified, phage-derived endolysins to kill bacteria was discovered in 1959 and, over the years, research on phage endolysins and their ability as new antibacterials has greatly progressed (29, 30). The unique nature of endolysins makes them highly attractive antibacterials for Gram-positive bacteria and provides several advantages over conventional antibiotics. Indeed, endolysins do not disturb the normal human microflora due to their high specificity, provide novel antimicrobial mechanisms, have a small chance of developing bacterial resistance and are able to kill colonizing pathogens on mucosal surfaces (31, 32). The potent effect of these phage encoded endolysins against different Gram-positive pathogens has been extensively reviewed (29, 33–35).

337 The majority of known endolysins is unable to cross the highly impermeable outer membrane of
338 Gram-negative species. The engineering of endolysins towards Artilysin®s allowed us to overcome this
339 barrier. Optimized fusions of phage endolysins with a peptide component capable of guiding the
340 endolysin through the bacterial outer membrane results in a strong bactericidal effect against Gram-
341 negative pathogens (11). Previous work described the development of Art-085 and its optimized
342 homolog Art-175, composed of a fusion between the SMAP-29 peptide and the N-terminus of the full-
343 length KZ144 endolysin containing three Cys→Ser mutations. Art-175 proved very successful in the
344 eradication of *P. aeruginosa* and its persister cells, and its potent antibacterial activity has since then
345 been demonstrated *in vivo* in dogs (11, 13, 14). The success of Art-175 prompted us to further expand
346 and verify this Artilysin® as a novel antimicrobial for the treatment of *A. baumannii*, another clinically
347 relevant pathogen within the order of the *Pseudomonadales*.

348 Compounds showing a broad activity without displaying cross-resistance with existing drug resistance
349 mechanisms show great promise as novel antibacterials. Therefore, a diverse panel of thirty-three *A.*
350 *baumannii* strains, including strains from different origins and with different antibiotic susceptibilities
351 was evaluated. Art-175 inhibits the growth of all these strains (MIC between 4 and 20 µg/ml). No
352 correlation between the MIC value of Art-175 and the source, isolation site, resistance spectrum
353 (aminoglycosides, quinolones and carbapenems) and the MIC value for colistin was observed,
354 demonstrating the lack of cross-resistance and the broad activity of Art-175. Addition of 0.2 mM EDTA
355 generally reduces the MIC value.

356 Four epidemiological reference strains that are resistant towards at least two classes of first-line
357 antibiotics (aminoglycosides, quinolones and carbapenems) were selected for further studies to
358 evaluate the *in vitro* efficiency of Art-175. In time-kill experiments, Art-175 shows a significantly

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increased reduction of cell number compared to two conventional antibiotics, ciprofloxacin and tobramycin. All therapeutics show a strain-dependent killing rate but a single dose of Art-175 eradicates stationary phase cells of all bacterial strains within 24 h (between 8 and 9 log reduction).

Different endolysins have been reported to have intrinsic antibacterial activity without the need for engineering, especially against *A. baumannii* (36, 37). The outer membrane of *A. baumannii* therefore appears to be less a barrier for large enzyme molecules than in other Gram-negative species. Endolysin PlyF307 derived from an *A. baumannii* prophage is effective against exponentially growing *A. baumannii* cells (>3 log units), but its activity is reduced when stationary phase cells are exposed to the antibacterial (approximately 1.5 log unit) (38). In fact, endolysins have been reported to be generally less active against stationary phase cells when compared to logarithmically growing cells (39). The complete elimination of stationary phase cells by Art-175, partially due to its anti-persister activity, is therefore one of the most remarkable features of Art-175.

Combination therapy has often been suggested for the treatment of Gram-negative infections, including *A. baumannii* (40, 41). Combinations of endolysins and antibiotics have been reported to be superior to antibiotics alone (42). To evaluate the potential of Art-175 as a supplement to existing antibiotic therapies, Art-175 was combined with ciprofloxacin and tobramycin, and its effect was compared to monotherapy.

The combined therapy results in an improved antibacterial effect for both mixtures, as compared to conventional antibiotic monotherapy. However, there is no significant improvement compared to Art-175 alone, suggesting that Art-175 kills the bacterial cells before either ciprofloxacin or tobramycin can exert their action.

The rapid mode of action of Art-175 is confirmed with the complete eradication of RUH134 (approximately 9 log reduction) after 2 hours of treatment, outcompeting both ciprofloxacin and tobramycin. A low concentration of EDTA, which chelates divalent cations that contribute to the stabilization of the outer membrane, further accelerates the killing rate. We introduced the use of microfluidic flow cells to immobilize bacteria to allow real-time observation. Using this method, we show that an immediate, rapid and intense lysis takes place. Under low osmotic pressure conditions (high ionic strength of the medium) and a lowered concentration of Art-175, the mode of action of Art-175 can be visualized in slow motion with the full enzymatic degradation of the shape-determining peptidoglycan sacculus followed by a destabilization of the cell and spontaneous lysis. Enzymatic degradation of the peptidoglycan layer is only possible when Art-175 has first passed the outer membrane. Since the endolysin KZ144 is not able to pass the outer membrane efficiently, this can be only explained by the fused SMAP-29 moiety that interferes with the electrostatic interactions between adjacent LPS molecules. This is in contrast to the receptor-mediated uptake mechanism as described for a chimeric endolysin, comprising the FyuA binding domain fused to the N-terminus of T4 lysozyme. This hybrid protein kills *Yersinia* and pathogenic *E. coli* strains, which express the FyuA receptor (43). Once passed the outer membrane, enzymatic degradation takes place, resulting in a rod to sphere transformation. Nevertheless, loss of the cell wall does not necessarily kill the cell, as has been extensively described for cell wall-deficient bacteria or L-forms, which are able to survive and even multiply if there is an appropriate isotonic environment (44, 45). The peptidoglycan layer can also be removed by peptidoglycan degrading enzymes (such as the commonly used hen egg white lysozyme) to prepare so-called protoplasts. These ‘naked’ cells remain viable, are competent to take up extracellular DNA and can reconstruct an intact cell wall after transformation. This process

requires that the cells are resuspended in an isotonic solution, i.e. a solution with the same osmolarity as inside the cell to eliminate the cellular osmotic pressure. This technique is commonly used in molecular microbiology to modify the genetic content of a bacterial cell. Therefore, the actual cause of death can be defined as a physical process called osmotic lysis. Depending on the osmolarity of the medium, we propose that osmotic lysis will take place immediately or only after extensive peptidoglycan degradation until the cells get too much destabilized by the internal osmotic pressure. Given this dependence on osmolarity, it would be interesting to evaluate Art-175 under serum conditions in future.

The presence of so-called persister cells is increasingly being recognized as an important cause of the chronic nature of bacterial infections (46, 47). Despite being a clinically relevant pathogen known to cause chronic infections, the observation of the persister phenotype in this species has only recently been described. 94.6% of the tested clinical isolates showed a detectable persister fraction, varying between 0.0007% and 10.1%, indicating that the persister phenotype is broadly distributed amongst *A. baumannii* strains (5). Unlike most traditional antibiotics, Art-175 does not require an active metabolism to exerts its antibacterial activity, making it an ideal candidate for novel anti-persister therapies. Treatment of stationary phase cultures of *A. baumannii* with high doses of tobramycin confirmed the presence of a persister fraction in RUH134 of about 0.0004%. Exposure of this isolated persister fraction to Art-175 (+ 0.5 mM EDTA) results in an efficient 2.4 log (2.7 log) reduction in surviving cells. However, in case of *P. aeruginosa* persisters, a complete elimination of the persister fraction could be achieved with similar doses. Despite the clear clinical relevance of persister cells, anti-persister therapies described in literature are still limited, Art-175 being the first molecule, to our knowledge, exerting an activity against *A. baumannii* persister cells.

No resistance development is observed after serial exposure of three different *A. baumannii* strains to sub-inhibitory concentrations of Art-175. On the contrary, repeated exposure to ciprofloxacin, a first-line antibiotic in healthcare settings, results in the quick recovery of highly resistant strains for both RUH875 and RUH134 with 512- and 265-fold increases in MIC values, respectively. Similar findings have been reported for endolysins (48, 49). The immutable nature of peptidoglycan and the high selection pressure for endolysin optimization during the long and intense co-evolution between phages and bacteria have been proposed as explanations for similar observations with endolysins and appear to hold true for Artilysin®s (14). Also, the 'extracellular' location of peptidoglycan excludes many potential resistance mechanisms such as efflux pumps and inactivation of the antibacterial (34).

In conclusion, Art-175 shows high and rapid *in vitro* bactericidal activity against two of the most dangerous Gram-negative pathogens posing risks to our current health care systems, *A. baumannii* and *P. aeruginosa*, both belonging to the order of *Pseudomonadales*. Art-175 clearly differentiates from other classes of antibiotics both in completeness of killing (including persisters), killing rate and the mode of action.

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600 **FIGURE legends**

601 **Figure 1: Antibacterial effect of Art-175 and combinations of conventional antibiotics with Art-175**
602 **on different *A. baumannii* reference strains.** Stationary phase cultures of respectively RUH875
603 (black), LUH5875 (dark grey), NCTC 13423 (light grey) and RUH134 (white) were treated for (A) 1 hour
604 or (B) 24 hours with ciprofloxacin (15 µg/mL), tobramycin (60 µg/mL), Art-175 (60 µg/mL) or the
605 combination therapy of Art-175 with these antibiotics. All concentrations correspond to a 30x MIC
606 value. Bacterial survival after 1 h and 24 h treatment is expressed in $\log_{10}$ (CFU/ml). ND = not
607 detected. (C) Time-kill curves were obtained for RUH134 with stationary phase cells that were treated
608 for 20 min, 40 min, 1 h or 2 h with buffer (control, black line), ciprofloxacin (filled circle; 15 µg/mL),
609 ciprofloxacin + Art-175 (open circle; 15 µg/mL and 120 µg/mL respectively), tobramycin (filled
610 triangle; 60 µg/mL), tobramycin + Art-175 (open triangle; 60 µg/mL and 120 µg/mL, respectively) or
611 Art-175 (open diamond; 120 µg/mL). All concentrations correspond to a 30x MIC value. Data points
612 represent the mean value (± SEM) of at least three independent repeats. (D) Stationary phase cells of
613 RUH134 were treated with increasing concentrations of Art-175 for 20 min, 40 min, 1h and 2h. The
614 control consists of buffer (black line), Art-175 was administered in 5x MIC (open circles; 20 µg/mL),
615 10x MIC (open triangles; 40 µg/mL), 20x MIC (open squares; 80 µg/mL) and 30x MIC (open diamonds;
616 120 µg/mL) concentrations. Data points represent the mean value (± SEM) of three independent
617 repeats.

618

619 **Figure 2: Real-time monitoring of osmotic lysis induced by Art-175.** Exponential phase cells of *A.*
620 *baumannii* RUH134 with (A) a high internal osmotic pressure (washed with 20 mM HEPES-NaOH pH
621 7.4 0.5 mM EDTA) or (B) a low internal osmotic pressure (washed with 20 mM HEPES-NaOH pH 7.4

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622 0.5 M NaCl 0.5 mM EDTA) were immobilized in a flow cell and exposed to Art-175 (0,4 mg/ml with 0.5
623 mM EDTA). A time-lapse series is presented with intervals of (A) 3 sec or B) 5 min. To visualize the
624 rapid lysis under high osmotic pressure conditions, three frames before contact with Art-175 are
625 shown (from -00:00:06). The flow of Art-175 is started at 00:00:00). Total duration is (A) 36 seconds
626 and (B) 1 h . Scale bars correspond to 2 µm. Movie S1A and S1B show the full time-lapse series.

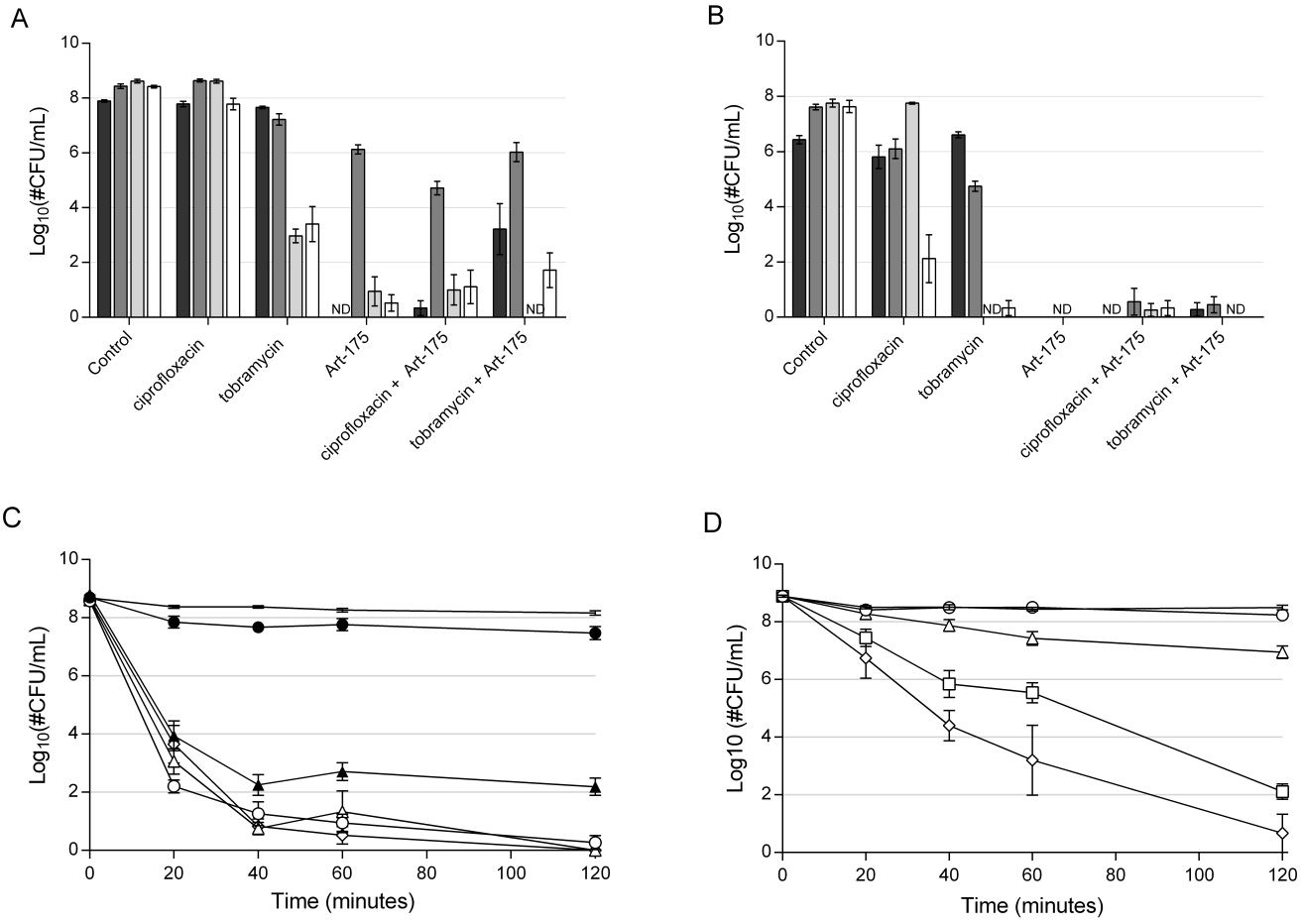
627

628 **Figure 3: Art-175 kills isolated persister cells.** Stationary phase cultures of RUH 134 were treated with
629 high doses of tobramycin to isolate the surviving persister fraction. The remaining persister cells were
630 treated with 30x MIC concentrations of ciprofloxacin (15 µg/mL), Art-175 (120 µg/mL) and an
631 equimolar dose of KZ144 (108 µg/mL), all in the absence and presence of 0.5 mM EDTA. As a control,
632 untreated persister cells and treatment with 0.5 mM EDTA and 30x MIC tobramycin (60 µg/mL) were
633 taken into account. Persister survival after 5 hours is expressed in $\log_{10}$ (CFU/ml). Mean values are
634 shown (± SEM) for at least 3 independent repeats.

635

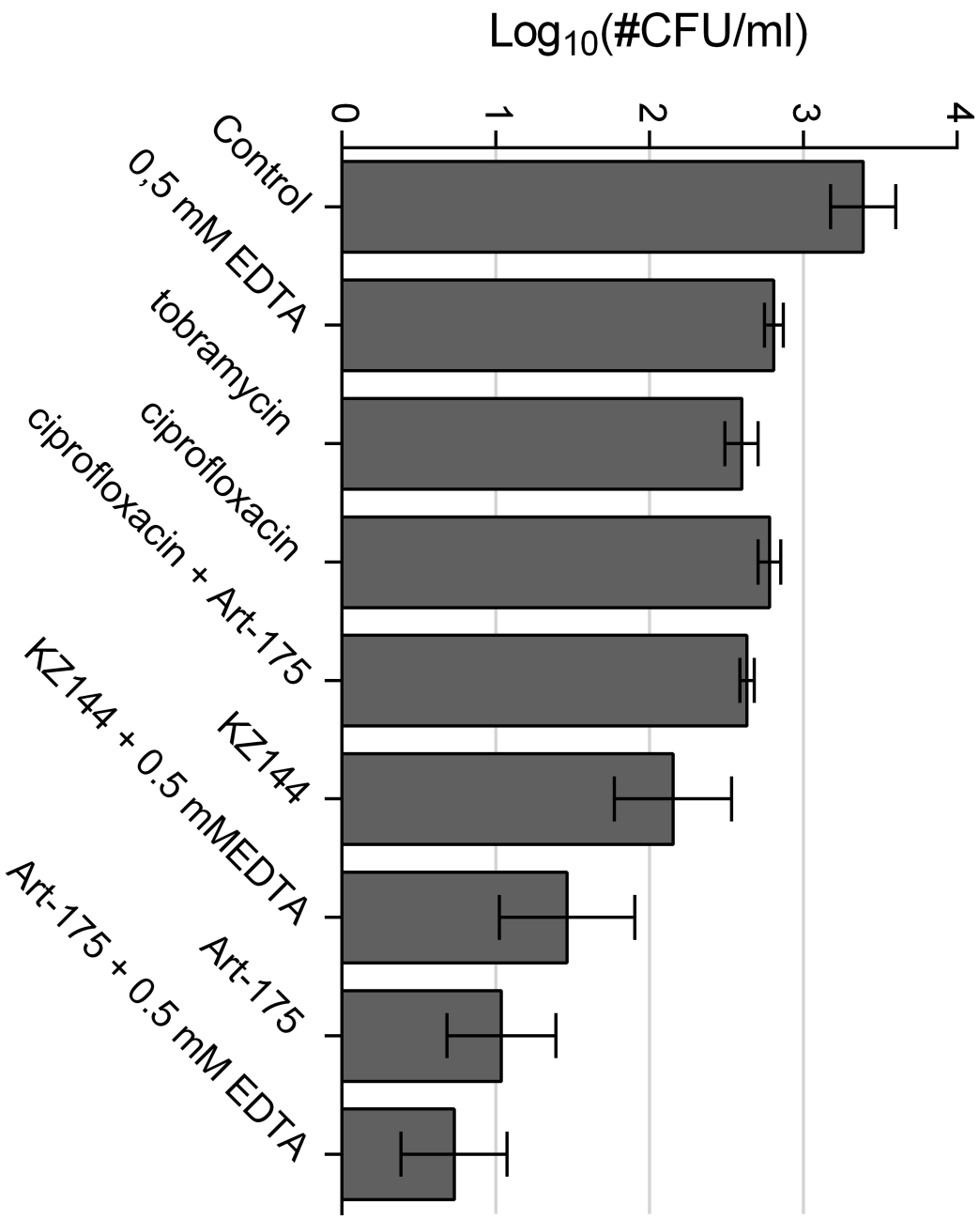
636 **Figure 4: Art-175 is highly refractory to resistance development.** *A. baumannii* strains RUH134
637 (triangle), RUH875 (circle) and LUH5875 (square) were serially treated with sub-inhibitory
638 concentrations of (A) ciprofloxacin or (B) Art-175 to select for decreased susceptibility. Values are
639 represented as the ratio of the MIC value after cycle i (MIC_i) over the MIC value at the start of the
640 experiment (MIC_0). After 20 cycles, the MIC of Art-175 increased maximally twofold, whereas the MIC
641 of ciprofloxacin increased 512- (RUH134 and RUH875) or 4-fold (LUH5875).

642



A

B


Log10(#CFU/ml)
0
1
2
3
4
Control
0,5 mM EDTA
tobramycin
ciprofloxacin
ciprofloxacin + Art-175
KZ144
KZ144 + 0.5 mMEDTA
Art-175
Art-175 + 0.5 mM EDTA