Modulation of antibiotic resistance and induction of a stress response in *Pseudomonas aeruginosa* by silver nanoparticles

Katarzyna Markowska, Anna M. Grudniak, Krzysztof Krawczyk, Izabela Wróbel and Krystyna I. Wolska

Department of Bacterial Genetics, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

The objective of this study was to characterize the effects of silver nanoparticles on *Pseudomonas aeruginosa*. Their interactions with several conventional antibiotics and ability to induce a stress response were examined. Interactions between silver nanoparticles (AgNPs) and antibiotics against free-living cells and biofilm of *P. aeruginosa* were studied using the chequerboard method and time-kill assays. The ability of AgNPs to induce a stress response was determined by evaluation of cellular levels of the DnaK and HtpG chaperones using SDS-PAGE and Western blot analysis. Synergistic activity against free-living *P. aeruginosa* between AgNPs and ampicillin, streptomycin, rifampicin and tetracycline, but not oxacillin, ciprofloxacin, meropenem or ceftazidime, was demonstrated by the chequerboard method. No such interactions were observed against *P. aeruginosa* biofilm. The results of time-kill assays confirmed synergy only for the AgNPs–streptomycin combination. AgNPs induced the expression of chaperone DnaK. No induction of the HtpG chaperone was detected. In conclusion, AgNPs not only display potent bactericidal activity against *P. aeruginosa*, but also act synergistically with several conventional antibiotics to enhance their effect against free-living bacteria as determined by the chequerboard method. The time-kill assay proved synergy between AgNPs and streptomycin only. The ability of AgNPs to induce the major chaperone protein DnaK may influence bacterial resistance to antimicrobials.

**INTRODUCTION**

Increasing antibiotic resistance among pathogenic bacterial species is a serious problem for public health and has stimulated research examining the antibacterial effects of alternative compounds and novel strategies (Kurek et al., 2011). The antibacterial activity of silver nanoparticles (AgNPs) is well known and they have many medical and technological applications (Edwards-Jones, 2009). The production of reactive oxygen species provoked by AgNPs causes the disruption of bacterial membranes (Singh et al., 2008), and they also interact with sulfur-containing membrane proteins (Rai et al., 2009). Proteomic analysis of the effects of AgNPs on *Escherichia coli* revealed that even short exposure to AgNPs can greatly influence the synthesis of several proteins (Lok et al., 2006). AgNPs are also active against bacterial biofilms (Kalishwaralal et al., 2010), so they may prove effective in combatting biofilm-mediated, drug-resistant and device-centred infections. It was shown that biofilm formation is inhibited by the ability of AgNPs to prevent the initial steps in their development, i.e. microbial adhesion to various surfaces (Monteiro et al., 2009). Besides the inherent antimicrobial activity of AgNPs, there is also the possibility of using them in combination with conventional antibiotics in order to improve their efficacy. Experiments performed using the disc diffusion assay provided evidence of synergy between AgNPs and several antibiotics, particularly against Gram-negative bacteria (Fayaz et al., 2010).

This study examined the effects of AgNPs in combination with several antimicrobial compounds on the antibiotic resistance and stress response of *Pseudomonas aeruginosa*. This ubiquitous opportunistic pathogen is able to form biofilms and is characterized by its multifactorial and increasing antibiotic resistance (Porras-Gómez et al., 2012). Thus, there is an urgent need for novel antimicrobial therapies and/or methods to enhance the activity of already ineffective antibiotics against this species. As the stress response is a determinant of antimicrobial resistance (Poole, 2012), the ability of AgNPs to induce this response can influence the susceptibility of *P. aeruginosa* to antibiotics.

**Abbreviations:** AgNP, silver nanoparticle; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; FICI, fractional inhibitory concentration index; MBIC, minimum biofilm inhibitory concentration; MEM, meropenem; OXA, oxacillin; RIF, rifampicin; STR, streptomycin; TET, tetracycline.
METHODS

Bacterial strain, culture media, chemicals and antibodies. *P. aeruginosa* ATCC 10145 was obtained from the Polish Collection of Microorganisms. *P. aeruginosa* STR<sup>R</sup> and RIF<sup>P</sup> spontaneous mutants were obtained by the standard method exploiting selection on plates supplemented with 512 µg streptomycin (STR) ml<sup>−1</sup> and 256 µg rifampicin (RIF) ml<sup>−1</sup>. Cultures were grown in Mueller–Hinton broth (MH; Biocorp Poland). As required, the medium was supplemented with antibiotics: ampicillin (AMP), oxacillin (OXA), STR, RIF, ciprofloxacin (CIP), tetracycline (TET), meropenem (MEM) and ceftazidime (CAZ) (Sigma–Aldrich) and/or AgNPs (Nano-Tech). The hydrocolloid of uncoated AgNPs was produced by a non-explosive high-voltage method (Polish Patent 3883399) using high-purity silver (99.9999 %) and ultrapure demineralized water. The concentration of nanoparticles in the hydrocolloid was 50 p.p.m. and the AgNp size ranged from 2 to 35 nm, according to transmission electron microscope evaluation (Chwalibog et al., 2010). For Western blot analysis, anti-DnaK and anti-HtpG rabbit polyclonal sera were used. The anti-DnaK serum was a kind gift from Professor Sabina Kędzierska-Mieszkowska (Institute of Biochemistry, University of Gdansk, Poland) and anti-HtpG serum was obtained from Eurogentec. Goat anti-rabbit IgG alkaline phosphatase conjugate secondary antibody was supplied by Sigma–Aldrich.

Determination of MIC and minimum biofilm inhibitory concentration (MBIC). MICs were determined using the broth microdilution technique performed in 96-well microtitre plates. Aliquots of MH containing series of twofold dilutions of AgNPs, AMP, OXA, STR, RIF, CIP, TET, MEM or CAZ were inoculated with *P. aeruginosa* (final cell density 1 × 10<sup>6</sup> c.f.u. ml<sup>−1</sup>) and incubated at 37 °C for 24 h. The lowest concentration of each tested agent that resulted in no visible turbidity was considered the MIC. To determine MBICs, a culture of *P. aeruginosa* was diluted 1:100 (final cell density 1 × 10<sup>5</sup> c.f.u. ml<sup>−1</sup>) in fresh medium and then mixed with MH supplemented with 0.45 % glucose and various concentrations of the tested agents in the wells of microtitre plates. Following incubation at 37 °C for 24 h, the lowest concentration of each substance that inhibited biofilm growth, as determined by the Crystal Violet staining method (Smith et al., 2008), was taken as the MBIC. Each determination was performed in triplicate.

Determination of drug combination activities by the checkerboard method. Combinations of AgNPs and separate antibiotics (AMP, OXA, STR, RIF, CIP, TET, MEM or CAZ), and also the individual antimicrobial agents, were mixed with *P. aeruginosa* culture (diluted to 1 × 10<sup>6</sup> c.f.u. ml<sup>−1</sup>) in fresh medium and then mixed with MH supplemented with 0.45 % glucose and various concentrations of the AgNPs and the antibiotics in the vertical and horizontal directions, respectively (Eliopoulos & Moellering, 1996). The plates were incubated at 37 °C for 24 h. To evaluate the impact of drug combinations on bacterial growth, the absorbance at 600 nm was measured using a Sunrise spectrophotometer and the data analysed with Magellan software (Tecan). To study the effect on *P. aeruginosa* biofilm formation, the microtitre plate wells were stained with Crystal Violet. Three independent replicates of both assays were performed. Fractional inhibitory concentration indices (FICIs) were calculated as follows: FICI = (MIC or MBIC of substance A in combination/MIC or MBIC of substance A alone) + (MIC or MBIC of substance B in combination/MIC or MBIC of substance B alone). The effect of a combination of two compounds was considered to be synergistic when the FICI value was ≤0.5 and antagonistic when it was >4, and no interaction was scored when it was in the range 0.5–4.0 (Odds, 2003).

Time-kill assay. An overnight culture of *P. aeruginosa* was diluted to 1 × 10<sup>6</sup> c.f.u. ml<sup>−1</sup> in MH supplemented with appropriate concentrations of AgNPs and STR, AMP, RIF or TET added alone or in combination, and then incubated at 37 °C. Aliquots of 0.1 ml were taken at the start of incubation and again after 1, 2, 3, 4, 8 and 24 h. Serially diluted samples (0.1 ml) were spread onto MH agar plates and colony numbers counted after 24 h incubation at 37 °C. A reduction in the viable cell count in the presence of both substances, in comparison with that obtained in the presence of the most active compound alone, of ≥2 log<sub>10</sub> units was interpreted as synergy (Matsumura et al., 1999). Kill curves were drawn after plotting log<sub>10</sub> c.f.u. against time. The experiment was performed three times.

Measurement of DnaK and HtpG induction by AgNPs using SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot analysis were performed as described by Grudniak et al. (2011) with modifications. An overnight culture of *P. aeruginosa* was diluted to 10<sup>6</sup> c.f.u. ml<sup>−1</sup> in fresh medium and incubated for 1 h at 37 °C. This culture was then divided into 10 ml aliquots that were supplemented with 0.15, 0.3 or 0.45 MIC of AgNPs, plus a negative control, and growth was continued to the exponential phase (OD<sub>600</sub>=0.6–0.8; spectrophotometer Ultraspec III, Pharmacia LKB). The cells were harvested by centrifugation (10 min, 4500 g), washed twice in Davies buffer (1.4 g K<sub>2</sub>HPO<sub>4</sub> l<sup>−1</sup>, 0.6 g KH<sub>2</sub>PO<sub>4</sub> l<sup>−1</sup>, 0.02 g MgSO<sub>4</sub>·7H<sub>2</sub>O l<sup>−1</sup>, 0.2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> l<sup>−1</sup>, 0.1 g C<sub>6</sub>H<sub>12</sub>N<sub>2</sub>O<sub>7</sub>; pH 7.2), then resuspended in 1 ml Davies buffer containing 0.2 % SDS (w/v) and incubated at 100 °C for 5 min. The protein concentration in the cell lysates was measured using the BCA assay (2,2′-bicinchoninic acid; Sigma–Aldrich), and then samples containing 50 µg of protein were analysed by SDS-PAGE. Following transfer of the resolved proteins to PVDF membrane, identical Western blots were reacted with the rabbit polyclonal sera: anti-DnaK diluted 1 : 4000 or anti-HtpG diluted 1 : 10 000. Reactive bands were disclosed by incubation with goat anti-rabbit IgG alkaline phosphatase conjugate secondary antibody diluted 1 : 15 000, followed by BCIP/NBT (5-bromo-4-chloro-3′-indolyphosphate-p-toluidine salt / nitro blue tetrazolium chloride) chromogenic substrate. This experiment was performed three times. The blots were analysed using the public domain software Image J (National Institutes of Health, 2013).

Statistical analysis. Data are means ± SD of three replicates or experiments. Statistical significance of the differences between experimental groups was calculated using the non-parametric Kruskal–Wallis test. A post hoc analysis using the Mann–Whitney U-test and Bonferroni correction was used to adjust the significance (P) value for the number of comparisons. Results where P<0.05 were considered statistically significant.

RESULTS

Antibacterial effect of AgNPs

Precise broth microdilution assays were determined for *P. aeruginosa* ATCC 10145. The low MIC (1 µg ml<sup>−1</sup>) and MBIC (4 µg ml<sup>−1</sup>) values of AgNPs confirmed the strong antimicrobial activity of this agent. MICs of antibiotics tested varied greatly, from 768 µg ml<sup>−1</sup> for OXA to 0.25 µg ml<sup>−1</sup> for CIP. MBICs of all antibiotics tested except CIP were higher than MICs. MBIC of CIP was equal to the respective MIC (Tables 1 and 2).

Combined effect of AgNPs and antibiotics on *P. aeruginosa*

The susceptibility of free-living *P. aeruginosa* to different combinations of AgNPs and antibiotics is presented in...
Table 1. AgNPs interacted synergistically with four of the six tested antibiotics: AMP, RIF, TET and STR. No interaction with OXA, CIP, MEM and CAZ was observed. Note that the last two compounds are used to treat *Pseudomonas* infections. None of the AgNP–antibiotic combinations were antagonistic. The results obtained for *P. aeruginosa* biofilms using the same method showed a lack of interaction between the silver nanoparticles and any of the antibiotics tested (Table 2). We also did not observe synergy between AgNPs and STR and RIF against free-living *P. aeruginosa* spontaneous mutants, resistant to 512 μg STR ml⁻¹ and 256 μg RIF ml⁻¹ (i.e. 21 times and 16 times higher concentrations, respectively, in comparison to the parental *P. aeruginosa* strain), suggesting that the synergistic interactions depend on the doses of the compounds used.

The time-kill experiments determined that only the combination with STR confirmed the synergy of these two antimicrobial agents against this strain of *P. aeruginosa* (Fig. 1a). Compared with the AgNPs and STR added separately, a combination of 0.25 μg AgNPs ml⁻¹ with 6 μg STR ml⁻¹ produced a decrease in c.f.u. of 2.7 log₁₀ (to 1.0 × 10⁷ c.f.u. ml⁻¹) was observed compared with both single compounds (Fig. 1a). For combinations of AgNPs with AMP, RIF and TET, no synergistic differences in bacterial survival were noticed after 8 h or 24 h of incubation (Fig. 1b–d).

**Induction of a stress response by AgNPs**

Western blot analysis of proteins extracted from late-exponential-phase cells of *P. aeruginosa* ATCC 10145 indicated that AgNP treatment substantially induced synthesis of DnaK (Fig. 2). Densitometric measurements showed that, on average, treatment of *P. aeruginosa* cells with AgNPs caused a 2.5- to threefold increase in the level of DnaK compared with untreated control cells. No significant increase in HtpG synthesis was observed (Table 3).

**DISCUSSION**

The results of chequerboard assays performed to identify interactions between AgNPs and several conventional antibiotics demonstrated the existence of synergy with AMP, STR, RIF and TET, but only against free-living cells and not biofilms of *P. aeruginosa*. From the medical point of view, the combination of AgNPs and antibiotics may be a potential strategy to combat *P. aeruginosa* infections.
of view, synergistic interactions are the most important since they allow the continued use of antibiotics against antibiotic-resistant isolates. The combined antibacterial activity of two compounds can utilize several strategies (Fischbach, 2011). There have been few reports concerning synergy between silver metal ions or nanoparticles and antibiotics. Fayaz et al. (2010) observed synergistic activity of AgNPs with several antibiotics, particularly AMP, against Gram-negative bacteria and proposed a model in which AgNPs–penicillin complexes interact with the bacterial cell wall and inhibit the formation of peptidoglycan cross-links, leading to bacterial lysis. Hwang et al. (2012) showed that AgNPs possess antimicrobial, including antibiofilm, effects and can act synergistically with several antibiotics. Their experiments revealed that the antibacterial activity was influenced by the ATP-associated metabolism rather than the permeability of the outer membrane. Synergy of AgNPs in combination with STR against E. coli (Ghosh et al., 2012) and with RIF against Bacillus subtilis (Konwarh et al., 2011) was also shown but only by the measurement of the growth inhibition zones on agar plates.

The antibiotics used in our study belong to several classes and have various cellular targets, modes of action and bacterial resistance mechanisms. Of four antibiotics displaying synergy with AgNPs, AMP inhibits the synthesis of peptidoglycan (the main component of the bacterial cell wall), STR and TET hamper various steps of translation and RIF inhibits initiation of transcription by binding to the beta subunit of RNA polymerase. For the two other antibiotics which do not act synergistically with AgNPs, CIP (a fluoroquinolone antibiotic) interferes with DNA gyrase and thus inhibits DNA replication, and OXA is a

**Fig. 1.** Time-kill curves showing the effect of a combination of antibiotic and AgNPs in comparison with the separate addition of each compound. (a) 0.25 MIC STR (6 μg ml⁻¹) + 0.25 MIC AgNPs (0.25 μg ml⁻¹). (b) 0.0625 MIC AMP (16 μg ml⁻¹) + 0.25 MIC AgNPs (0.25 μg ml⁻¹). (c) 0.25 MIC TET (2.5 μg ml⁻¹) + 0.25 MIC (0.25 μg ml⁻¹) AgNPs. (d) 0.25 MIC RIF (4 μg ml⁻¹) + 0.125 MIC AgNPs (0.125 μg ml⁻¹). Mean ± SD values of triplicate cultures.
member of the broad beta-lactam family inhibiting cell wall synthesis (for a review see van Hoek et al., 2011). As the combined antimicrobial activity can utilize several strategies, the underlying basis of the observed synergy can differ.

No synergy was reported for any antibacterial combination tested when studying the susceptibility of \emph{P. aeruginosa} biofilm. Bacterial biofilms are generally less susceptible to AgNPs than planktonic cells, probably due to the extracellular matrix coating cells in a biofilm, aggregation of cells to reduce their exposed surface and the retardation of AgNP diffusion caused by nanosilver aggregation and the resulting increase in mean particle size (Choi et al., 2010). The same reasons can be used to explain the absence of inhibitory cooperation.

\textbf{Table 3.} Effect of AgNPs on the induction of selected \emph{P. aeruginosa} heat-shock proteins

The amount of each protein in the control sample (no AgNPs added) was arbitrarily assigned the value of 1. Results represent mean ± SD values from densitometric analysis of three separate Western blots. *$P<0.05$.

<table>
<thead>
<tr>
<th>AgNP treatment</th>
<th>Relative amount of DnaK</th>
<th>Relative amount of HtpG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no AgNPs)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.15 MIC AgNPs</td>
<td>2.43* ± 0.22</td>
<td>1.18 ± 0.22</td>
</tr>
<tr>
<td>0.3 MIC AgNPs</td>
<td>2.50* ± 0.35</td>
<td>1.17 ± 0.15</td>
</tr>
<tr>
<td>0.45 MIC AgNPs</td>
<td>3.03* ± 0.67</td>
<td>1.02 ± 0.22</td>
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The time-kill experiments were performed for antimicrobial combinations showing synergy in the chequerboard assay. Synergy was confirmed only for AgNPs in combination with STR but not with AMP, RIF and TET. Chequerboard and time-kill methods, although time-consuming and labour-intensive, are still, along with the E-test, the most widely used techniques to assess synergy. A variety of investigators have found that these two methods are not interchangeable (White et al., 1996). For example Bonapace et al. (2000), studying antibiotic synergy against \emph{Acinetobacter baumannii}, found that the agreement between these two methods was only 51% (range 30–67%). In view of the above, the difference between synergistic interactions found by us was not unexpected.

In the present study, we also examined the ability of AgNPs to induce a stress response by measuring cellular levels of the DnaK and HtpG chaperones. These proteins are representative indicators of stress conditions and are induced by a variety of stressors including heat, ethanol and antibiotics (Yura et al., 1996). DnaK and HtpG play roles in the correct folding of newly translocated polypeptides, disaggregation of misfolded proteins, suppressing their aggregation, unfolding of peptides for translocation and also heat shock signalling (Saibil, 2008).

We found that the presence of AgNPs caused an increase in levels of DnaK by 2.5–3-fold, indicating their ability to induce a stress response. However, no induction of HtpG was observed in \emph{P. aeruginosa}. It should be noted that the mode of induction of HtpG by heat or chemical agents in \emph{E. coli} has several unique features compared to the induction of other chaperones (Mason et al., 1999). The induction of chaperone synthesis by antibiotics is well documented (Cardoso et al., 2010) but there have been few reports on the effects of AgNPs on cellular chaperone levels. The ability of AgNPs to induce two inclusion body chaperones, IbpA and IbpB, was demonstrated by a proteomic analysis of \emph{E. coli} K-12 strain MC1655.6 (Lok et al., 2006).

It is well known that proteins, such as DnaK, induced in bacterial cells exposed to antibiotics affect their susceptibility to these agents. It was shown that mutations in dnaK increased \emph{E. coli} susceptibility to fluoroquinolones (Yamaguchi et al., 2003). Recently a protective effect of DnaK, and especially GroEL/GroES chaperons, against aminoglycoside antibiotics was also reported (Goltermann et al., 2013). It is possible that the ability of AgNPs to induce DnaK could diminish its antimicrobial activity and also influence the activity of other antimicrobials applied simultaneously.

In conclusion, we have demonstrated the modulation of antibiotic susceptibility in \emph{P. aeruginosa} by AgNPs. AgNPs acted synergistically with AMP, STR, RIF and TET against free-living cells of \emph{P. aeruginosa} as determined by the chequerboard method. The time-kill assay proved the presence of synergy between AgNPs and STR only. By contrast, no effect on a \emph{P. aeruginosa} biofilm was observed. Treatment with AgNPs enhanced cellular levels of the...
chaperone DnaK but not HtpG. The ability of AgNPs to influence chaperone concentration may be correlated with the susceptibility of *P. aeruginosa* to nanosilver and antibiotics.

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**REFERENCES**


