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Cellular pharmacokinetics and intracellular activity against *Listeria monocytogenes* and *Staphylococcus aureus* of chemically modified and nanoencapsulated gentamicin

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Objectives: The aim of this study was to investigate different hydrophobic gentamicin formulations [gentamicin-bis(2-ethylhexyl) sulfosuccinate (GEN-AOT), microstructured GEN-AOT (PCA GEN-AOT) and GEN-AOT-loaded poly(lactide-co-glycolide) acid (PLGA) nanoparticles (NPs)] in view of improving its therapeutic index against intracellular bacteria. The intracellular accumulation, subcellular distribution and intracellular activity of GEN-AOT and NPs in different monocytic–macrophagic cell lines were studied.

Methods: Human THP-1 and murine J774 phagocytic cells were incubated with GEN-AOT formulations at relevant extracellular concentrations [from 1× MIC to 18 mg/L (human C_{max})], and their intracellular accumulation, subcellular distribution and toxicity were evaluated and compared with those of conventional unmodified gentamicin. Intracellular activity of the formulations was determined against bacteria showing different subcellular localizations, namely *Staphylococcus aureus* (phagolysosomes) and *Listeria monocytogenes* (cytosol).

Results: GEN-AOT formulations accumulated 2-fold (GEN-AOT) to 8-fold (GEN-AOT NPs) more than gentamicin in phagocytic cells, with a predominant subcellular localization in the soluble fraction (cytosol) and with no significant cellular toxicity. NP formulations allowed gentamicin to exert its intracellular activity after shorter incubation times and/or at lower concentrations. With an extracellular concentration of 10× MIC, a 1 log_{10} decrease in *S. aureus* intracellular inoculum was obtained after 12 h instead of 24 h for NPs versus free gentamicin, and a static effect was observed against *L. monocytogenes* at 24 h with NPs, while free gentamicin was ineffective.

Conclusions: GEN-AOT formulations yielded a high cellular accumulation, especially in the cytosol, which resulted in improved efficacy against both intracellular *S. aureus* and *L. monocytogenes*.

Keywords: nanoparticles, intracellular bacteria, drug accumulation, subcellular distribution

Introduction

Despite their highly bactericidal character, aminoglycoside antibiotics are considered poorly active against intracellular bacteria,[1,2] which has been attributed to their inappropriate cellular pharmacokinetic profile. Because of their high hydrophilicity, aminoglycosides penetrate cells only slowly and, once inside them, remain confined in the lysosomal compartment, where their activity is reduced by the acidic pH.[3] Considerable efforts are therefore made to enhance their cellular concentration.

Drug delivery system-based approaches are being explored in this context.[4] Although they have shown very promising results, their *in vivo* stability and drug loading efficiency in the carriers remain important issues. A new approach consists in the chemical modification of gentamicin with the anionic surfactant bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) (GEN-AOT) and its micronization by precipitation using a compressed fluid-based methodology (precipitation with a compressed antisolvent, PCA) to obtain a microstructured GEN-AOT (PCA GEN-AOT), or incorporation in poly(lactide-co-glycolide) (PLGA) nanoparticles (NPs) with a very high encapsulation efficiency.[5]

We compared the cellular pharmacokinetics and pharmacodynamics of these new formulations using bacteria

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thriving in different intracellular locations, namely *Staphylococcus aureus* (phagolysosomes) and *Listeria monocytogenes* (cytoplasm).

Materials and methods

Materials

Gentamicin sulphate and AOT were purchased from Sigma-Aldrich (St Louis, MO, USA) and PLGA 502H (Resomer® RG 502H, PLGA 50:50, 13.7 kDa) and 752H (Resomer® RG 752H, PLGA 75:25, 17 kDa) were supplied by Boehringer Ingelheim (Ingelheim, Germany). Reagents for cell culture were from Invitrogen Inc. (Carlsbad, CA, USA) and bacterial culture medium was from Becton Dickinson (Franklin Lakes, NJ, USA). Other reagents were obtained from Sigma-Aldrich or Merck (Madrid, Spain).

Preparation of GEN-AOT, PCA GEN-AOT and GEN-AOT NPs

All gentamicin formulations were prepared as previously described. The ionic complex of gentamicin and the anionic surfactant AOT was obtained by the hydrophobic ion pairing ('HIP') method[6] and was either micronized by PCA technology[5] or encapsulated into PLGA 502H or 752H NPs by the oil-in-water emulsion solvent evaporation method[5] (nominal drug loading of 20 mg per 200 mg of polymer).

Cell culture conditions and viability assessment

The J774 macrophage cell line (ATCC TIB-67) and human THP-1 monocytes (ATCC TIB-202) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Differentiation of the THP-1 cells into adherent macrophages (A-THP-1) was obtained by incubation of the cells with 75 ng/mL phorbol 12-myristate 13-acetate ('PMA') in complete RPMI medium for 48 h. Cell viability was checked in cells exposed for 24 h to 18 mg/L gentamicin formulations [see Figure S1, available as Supplementary data at *JAC* Online; assays used were release of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the culture medium (LDH assay)[7] and formation of blue formazan crystals by mitochondrial dehydrogenases in metabolically active cells (MTT assay)[8]].

Cellular accumulation and fractionation studies

The cellular accumulation of the antibiotic formulations was studied in THP-1 cells, A-THP-1 cells and J774 cells as previously reported.[9,10] Their subcellular distribution was studied in J774 cells after 24 h of incubation using the cell fractionation procedure described previously.[11] Gentamicin content was quantified by microbiological assay using antibiotic medium 11 and *Bacillus subtilis* ATCC 6633 as test organism (limit of detection 0.125 mg/L, linear response between 0.125 and 64 mg/L; $r^2=0.989$)[7] and expressed by reference to the total cell protein content (determined by the Lowry method). Cellular accumulation was estimated using a conversion factor of 5 μL cell volume/mg of cell protein.[12,13]

Bacterial strains, susceptibility testing and cell infection studies

MICs and MBCs were determined in Mueller–Hinton broth (for methicillin-susceptible *S. aureus* strain ATCC 25923, methicillin-resistant *S. aureus* strain ATCC 33591 and *Pseudomonas aeruginosa* strain PAO1) or trypticase soy broth (for *L. monocytogenes* serotype 1/2a strains EGD and 56[14]) by broth microdilution, according to the CLSI[15] recommendations. MICs for *S. aureus* ATCC 25923 were also determined with Mueller–Hinton broth adjusted to different pHs.

Antibiotic intracellular activity was evaluated in J774 cells infected with *S. aureus* ATCC 25923 (bacteria/cell ratio of 4) or *L. monocytogenes* strain 56 (bacteria/cell ratio 5) using fully validated procedures (described in detail in Carryn *et al.*[1] and Seral *et al.*[2]). Intracellular growth of the bacteria was evaluated after 12 or 24 h of incubation in control conditions (gentamicin at 0.5× MIC to prevent extracellular bacterial growth) or in the presence of antibiotic formulations.

Statistical analysis

Data analysis and graphical presentation were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Statistical comparison between different groups was performed using the Mann–Whitney *U*-test.

Results and discussion

Micronized GEN-AOT and GEN-AOT polymeric NPs

Micronization of GEN-AOT (PCA GEN-AOT) resulted in a powdered solid with a mean particle diameter of 1 μm and a zeta potential of around −1 mV. GEN-AOT-loaded PLGA 502H and PLGA 752H NPs presented mean diameters of 263±10 nm and 269±24 nm and a zeta potential of −3.3±0.5 mV and −3.5±0.9 mV, respectively. Encapsulation efficiencies of 100% were achieved for both NP formulations with drug loadings of 21.9±0.5 and 22.7±0.7 μg of gentamicin/mg of NPs for PLGA 502H and PLGA 752H NPs, respectively.

Kinetics of cellular accumulation

The cellular uptake of gentamicin has been extensively studied in different cell lines and conditions,[3] and was found to be low in phagocytic cells.[16] We therefore firstly exposed cells for 24 h to an extracellular concentration of each formulation corresponding to 18 mg/L gentamicin (human C_{max} after a conventional dose).[17] Macrophages accumulated more antibiotic than monocytes (see Figure S2, available as Supplementary data at *JAC* Online) and were therefore used for kinetic experiments (Figure 1). Gentamicin accumulation proceeded in a slow and linear fashion ($r^2=0.988$) (Figure 1c) to reach an apparent cellular to extracellular concentration ratio of 1.5 at 24 h with no appearance of a plateau (Figure 1a and b). This compares very well with the rate of uptake of horseradish peroxidase, a fluid-phase endocytosis tracer, in J774 macrophages[10] and is consistent with the mechanism of pinocytosis proposed to explain the slow accumulation of gentamicin.[13,18] Non-encapsulated GEN-AOT also followed linear kinetics of accumulation. Yet its rate of uptake was 1.5 to 2-fold higher (Figure 1c). This could be attributed to the lower charge of the ion pair, which would increase the partition coefficient of gentamicin, as reported for cisplatin[19] and for antibiotics such as ampicillin and erythromycin.[20–22] The grossly linear kinetics of uptake of GEN-AOT could fit with this model, but could also result from a slow endocytic process for which the plateau of accumulation has not yet been reached, as is the case for gentamicin. Incorporation of GEN-AOT into polymeric NPs further increased both antibiotic uptake rate and efficiency, especially for NPs formulated with 752H, the more hydrophobic PLGA copolymer. It is known that uptake of particles by phagocytic cells is critically dependent on their physicochemical properties.[23] For PLGA particles, it is inversely related to polymer hydrophilicity.[24,25] GEN-AOT 752H NPs allowed gentamicin to accumulate >10-fold in both macrophage cell lines (see

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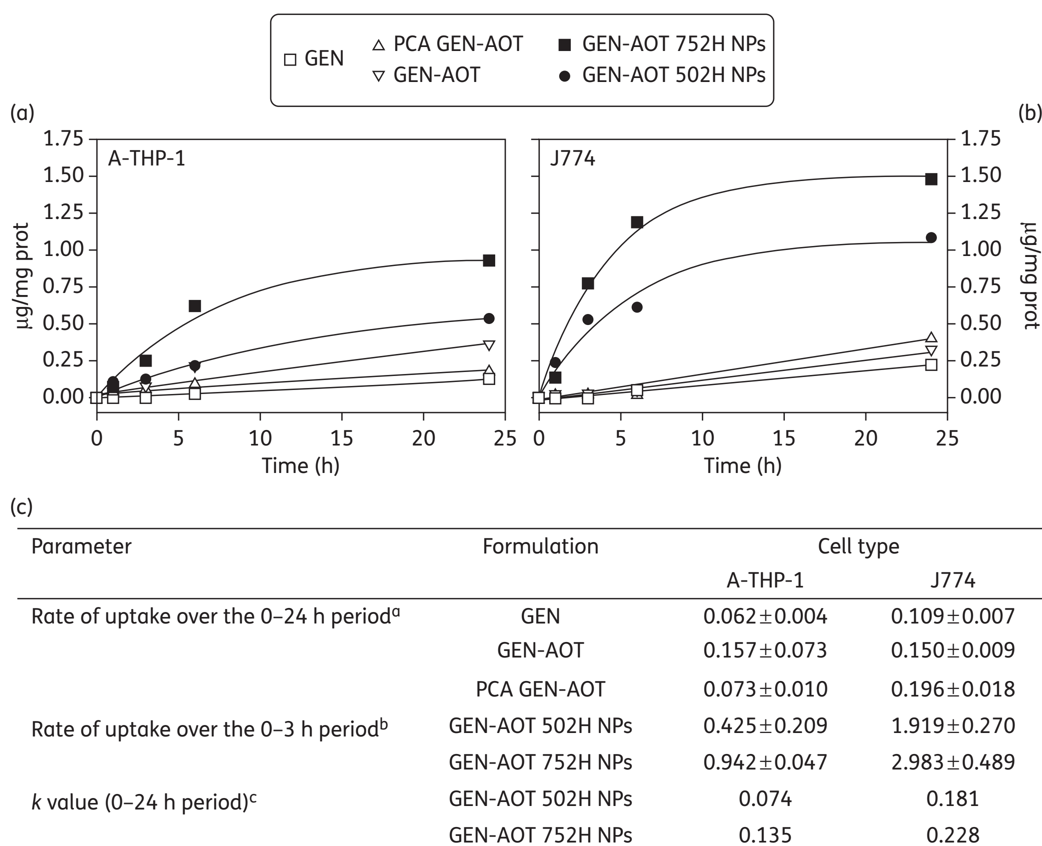

Parameter	Formulation	Cell type	
		A-THP-1	J774
Rate of uptake over the 0–24 h period[a]	GEN	0.062±0.004	0.109±0.007
	GEN-AOT	0.157±0.073	0.150±0.009
	PCA GEN-AOT	0.073±0.010	0.196±0.018
Rate of uptake over the 0–3 h period[b]	GEN-AOT 502H NPs	0.425±0.209	1.919±0.270
	GEN-AOT 752H NPs	0.942±0.047	2.983±0.489
k value (0–24 h period)[c]	GEN-AOT 502H NPs	0.074	0.181
	GEN-AOT 752H NPs	0.135	0.228

Figure 1. Accumulation kinetics of 18 mg/L gentamicin (GEN), GEN-AOT and its microstructured (PCA GEN-AOT) and encapsulated (GEN-AOT 502H NPs and GEN-AOT 752H NPs) forms in A-THP-1 cells (a) and J774 cells (b) over 24 h. Data are expressed as μg of internalized drug/mg of cell protein. (c) Rates of uptake of gentamicin and its formulations in A-THP-1 and J774 cells. Calculations were made based on mathematical regressions of the data presented. All values are expressed as accumulation factor per hour [(Cc/Ce)/h]; see Figure S2 for accumulation factors at 24 h. [a]Slope of the linear regression correlating gentamicin accumulation with time (0–24 h timepoints). [b]Slope of the linear regression correlating gentamicin accumulation with time during the linear phase of the uptake (0–3 h timepoints). [c]Calculated from one phase exponential association (0–24 h timepoints).

Figure S2 for accumulation factors of the different formulations at 24 h). This rate of uptake remains ∼10-fold lower than that of the lipoglycopeptide antibiotic oritavancin, which is thought to enter cells by adsorptive endocytosis,[10] suggesting that binding at the cell surface probably differs.

Subcellular distribution of gentamicin and GEN-AOT formulations

Cellular accumulation *per se* is not always predictive of antibiotic intracellular efficacy, notably because a sufficient amount of the drug also needs to reach the infected compartment.[26,27] Figure 2 shows the subcellular distribution of the formulations together with those of the marker enzymes cytochrome *c* oxidase (for mitochondria), *N*-acetyl-β-glucosaminidase (for lysosomes) and LDH (for soluble proteins) in the control cells. No differences were found in the subcellular distribution profile of the markers between non-treated and treated J774 cells, indicating that the treatments did not affect the biophysical properties and integrity of the studied organelles or the distribution of the soluble proteins (data not shown). As expected from its lysosomotropic nature, gentamicin showed a subcellular distribution that was very close to that of *N*-acetyl-β-glucosaminidase, predominantly localized in the granular fraction (77% of cell-associated drug). This intracellular disposition is not specific to macrophages as it is also reported in other non-phagocytic cell lines,[28–31] and is thought to be, together with the decreased activity of gentamicin at acidic pH, the reason for its low efficacy against a number of intracellular bacteria.[1,32,33] Interestingly, ion pairing and encapsulation processes altered the distribution profile of gentamicin inside the cells and allowed higher accumulation in their soluble fraction (containing the cytosol and the soluble proteins). Thus, in cells incubated with GEN-AOT, 45% of cell-associated drug was recovered in the soluble fraction, leading to a 25-fold increase in gentamicin accumulation in this fraction. Again, this could be explained by the non-ionized character of GEN-AOT, which could facilitate the translocation of the drug from the lysosomes to the cytoplasm or its diffusion through the pericellular membrane. In cells incubated with PLGA 502H and 752H NPs, the accumulation of gentamicin was 2- to 3-fold higher in the organelles and 74- to 124-fold higher in the soluble fraction than in cells exposed to free gentamicin. It has been proposed that the acidic pH of lysosomes may cause an inversion of NP surface charge, which may favour the interaction

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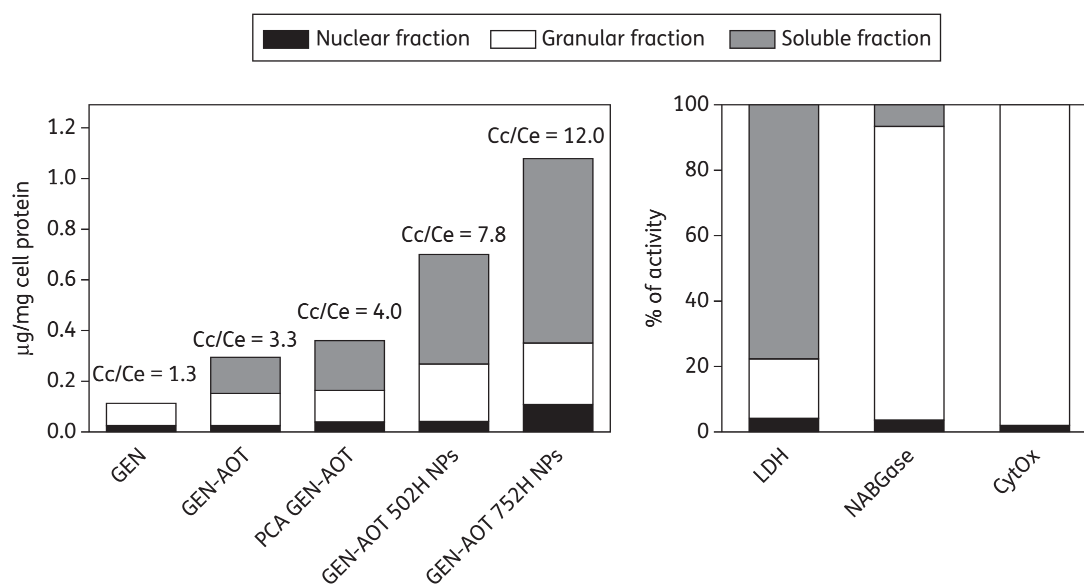

Figure 2. Subcellular distribution of gentamicin (GEN), GEN-AOT, PCA GEN-AOT and GEN-AOT-loaded PLGA NPs (GEN-AOT 502H NPs and GEN-AOT 752H NPs) in J774 cells after 24 h of incubation. The left-hand panel shows the drug content in the different fractions expressed as μg/mg of cell protein in the unfractionated homogenate. Total cellular accumulation of the drug, expressed as the cellular-to-extracellular drug concentration (Cc/Ce), is indicated at the top of each bar. The right-hand panel shows the distribution of the marker enzymes LDH, *N*-acetyl-β-glucosaminidase (NABGase for lysosomes) and cytochrome *c* oxidase (CytOx for mitochondria) in non-treated J774 cells. Enzymes were assayed for each fractionation experiment, but as no substantial changes were found only one set of data is shown.

of NPs with the lysosomal membrane and facilitate their escape into the cytosol.[34,35]

Antibiotic susceptibility studies and effect of acidic pH

MIC and MBC values of gentamicin and its formulations are shown in Table S1 (available as Supplementary data at *JAC* Online). No marked differences (one dilution above or below) were found between values measured for gentamicin and the different formulations, confirming that AOT coupling and encapsulation into polymeric NPs did not affect antibiotic potency or bactericidal character. It is noteworthy that the intrinsic activity of all GEN-AOT formulations against *S. aureus* was less affected by acidic pH than that of gentamicin itself (2–3 $\log_2$ dilutions increase between pH 7.4 and 5.0 instead of 5 dilutions; see Figure S3, available as Supplementary data at *JAC* Online). The masking of the cationic amino groups of gentamicin after ion pairing could prevent, at least partially, the protonation of the antibiotic at low pH, and therefore result in a smaller loss of antibacterial activity.

Intracellular activity of the antibiotic against S. aureus and L. monocytogenes

Because aminoglycosides are concentration-dependent antibiotics, the dramatic increase in cellular concentration may contribute to improvement of their intracellular activity, especially against cytosolic bacteria. We therefore examined the activity of gentamicin and its formulations using for comparison *S. aureus* (phagolysosomal) and *L. monocytogenes* (cytosolic). In a first experiment, intracellular activity was evaluated at a fixed extracellular concentration (18 mg/L) and incubation time (12 h for *L. monocytogenes* and 24 h for *S. aureus*). These conditions made it possible to achieve a similar intracellular proliferation rate in both cases (∼1.5 $\log_{10}$ unit increase in cfu/mg of protein) and, therefore, to compare activity against similar inocula (Figure 3, left-hand panels). In these conditions, all formulations yielded similar intracellular reductions of *S. aureus* infection (1.1–1.45 $\log_{10}$ unit reduction). This corresponds to the maximal effect that can be achieved in cells exposed to gentamicin extracellular concentrations >10 mg/L (50× MIC),[36] so that a further increase in its intracellular concentration does not add much to the intracellular effect. In contrast, GEN-AOT treatments improved gentamicin intracellular activity against *L. monocytogenes*. GEN-AOT and PCA GEN-AOT significantly decreased intracellular bacterial growth compared with control cells and cells incubated with gentamicin alone. GEN-AOT 502H NPs allowed a static effect to be reached, and GEN-AOT 752H NPs caused a slight decrease in intracellular inoculum. These data nicely correlate with the commensurate increase in gentamicin concentration observed in the soluble fraction of cells incubated with these formulations.

In the next experiment, we followed activity over time and upon incubation with increasing concentrations of either free gentamicin or GEN-AOT 752H NPs. Against intracellular *S. aureus*, gentamicin was bacteriostatic at 2× MIC and reached its maximal effect at 24 h at 10× MIC. In contrast, a significant decrease in intracellular inoculum was observed when cells were incubated for 12 h with NPs. Against *L. monocytogenes*, gentamicin was inactive whatever the time of incubation or the concentration used. In contrast, NPs markedly reduced intracellular growth at an extracellular concentration of 10× MIC. These data confirm the time- and concentration-dependent character of gentamicin activity.[2,36,37] They also suggest that

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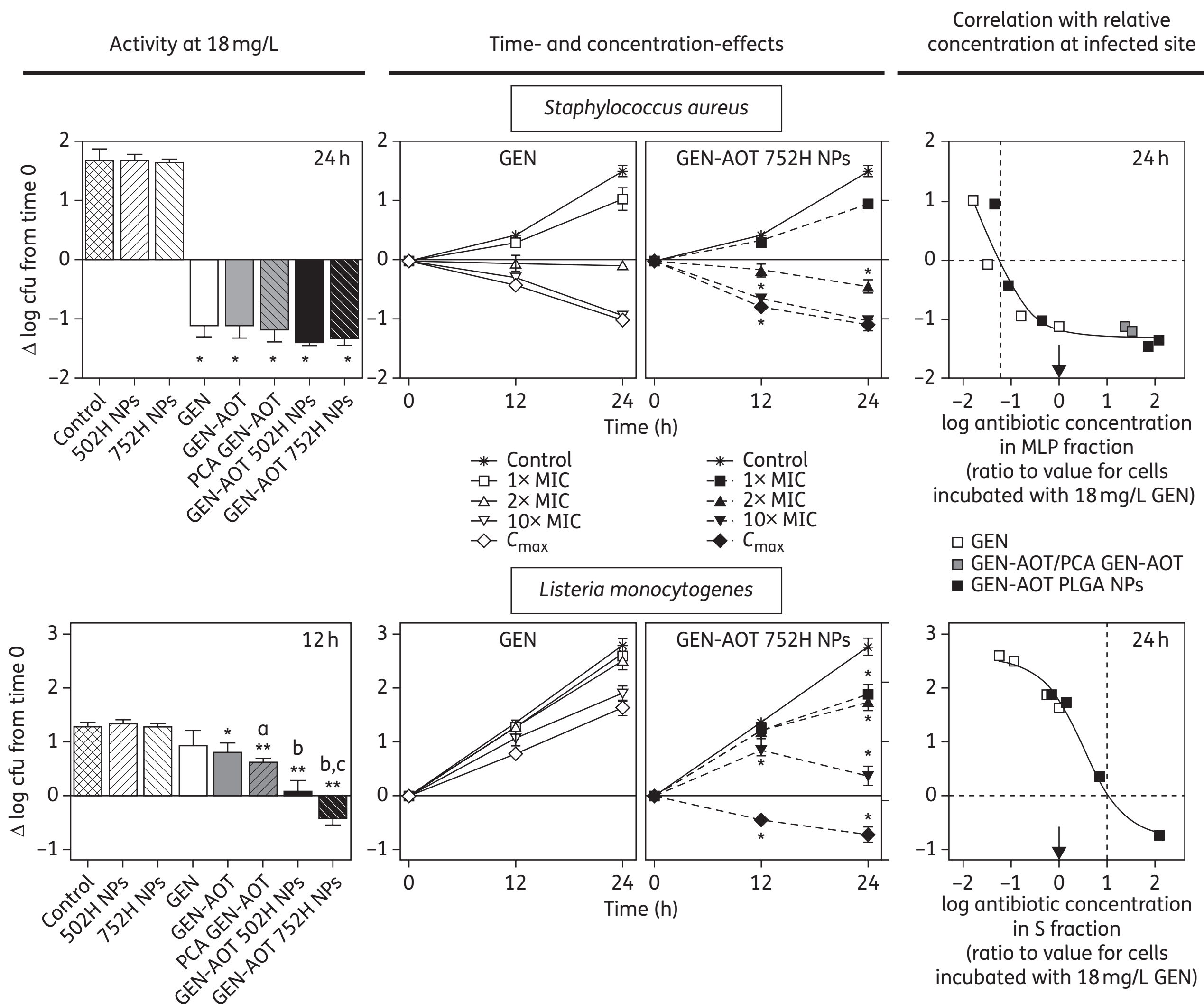

Figure 3. Activity of gentamicin (GEN) and its different formulations against intracellular *S. aureus* ATCC 25923 (top) and intracellular *L. monocytogenes* strain 56 (bottom). Activity is expressed as the change in the number of cfu per mg of cell protein from the initial inoculum. Data are the means and standard deviations of at least four independent determinations. Left-hand panels: infected cells were exposed to a fixed concentration of 18 mg/L gentamicin for 24 h (*S. aureus*) or 12 h (*L. monocytogenes*). $^*P<0.05$ or $^{**}P<0.01$ when compared with the untreated control; a=$P<0.05$ or b=$P<0.01$ when compared with gentamicin; c=$P<0.01$ when compared with GEN-AOT. Middle panels: infected cells were exposed to increasing extracellular concentrations of free gentamicin or of GEN-AOT 752H NPs for 12 or 24 h. $^*P<0.05$ for GEN-AOT PLGA 752H NPs when compared with gentamicin under the same concentration and exposure time conditions. Right-hand panels: correlation between the activity of gentamicin and its different formulations at 24 h and the concentration of gentamicin reached in the infected compartment. Concentrations are expressed as the ratio to the concentration reached in cells incubated with gentamicin in the granular fraction (MLP fraction) for *S. aureus* and in the soluble fraction (S fraction) for *L. monocytogenes*, as calculated from the data presented in Figure 2 [log scale; 0 corresponds to the subcellular concentration of gentamicin after 24 h of incubation with 18 mg/L of the free drug (highlighted by the arrow)]. The horizontal broken line shows a static effect and the vertical broken line shows the subcellular concentration needed to reach this static effect (C_s). The data were fitted to sigmoidal regressions: $r^2=0.8726$ for *S. aureus* and $r^2=0.9715$ for *L. monocytogenes*.

NPs can improve the activity of gentamicin not only against *L. monocytogenes*, but also against *S. aureus*, allowing it to control intracellular growth at lower concentrations and for shorter incubation times.

The right-hand panels of Figure 3 illustrate the correlation between the intracellular activity and the relative concentration of gentamicin in the infected subcellular fraction after 24 h of incubation with the different formulations. To better evidence the capacity of formulations to increase antibiotic concentration and activity, subcellular concentrations were expressed as the ratio to the value measured in cells incubated with 18 mg/L free gentamicin. Against both bacteria, the data fitted a sigmoidal regression, allowing us to calculate the intracellular static concentration, i.e. the gentamicin concentration in the infected fraction needed to prevent bacterial growth. Against *S. aureus*, the figure shows that most of the concentration effect takes place for lysosomal gentamicin concentrations that are 100- to 10-fold lower than are obtained in cells incubated with 18 mg/L free gentamicin,

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with the static effect obtained for cells incubated with 1 mg/L. If it is considered that lysosomes may represent ~2% of cell volume, and using accumulation data from Figure 2, this would correspond to a drug concentration in these organelles of ~50 mg/L (12× MIC at pH 5.4) [calculated as gentamicin concentration in the considered fraction (in μg/mg of protein)×cell volume (in mL/mg of protein)$^{-1}$×percentage of cell volume represented by the considered fraction×C_s (as a ratio to the concentration of gentamicin in the considered fraction for cells incubated with 18 mg/L), with cell volume estimated to be 0.005 mL/mg of protein, the gentamicin concentration in the considered fraction obtained from data in Figure 2, the percentage of cell volume estimated to be 2% for lysosomes and 70% for cytosol, and C_s interpolated from the sigmoidal regression presented in the right-hand panels of Figure 3 (highlighted by the vertical broken lines)]. Against *L. monocytogenes*, the effect of concentration is manifest in the range of cytosolic concentrations obtained upon incubation with NPs at increasing concentrations, while free gentamicin is clearly suboptimal in the range of concentrations tested. Accordingly, a static effect is observed for a cytosolic concentration that is 6-fold higher than that which can be obtained upon incubation with gentamicin at its C_{max}. If it is considered that the cytosol represents ~70% of the cell volume, this means that a concentration of 18 mg/L (18× MIC at pH 7.4) is needed to prevent *Listeria* growth. These data therefore suggest that the intracellular medium defeats the activity of gentamicin and/or that bacterial responsiveness is reduced in the intracellular environment, making gentamicin 10-fold less potent than it is extracellularly. By increasing the gentamicin concentration, NPs compensate for this loss of potency.

Conclusions

Although this study was not designed to evaluate the therapeutic potential of new formulations of gentamicin, the results presented here indicate that GEN-AOT and its polymeric nanocarriers, especially GEN-AOT 752H NPs, allow gentamicin to accumulate to higher levels inside the cells and to distribute in both lysosomal and cytosolic compartments, which results in an improved intracellular activity against intracellular bacteria thriving in the cytosol, such as *L. monocytogenes,* and those thriving in the lysosomes, such as *S. aureus*. These NPs may therefore make it possible to reduce both the required dose and the administration frequency of gentamicin, with potential subsequent reduction of its toxicity.

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Transparency declarations

None to declare.

Supplementary data

Figures S1, S2 and S3, and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Figure S1. Effect of 18 mg/L gentamicin (GEN) and the different GEN-AOT formulations on the viability of human THP-1 monocytes, A-THP-1 cells and murine J774 macrophages as determined by the LDH release assay (top) and MTT cytotoxicity assay (bottom) after 24 h of incubation. Data are expressed as the means and standard deviations of three independent determinations. MeOH, methanol.

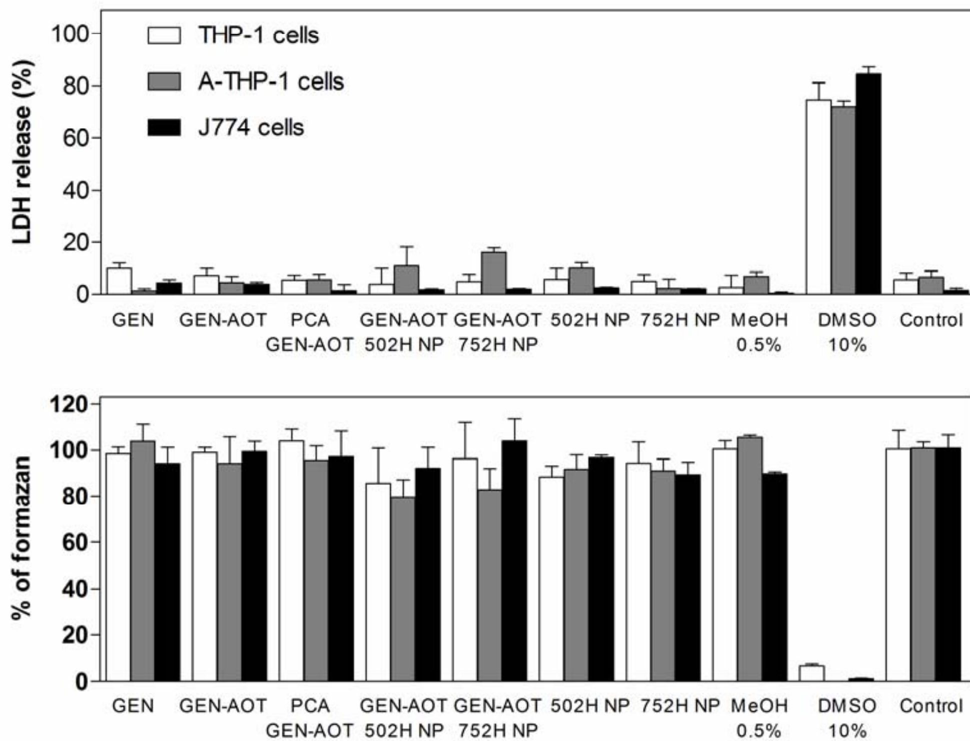

Figure S2. Cellular accumulation of gentamicin in THP-1, A-THP-1 or J774 cells incubated for 24 h with 18 mg/L gentamicin or the formulations containing the same antibiotic concentration. Data are expressed as the means and standard deviations of three independent determinations. Statistical analysis: **P*<0.05 or ***P*<0.01 when compared with gentamicin (GEN); a=*P*<0.05 when compared with GEN-AOT; b=*P*<0.05 when compared with GEN-AOT-loaded PLGA 502H nanoparticles (GEN-AOT 502H NP).

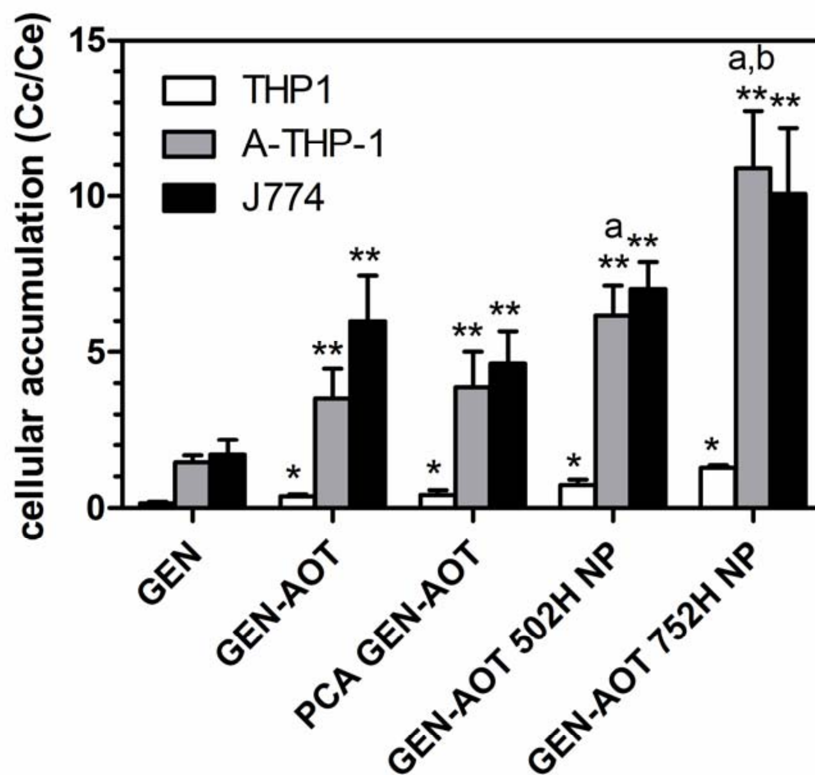

Figure S3. Effect of pH on the MICs of gentamicin (GEN) and GEN-AOT treatments against *S. aureus* strain ATCC 25923. Results are expressed as the MIC in mg/L of antibiotic.

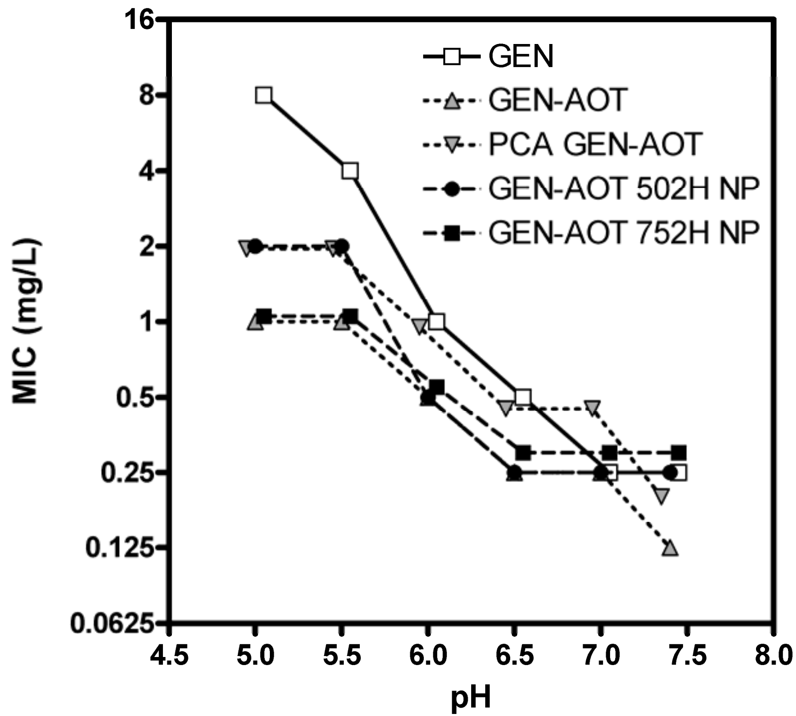

Table S1. MICs and MBCs (mg/L) of gentamicin (GEN) and different GEN-AOT formulations for various bacterial isolates

	S. aureus ATCC 25923 (MSSA)[a]		*S. aureus* ATCC 33591 (MRSA)[b]		*P. aeruginosa* PAO1[c]		*L. monocytogenes* EGD[d]		*L. monocytogenes* 56[e]	
Antimicrobial	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
GEN	0.25	1	2	4-8	0.5	2	0.5	1	1-2	4
GEN-AOT	0.125	0.5	1	2	0.25-0.5	1	0.25-0.5	0.5	1	2-4
PCA GEN-AOT	0.125-0.25	0.5	1-2	2-4	0.25-0.5	1-2	0.25-0.5	0.5	1	2-4
GEN-AOT 502H NPs	0.25	0.5	1	2	0.25-0.5	1	0.25-0.5	0.5	1	2-4
GEN-AOT 752H NPs	0.25	0.5	1	2	0.25-0.5	1	0.25-0.5	0.5	1	2-4
502H NPs	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
752H NPs	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
AOT	32	>32	>32	>32	32	>32	32	>32	>32	>32

[a]MSSA, methicillin-susceptible *S. aureus* obtained from ATCC (Manassas, VA, USA).
[b]MRSA, methicillin-resistant *S. aureus* obtained from ATCC (Manassas, VA, USA).
[c]Obtained from ATCC (Manassas, VA, USA).
[d]Provided by P. Berche (Hôpital Necker, Paris, France).
[e]Provided by Dr I. García-Jalón (Department of. Microbiology, University of Navarra, Pamplona, Spain).