Title:

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

Authors: Edurne Imbuluzqueta[1], Sandrine Lemaire[2], Carlos Gamazo[3], Elisa Elizondo[4,5], Nora Ventosa[4,5], Jaume Veciana[4,5], Françoise Van Bambeke[2†], María J. Blanco-Prieto[1†*]

[1]Department of Pharmacy and Pharmaceutical Technology, University of Navarra, 31008 Pamplona, Spain

[2]Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain B-1200 Brussels, Belgium

[3]Department of Microbiology, University of Navarra, 31008 Pamplona, Spain

[4]Department of Molecular Nanoscience and Organic Materials. Institute of Materials Science (ICMAB-CSIC), Bellaterra, 08193, Spain

[5]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)

Address for correspondence:

María J. Blanco Prieto, Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain. Tel.: +34 948425600; Fax: +34 948425649. E-mail: address: mjblanco@unav.es

† Joint senior authors contributed equally to this study.

Running title: Intracellular activity of GEN-AOT nanoparticles

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SYNOPSIS

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(lactid-co-glycolic) 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 X MIC to 18 mg/L [human Cmax]), 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- (GEN-AOT) to 8- fold (GEN-AOT NP) 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 time and/or at lower concentrations. With an extracellular concentration of 10 X MIC, a 1 $\log_{10}$ decrease in *S. aureus* intracellular inoculum was obtained after 12 h instead of 24h for NPs vs. 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 an improved efficacy against both intracellular *S. aureus* and *L monocytogenes.*

INTRODUCTION

Despite their high bactericidal character, aminoglycoside antibiotics are considered as poorly active against intracellular bacteria,[1, 2] which has been attributed to their inappropriate cellular pharmacokinetic profile. Because of their high hydrophilicity, aminoglycosides only penetrate slowly cells 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 (DDS)-based approaches are being explored in this context.[4] Although they have shown very promising results, *in vivo* stability and drug loading efficiency in the carriers remain however 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 through a compressed fluid-based methodology (Precipitation with a Compressed Antisolvent, PCA) to obtain a microstructured GEN-AOT (PCA GEN-AOT), or incorporation in poly(lactid-co-glycolic) (PLGA) nanoparticles (NPs) with a very high encapsulation efficiency.[5]

We have compared here the cellular pharmacokinetics and pharmacodynamics of these new formulations using bacteria sojourning in different intracellular locations, namely *Staphylococcus aureus* (phagolysosomal) and *Listeria monocytogenes* (cytoplasmic).

MATERIALS AND METHODS

Materials

Gentamicin sulphate and Bis(2-ethylhexyl) sulfosuccinate sodium salt (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 from Becton Dickinson (Franklin Lakes, NJ, USA). Other reagents were obtained from Sigma-Aldrich or Merck (Madrid, Spain).

Preparation of gentamicin-AOT, microstructured gentamicin-AOT and gentamicin-AOT nanoparticles

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 Precipitation with a Compressed Antisolvent (PCA) technology[5] or encapsulated into PLGA 502H or 752H nanoparticles (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

J774 macrophage cell line (ATCC TIB-67) and human THP-1 monocytes (ATCC TIB-202) were maintained in RPMI 1640 medium supplemented with 10 % foetal calf serum at 37 ºC 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 1 in Supplemental Material; assays used: release of the cytoplasmic enzyme lactate dehydrogenase 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

Minimal inhibitory (MIC) and bactericidal concentrations (MBC) were determined in Mueller-Hinton broth (for methicillin-sensitive *S. aureus* strain ATCC 25923, methicillin-resistant strain

ATCC 33591 and *P. aeruginosa* strain PAO1) or Trypticase Soy broth (for *L. monocytogenes* serotype 1/2a strains EGD and 56[14]) by broth microdilution, according to the Clinical and Laboratory Standards Institute[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 details in ref. 1, 2) Intracellular growth of the bacteria was evaluated after 12 h or 24 h incubation in control conditions (gentamicin at 0.5 x 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 Mann-Whitney U test.

RESULTS AND DISCUSSION

Micronized gentamicin-AOT and gentamicin-AOT polymeric nanoparticles (NP)

Micronization of GEN-AOT (PCA GEN-AOT) resulted in a powdered solid with a mean 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 gentamicin/mg of NP 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 Cmax after conventional dose).[17] Macrophages accumulated more antibiotic than monocytes (see Figure S.2 [supplementary material]) and were therefore used them for kinetic

experiments (Figure 1). Gentamicin accumulation proceeded in a slow and linear fashion (r^2 = 0.988) (Figure 1, panel C) to reach an apparent cellular to extracellular concentration ratio of 1.5 at 24 h with no appearance of a plateau (Figure 1, panels A and B). This compares very well with the rate of uptake of horseradish peroxydase, a fluid phase endocytosis tracer, in J774 macrophages[10] and is coherent with the mechanism of pinocytosis proposed to explain gentamicin slow accumulation.[13, 18] Non-encapsulated GEN-AOT also followed a linear kinetics of accumulation. Yet, its rate of uptake was 1.5 to 2-fold higher (Figure 1, panel C). 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 physico-chemical properties.[23] For PLGA particles, it is inversely related to polymer hydrophilicity.[24, 25] GEN-AOT 752H NPs allowed gentamicin to accumulate more than 10-fold in both macrophage cell lines (see Figure 2 in supplemental material for accumulation factors of the different formulations at 24h). This rate of uptake remains about 10 times 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 are probably different.

Subcellular distribution of gentamicin and gentamicin-AOT formulations

Cellular accumulation per se is not always predictive of antibiotic intracellular efficacy notably because the drug also needs to reach in sufficient amount 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 lactate dehydrogenase (for soluble proteins) in the control cells. No differences were found in subcellular distribution profile of the markers in 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 responsible of the 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 for a 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 cell 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 of NP with the lysosomal membrane and facilitate their escape into cytosol.[34, 35]

Antibiotic susceptibility studies and effect of acidic pH

MIC and MBC values of gentamicin and its formulations are shown in Table S.1 (supplementary material). 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. Noteworthy, 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 S.3 [Supplementary material). 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 improve their intracellular activity, especially against cytosolic bacteria. We therefore examined the activity of gentamicin and its formulations using in comparison *S. aureus* (phagolysosomial) and *L. monocytogenes* (cytosolic). In a first experiment, intracellular activity was evaluated at fixed extracellular concentration (18 mg/L) and incubation time (12 hours for *L. monocytogenes* and 24 hours for *S. aureus*). These conditions allowed to achieve in both cases a similar intracellular proliferation (approx. 1.5 $\log_{10}$-unit increase in CFU/mg of protein) and, therefore, to compare activity towards similar inocula (Figure 3, left panels). In these conditions, all formulations yielded similar intracellular reduction of the *S. aureus* infection (1.1 to 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 x MIC),[36] so that a further increase in its intracellular concentration does not add much to the intracellular effect. On the contrary, GEN-AOT treatments improved gentamicin intracellular activity against *L. monocytogenes*. GEN-AOT and PCA GEN-AOT significantly decreased the intracellular bacterial growth compared to the control cells or cells incubated with gentamicin alone. GEN-AOT 502H NPs allowed to reach a static effect, 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 a 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 X MIC and reached its maximal effect at 24 h already at 10 x MIC. In contrast, a significant decrease in intracellular inoculum was already observed when cells were incubated 12 h with NPs. Against *L. monocytogenes*, gentamicin was inactive whatever the time of incubation or the concentration used. On the contrary, NPs markedly reduced intracellular growth for an extracellular concentration of 10 x MIC. These data confirm the time- and concentration-dependent character of gentamicin activity.[2, 36, 37] They also suggest that NPs can improve its activity not only against *L. monocytogenes,* but also against *S. aureus,*

allowing it to control intracellular growth al lower concentrations and already for shorter incubation times.

The right 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 interest of formulations in increasing antibiotic concentration and activity, subcellular concentrations are expressed as the ratio to the value measured in cells incubated with 18 mg/L free gentamicin. Against both bacteria, data fitted sigmoidal regression allowing us to calculate intracellular static concentration, i.e. 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 100-10X lower that obtained in cells incubated with 18 mg/L free gentamicin, with the static effect obtained for cells incubated with 1 mg/L. If considering that lysosomes may represent about 2 % of cell volume, and using accumulation data from Figure 2, this would correspond to a drug concentration in these organelles of approx. 50 mg/L (12 X MIC at pH 5.4).[a] 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 what can be obtained upon incubation with gentamicin at its Cmax. If considering that the cytosol represents about 70 % of the cell volume, this means that a concentration of 18 mg/L (18 X 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 X less potent than extracellularly. By increasing gentamicin concentration, NP compensate for this loss of potency.

[a] Calculated as (gentamicin concentration in the considered fraction [in µg/mg prot]) x (cell volume [in ml/mg prot])$^{-1}$ x % of cell volume represented by the considered fraction x Cstat [in ratio to the concentration of gentamicin in the fraction for cells incubated with 18 mg/L), with cell volume estimated to 0.005 mL/mg prot, gentamicin concentration in the considered fraction obtained from data in Figure 2, % of cell volume estimated to 2 % for lysosomes and 70 % for cytosol, and Cstat interpolated from the sigmoidal regression presented in the right panels in Figure 3 (highlighted by the vertical dotted lines)

To conclude, although 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 *Listeria monocytogenes* and those sojourning in the lysosomes, 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.

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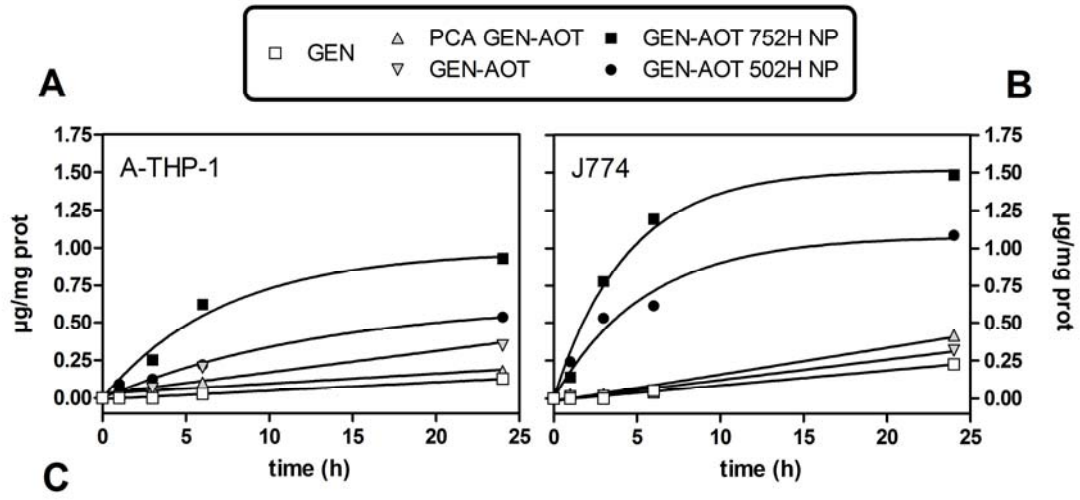
Figures:



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 NP	0.425 ± 0.209	1.919 ± 0.270
	GEN-AOT 752H NP	0.942 ± 0.047	2.983 ± 0.489
k value (0-24h period)[c]	GEN-AOT 502H NP	0.074	0.181
	GEN-AOT 752H NP	0.135	0.228

Figure 1: Accumulation kinetics of 18 mg/L of gentamicin (GEN), gentamicin-AOT (GEN-AOT) and its microstructured (PCA GEN-AOT) and encapsulated forms (502H NP and 752H NP) in A-THP-1 cells (A) and J774 cells (B) over 24 hours. Data are expressed as internalized drug micrograms per milligrams of cell protein. Lower panel (C) shows the 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 S.2 in supplemental material for accumulation factors at 24 h). [a] slope of the linear regression correlating gentamicin accumulation with time (0-24h time points); [b] slope of the linear regression correlating gentamicin accumulation with time during the linear phase of the uptake (0-3 h time points); [c] calculated from one phase exponential association (0-24h time points)

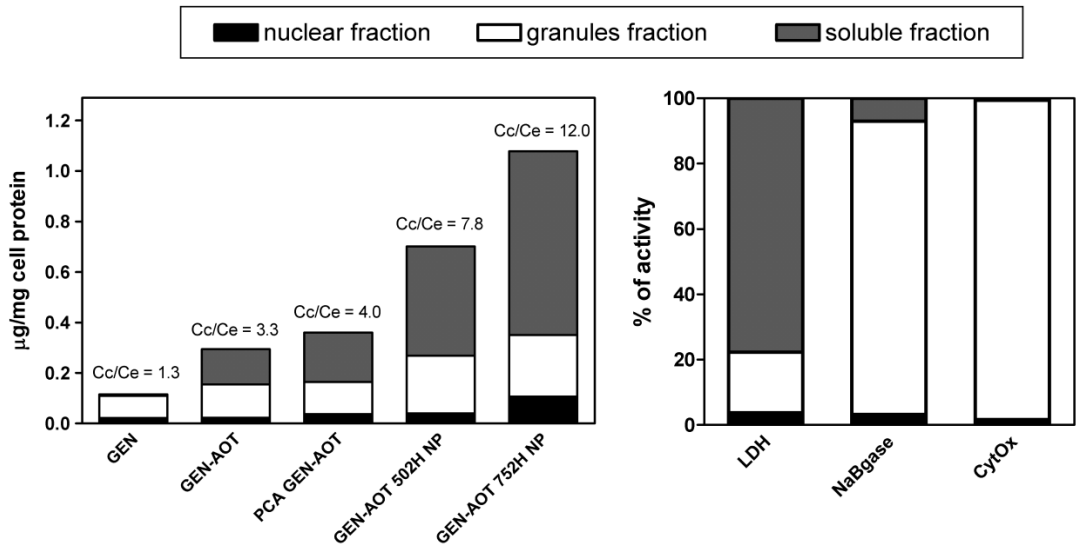


Figure 2: Subcellular distribution of gentamicin (GEN), gentamicin-AOT (GEN-AOT), microstructured gentamicin-AOT (PCA GEN-AOT) and gentamicin-AOT loaded PLGA nanoparticles (NP) in J774 cells after 24 h incubation. The left panel shows the drug content in the different fractions expressed as µg/mg of cell protein in the unfractionated homogenate. Total cellular accumulation of the drug, expressed as the cellular-to-extracellular drug concentration (Cc/Ce), is also indicated on the top of each bar. The panel on the right shows the distribution of the marker enzymes lactate dehydrogenase (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.

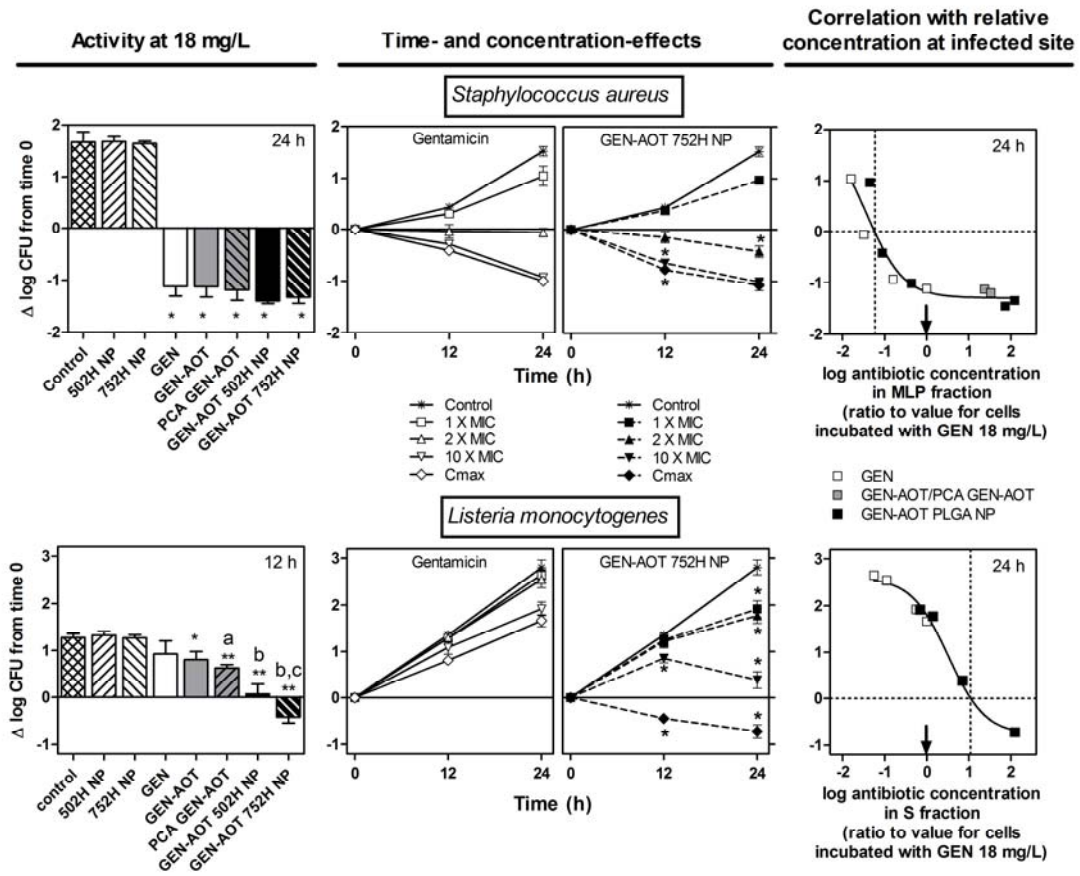


Figure 3: Activity of gentamicin and its different formulations against intracellular *S. aureus* ATCC 25923 (top) or intracellular *L. monocytogenes* strain 56 (bottom). Activity is expressed as the change in the number of cfu per mg of cell protein from the initial inoculum. Data are mean and standard deviation of at least four independent determinations.

Left panel: infected cells were exposed to a fixed concentration of 18mg/L gentamicin during 24h (*S. aureus*) or 12h (*L. monocytogenes*). Statistical analysis: * = $p < 0.05$ or ** = $p < 0.01$ when compared with the untreated control; a = $p < 0.05$ or b = $p < 0.01$ when compared with GEN; c = $p < 0.01$ when compared to GEN-AOT.

Middle panel: infected cells were exposed to increasing extracellular concentrations of free gentamicin or of GEN-AOT 752H NP during 12 or 24 h. Statistical analysis: * = $p < 0.05$ for gentamicin-AOT PLGA 752H nanoparticles when compared with gentamicin at the same concentration and exposure time conditions.

Right panel: 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 granules 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 incubation with 18 mg/L of the free drug [highlighted by the arrow]). The horizontal dotted line shows a static effect; the vertical dotted line shows the subcellular concentration needed to reach this static effect. The data were fitted to sigmoidal regressions; R^2: 0.8726 for *S. aureus* and 0.9715 for *L. monocytogenes.*

Abbreviations: 502H NP = non-loaded PLGA 502H nanoparticles, 752H NP = non-loaded PLGA 752H nanoparticles, GEN= gentamicin, GEN-AOT = gentamicin-AOT, PCA GEN-AOT = microstructured gentamicin-AOT, GEN-AOT 502H NP = gentamicin-AOT loaded PLGA 502H nanoparticles, GEN-AOT 752H NP = gentamicin-AOT loaded PLGA 752H nanoparticles.

Supplementary material:

Table S.1: Minimal inhibitory (MIC) and bactericidal (MBC) concentrations (mg/L) of gentamicin (GEN) and different gentamicin-AOT (GEN-AOT) formulations against various bacterial isolates.

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

[a]MSSA, methicillin-sensitive *Staphylococcus aureus* obtained from American Tissue Cell Collection (Manassas, VA, USA)
[b]MRSA, methicillin-resistant *Staphylococcus aureus* obtained from American Tissue Cell Collection (Manassas, VA, USA)
[c]Obtained from American Tissue Cell Collection (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)

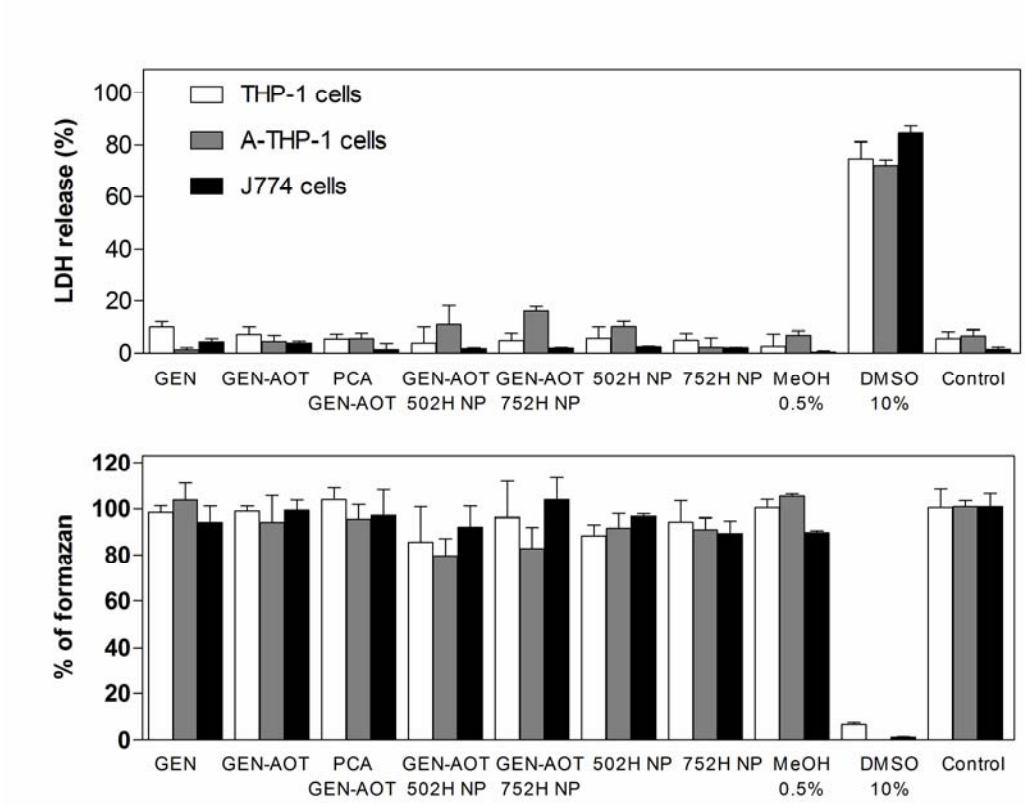


Figure S.1: Effect of 18 mg/L of gentamicin (GEN) and the different gentamicin-AOT (GEN-AOT) formulations on the viability of human THP-1 monocytes, A-THP-1 cells and murine J774 macrophages as determined by the lactate dehydrogenase (LDH) release assay (top) and MTT cytotoxicity assay (bottom) after 24 h incubation. Data are expressed as mean and standard deviations of three independent determinations. Abbreviations: GEN = gentamicin, GEN-AOT = gentamicin-AOT, PCA GEN-AOT = microstructured gentamicin-AOT, GEN-AOT 502H NP = gentamicin-AOT loaded PLGA 502H nanoparticles, GEN-AOT 752H NP = gentamicin-AOT loaded PLGA 752H nanoparticles, 502H NP = non-loaded PLGA 502H nanoparticles, 752H NP = non-loaded PLGA 752H nanoparticles, MeOH = methanol.

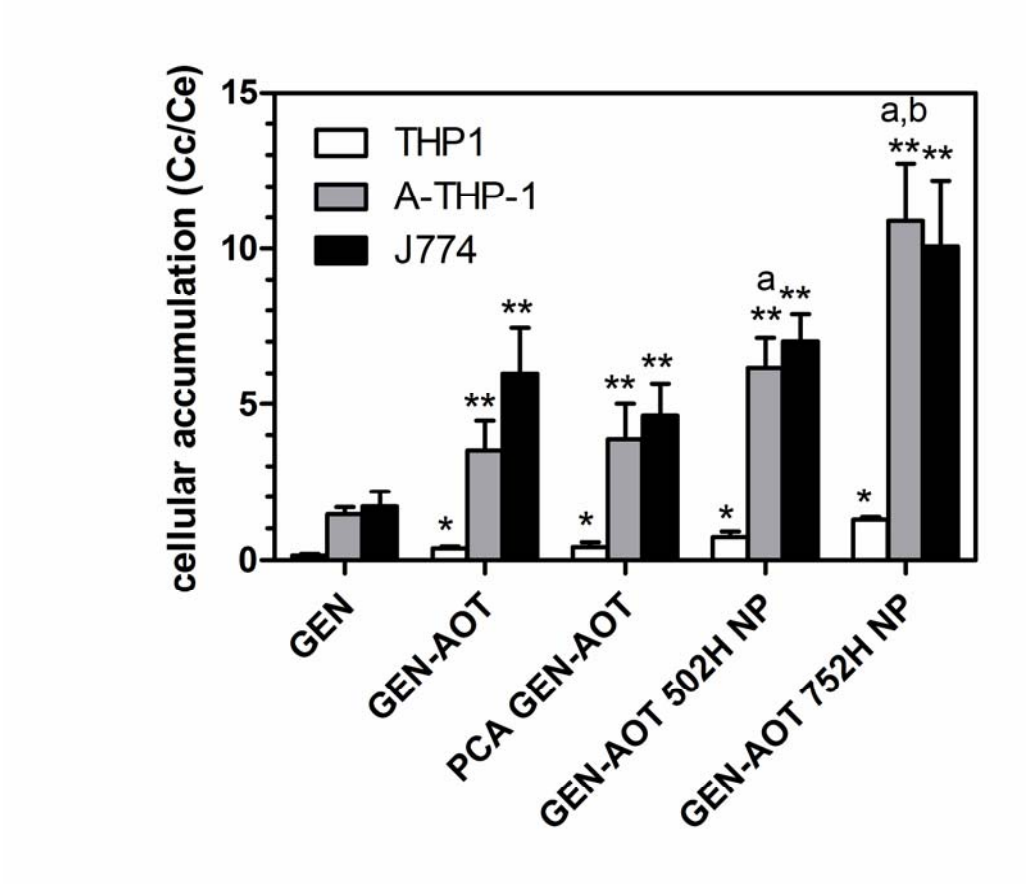


Figure S.2: Cellular accumulation of gentamicin in THP-1, A-THP-1 or J774 cells incubated during 24 h with 18 mg/L gentamicin or the formulations containing the same antibiotic concentration. Data are expressed as the mean and standard deviations of three independent determinations. Statistical analysis: * = $p < 0.05$ or ** = $p < 0.01$ when compared with gentamicin (GEN); a = $p < 0.05$ when compared with gentamicin-AOT (GEN-AOT); b = $p < 0.05$ when compared to gentamicin-AOT loaded PLGA 502H nanoparticles (GEN-AOT 502H NP); ns = no significant differences between the different cell lines for the same gentamicin treatment. Abbreviations: GEN= gentamicin, GEN-AOT= gentamicin-AOT, PCA GEN-AOT = microstructured gentamicin-AOT, GEN-AOT 502H NP = gentamicin-AOT loaded PLGA 502H nanoparticles, GEN-AOT 752H NP = gentamicin-AOT loaded PLGA 752H nanoparticles.

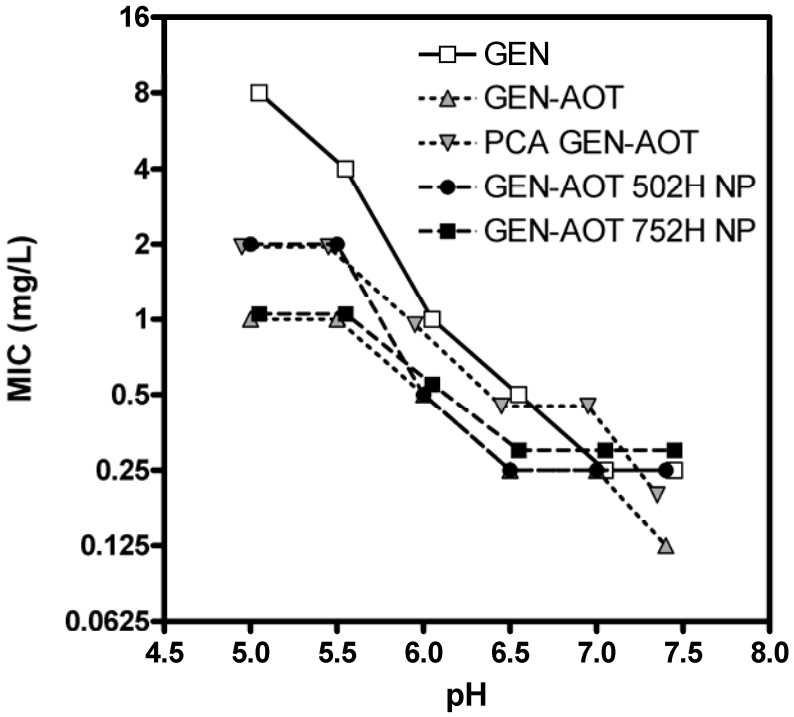


Figure S.3: Effect of pH on the minimal inhibitory concentrations (MIC) of gentamicin (GEN) and gentamicin-AOT (GEN-AOT) treatments against *Staphylococcus aureus* strain ATCC 25923. Results are expressed as the MIC in mg/L of antibiotic.