Apoptosis induced by aminoglycosides in LLC-PK1 cells: comparison between neomycin, gentamicin, amikacin, and isepamicin using electroporation.

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Running title: Apoptosis induced by aminoglycosides

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ABSTRACT

Aminoglycosides were compared for apoptosis induction (DAPI staining, activation of caspase 3) in renal LLC-PK1 cells. Amikacin caused less apoptosis than gentamicin in incubated cells. In electroporated cells, neomycin B and gentamicin caused apoptosis in the 0.06-0.1 mM range, isepamicin required larger concentrations (0.2 mM), and amikacin was without effect.
Multi-resistance in Gram-negative bacteria (1,11) and lack of truly novel compounds (24) calls for improvement of formerly established antibiotics. Aminoglycosides (7) offer possibilities in this context (16,23,26) but their nephrotoxic potential remains of concern (7). Aminoglycosides accumulate in kidney proximal tubular cells by receptor-mediated endocytosis (14,19) and trigger a sequence of alterations that include apoptosis (4,13,25). Gentamicin-induced apoptosis can be reproduced with cultured renal LLC-PK1 cells (3,4,9), either by incubating them with large drug concentrations or by electroporating them at low concentrations (20). Amikacin, which resists inactivation by several aminoglycoside-modifying enzymes (12), has been shown to cause less renal apoptosis than gentamicin in animals treated at therapeutically-relevant doses (4,8,10). In the present study, we have examined whether amikacin could also be differentiated from gentamicin for apoptosis using incubated and electroporated cells. In the latter model, we included neomycin B (a well-known nephrototoxic aminoglycoside [7]), and isepamicin (which shares many of the properties of amikacin including its lower potential of causing nephrotoxicity [13,15]).

All methods and products were as previously described (20,22), except for minor modifications (see Supplementary Material). Cell-associated aminoglycosides were measured by a microbiological technique (20; linear response for both gentamicin and amikacin [R² > 0.99]). All aminoglycosides were obtained as pure compounds (microbiological standards from the original manufacturer) or purchased from Sigma-Aldrich or Serva Fine Chemicals GmbH (Heidelberg, Germany). Gentamicin and amikacin were also obtained as the products registered for clinical use in Belgium.
All concentrations expressed as free base (see Supplemental Material for structures with molecular weights). Statistical analyses were made using GraphPad Prism® version 4.02 and GraphPad Instat® version 3.06 (GraphPad Prism Software, San Diego, CA).

Figure 1 shows data obtained with cells incubated with gentamicin or amikacin. Gentamicin (2 mM [926 mg/L]) caused a marked, time-dependant increase in the percentage of apoptotic cells (as in [5]), whereas amikacin (6 mM [3.516 g/L]) was without effect at day 1 and 2, and caused only a small increase at day 3. In parallel, gentamicin caused also marked increase in caspase-3 activity at day 1, followed by a maximum at day 2 and a decrease thereafter. Caspase-3 activity was slightly lower or similar to that of controls in cells incubated with amikacin. Apoptosis, measured after 2 days of incubation, proceeded on a concentration-dependent manner with gentamicin (0-3 mM [0-1.389 g/L]), whereas amikacin was without significant effect up to 9 mM [5.274 g/L]. Accumulation of both drugs measured at 48 h was linearly related to their extracellular concentration with slopes of 11.9 ± 0.9 nmol x mg prot.\(^{-1}\) x mM\(^{-1}\) for gentamicin and 7.68 ± 0.51 for amikacin. As a result, cells incubated with amikacin had actually a 1.9-fold larger drug molar content than those incubated with gentamicin when compared at an extracellular concentration molar ratio of 3:1 (corresponding to their most common dosage ratio in humans [gentamicin, 4 mg/kg (8.56 µmol/kg); amikacin, 15 mg/kg (25.6 µmol/kg)]). Lactate dehydrogenase release (index of necrosis [20]), remained non-significantly different from matching controls in all conditions.

In the next series of experiments, cells were electroporated in the presence of increasing concentrations of neomycin B, gentamicin, isepamicin, or amikacin. As shown in Fig. 2, neomycin B and gentamicin caused a marked increase in apoptosis for concentrations (during electroporation) spanning between 0.064 and 0.128 mM, with a maximum at 0.064 mM (39.2 mg/L) for neomycin B and at around 0.1 mM
(46.7 mg/L) for gentamicin (the bell-shaped curve of apoptosis vs. concentration is due to the development of necrosis once the concentration reaches a critical threshold; see [20] for a discussion). Isempamicin showed a considerably less marked effect and larger concentrations (between 0.192 and 0.384 mM [109-218 mg/L]). Amikacin was without effect at all concentrations tested (results similar to those described here were obtained with the clinical forms of gentamicin and amikacin; see Supplementary Material). The apparent cell concentrations in gentamicin and amikacin were determined 1 h after electroporation and were linearly related to their extracellular concentrations ($R^2 > 0.992$) but with a larger slope for amikacin compared to gentamicin ($53.3 \pm 1.7$ vs. $26.7 \pm 1.5$ nmol x mg prot. $^{-1}$ x mM$^{-1}$ [p < 0.001]; the slope for gentamicin was similar to that previously reported (20)).

The present study extends to cultured and electroporated cells our observations made in rats and which showed that amikacin induces less apoptosis than gentamicin when tested at clinically-relevant dosages (4). In our conditions of culture, LLC-PK1 cells take up aminoglycosides only slowly and to a limited extent (20,22), making necessary to use extracellular concentrations that largely exceed those observed in blood in vivo. Electroporation, a method now widely used for gene transfer and drug delivery in the cytosol of eukaryotic cells without loss of viability [6], makes possible (i) to compare drugs at more clinically-relevant concentrations (the percentage of apoptotic cells being already about 7-fold larger than in controls for a gentamicin concentration as low as 0.03 mM [approx. 14 mg/L]); (ii) to confirm the low apoptogenic potential of amikacin in comparison with gentamicin, while demonstrating that it is not related to a lower drug accumulation. The common behaviors of neomycin B and gentamicin, on the one hand, and of amikacin and iseMPamicin on the other hand suggest specific interactions of these drugs with those intracellular constituents susceptible to trigger apoptosis (18,20,22). These should be further explored though systematic structure-activity relationship studies, but it
already appears that the number of ionisable groups (and perhaps also their position) could be critical (see Supplementary Material).

Apoptosis is an established mechanism of renal drug-induced toxicity [21] that develops at lower dosages than necrosis (2,4,17). Although the renal toxicity of aminoglycosides may involve other mechanisms than apoptosis (7,21) making clinically-pertinent drug ranking quite complex, the method developed here may help in further refining our approach towards the selection of safer derivatives. Generally speaking, it may also prove useful for the study of other drugs which, under normal conditions, would only reach slowly or poorly their intracellular pharmacological or toxicological target.
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References


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Figure 1

- Top graph: Percentage of apoptotic cells (DAPI+) over time (h) for different concentrations of GEN (2 mM) and AMK (6 mM) compared to controls.
- Middle graph: Caspase-3 activity (% of control) over time (h) for GEN (2 mM) and AMK (6 mM) compared to controls.
- Bottom graph: Percentage of apoptotic cells (DAPI+) at different aminoglycoside concentrations (GEN and AMK) over time (h).
Apoptosis (upper and lower panels) and increase of caspase-3 activity (middle panel) in LLC-PK1 cells incubated in the absence of aminoglycoside (open squares) or in the presence of gentamicin (closed squares) or amikacin (open triangles). Upper panel: cells were incubated for up to 3 days without or with gentamicin 2 mM (0.926 g/L) or amikacin 6 mM (3.51 g/L) and the percentage of apoptotic nuclei determined by microscopic examination after DAPI staining (camptothecin 14.3 µM [5 mg/L] was used as positive controls and yielded values of 43.1 ± 1.8, 40.8 ± 2.2, and 48.7 ± 2.8 at 24, 48 and 72h, respectively). Middle panel: same conditions of incubation as in the upper panel. Caspase-3 activity was assayed Ac-DEVD-AMC (See Supplementary Material). Cells incubated with camptothecin (known to cause massive apoptosis in LLC-PK1 cells [22]) yielded values of 2767 ± 213, 653 ± 41, and 150 ± 35 % of control values at 24, 48, and 72 h, respectively. Lower panel: cells were incubated for 48 h without aminoglycoside or with gentamicin (1-3 mM; 0.463-1.39 g/L) or amikacin (3-9 mM; 1.76-5.26 g/L). All values are means ± SD (n = 3). Statistical analysis (two-tailed ANOVA) for differences between treated cells and matched controls (upper and middle panels) or between cells incubated with and without aminoglycoside (lower panel) : *, p < 0.05; **, p < 0.01; ***, p < 0.001. All comparisons between gentamicin and amikacin are made at a 1:3 molar ratio to correspond to the daily dosage ratios of these drugs for common therapeutic applications (see text).
Figure 2

Apoptosis in electroporated cells. Cells were electroporated in the absence (controls) or in the presence of neomycin B, gentamicin, isepamicin, or amikacin, returned to aminoglycoside-free medium, and apoptotic nuclei enumerated 24 h later. Values are means ± SD (n=3). Statistical analysis (two-tailed ANOVA; p < 0.01): all values for neomycin B and gentamicin, except those observed for the largest concentration tested (0.256 mM), are significantly different from controls; isepamicin: values observed for 0.192, 0.288, and 0.384 mM are significantly different from controls; amikacin: no difference from controls. 0.12 mM corresponds to approx. 74 mg/L for neomycin B, 56 mg/L for gentamicin (taking into account the respective contents of the commercial gentamicin in C1, C1a and C2 components, 68 mg/L for isepamicin, and 70 mg/L for amikacin. See supplementary material for structures of tested compounds.