Gentamicin (GEN) causes apoptosis at low concentrations in LLC-PK1 cells subjected to electroporation (EP).

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ABSTRACT

BACKGROUND: GEN accumulates in lysosomes ok kid.prox.tub.cells by recept.-mediated endocytosis and causes apoptosis (APO) in rats treated with clin.-relevant doses (AAC 44:665-75, 2000). GEN-induced APO can be reproduced with cult. Cells, but large extracell. Conc. (1-3 mM/0.4-1.2g/L) are needed, presumably because of low endocytic activity (Toxicol. Sci. 56:229-239, 2000). In LLC-PK1, GEN-induced APO involves a permeabilization of lysosomes (GEN release in the cytosol?), followed by mitochondrial pathway and caspase activation (Toxicol Appl Pharmacol E-pub 19 Feb. 2005). EP allows direct access to cytosol for membrane-impermeant drugs. We, therefore, examined wether EP would not sensitize cells to GEN-induced APO.

METHODS: EP: Cells were subjected to 8 pulses (1msec each) at 800 V/cm (sq. waves) in the presence of GEN (3 µM-3 mM [1.2 mg/L-1.2 g/L]), returned to drug-free medium, and examined after 8 h for Bax content (marker of mitochondrial pathway activation [western blot] and after 24 h for APO (DAPI staining) and necrosis (LDH release). In parallel, non-electroporated cells (NEP) were maintained in the cont.presence of GEN for 8 and 24 h. All exper. Were performed in triplicate.

RESULTS:

EP-treated cells showed signif. Incr. of Bax (8 h) at GEN 30 µM, whereas inc. at 1 mM for 8 hours was required for NEP to show Bax incr. APO and necrosis developped as follows (*P<0.05 EP vs NEP):

Parameter	Treat.	GEN concentr. (mM)				
		0	0.003	0.03	0.3	3
Apoptosis (% of cells)	EP	4.0±0.1	5.7±0.1	15.4±1.1*	19.1±1.8*	7.9±0.6*
	NEP	3.5±0.8	3.5±1.0	3.4±1.1	2.6±1.8	10.9±0.6*
Necrosis (% of cells)	EP	8.3±4.6	9.5±13.4	9.4±4.0	27.9±9.0*	69.5±5.6*
	NEP	1.9±2.7	1.8±2.1	4.0±0.8*	2.0±2.8	0±1.4

CONCLUSIONS: EP triggers APO at GEN conc. About 100-fold lower than in cells incubated with GEN, suggesting that GEN delivery to cytosol is the cause of apoptosis. In NEP-cells, lysosomal seqsuestration (through endocytosis) may actually protect them from the direct, toxic effect of GEN).

INTRODUCTION

Aminoglycoside antibiotics cause acute renal failure in patients, associated with histological and functional signs of proximal tubules toxicity. The underlying molecular mechanisms remain, however, poorly defined (Gilbert, 2005).

Aminoglycosides enter proximal tubular cells by pinocytosis from the luminal pole and accumulate in lysosomes where they cause a conspicuous phsopholipidosis (Tulkens, 1986).

Besides lysosomal changes, however, proximal tubules of animals treated with gentamicin also show clear signs of apoptosis (El Mouedden et al., 2000a). This can be reproduced in vitro with both renal and non-renal cells, which, however, must be exposed to large concentrations of gentamicin because of inefficient drug uptake (El Mouedden et al., 2000b).

Using LLC-PK1 cells, we observed that gentamicin causes an early permeabilization of lysosomes before activation of the mitochondrial pathway of apoptosis can be detected (Servais et al., 2005). This raised the question as to whether the lysosomal sequestration of gentamicin would not actually protect cells from its apoptogenic effect.

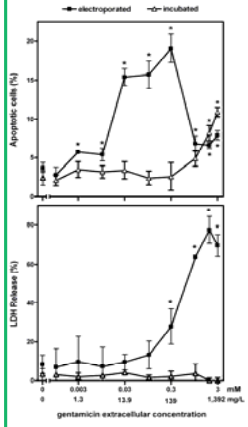
AIM OF THE STUDY

- To assess if the cytosolic delivery of GEN by electroporation makes cells more susceptible to develop apoptosis than if incubated with the drug.

- To analyze if the pro-apoptotic Bax protein, present in the cytosol and acting upstream the mitochondrial pathway, is involved GEN-induced apoptosis.

RESULTS

Development of apoptosis and necrosis as a function of GEN concentration in electroporated vs. incubated cells

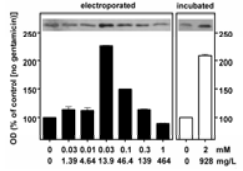


EP of GEN triggers apoptosis for GEN concentrations **100-fold lower** than those required for cells incubated with GEN

GEN triggers necrosis in electroporated cells as from 300 µM, while not affecting incubated cells

Fig.1: Percentage of apoptotic cells (upper panel) (assessed by DAPI staining) and necrotic cells (lower panel) (assessed by LDH release) for EP cells (dark squares) and incubated cells (open triangles).

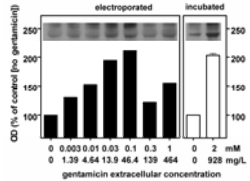
Involvement of Bax in GEN-induced apoptosis after electroporation or incubation.



GEN-induced apoptosis is associated with an increase in Bax cell content both in electroporated and incubated cells

Fig.3: Detection of Bax by western blot analysis in LLC-PK1 cells lysates. EP cells were subjected to electroporation in the presence of GEN and returned to drug-free medium for 8 h at 37°C before being collected and processed. Incubated: cells were maintained at 37°C for 8 h in the presence of GEN.

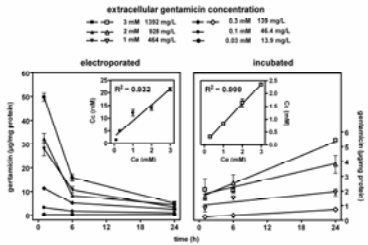
Increase of ubiquitinated forms of Bax in GEN-induced apoptosis after electroporation or incubation.



Bax accumulation in cells electroporated or incubated with GEN is associated with an increase of its ubiquitinated forms (normally processed by the proteasome)

Fig.4: Detection of ubiquitinated-Bax by western blot analysis in LLC-PK1 cells lysates. EP cells were subjected to electroporation in the presence of GEN and returned to drug-free medium for 8 h at 37°C before being collected and processed. Incubated cells were maintained at 37°C for 8 h with GEN.

Cellular GEN content in electroporated vs. incubated cells.



Cells electroporated with GEN show a high and immediate accumulation of GEN (proportional to the extracellular GEN content (ratio ~ 6). The cellular concentration decrease thereafter rapidly.

For cells incubated with GEN, the uptake of GEN proceeds almost linearily with time with a stable clearance of 4.44 µl/mg protein/24H (typical value for fluid phase pinocytosis).

Fig.2: Cellular content of GEN as function of time and extracellular GEN concentration.
For EP, cells were electroporated with increasing concentrations and returned to drug free medium up to 24h (left panel), The incubated cells (right panel) were left in continuous contact of GEN up to 24 h at 37°C. The insets in both panels show the relation between extracellular and cellular content of GEN after 1h (for EP) and after 24h for incubated cells.

METHODS

Treatment of cells:

Electroporation: Subconfluent cells were detached by trypsinization, centrifuged at 1000 rpm and resuspended in electroporation buffer (10 mM phosphate buffer pH 7.2, 250 mM sucrose and 1 mM $MgCl_2$), in the presence of increasing GEN concentrations. Cells were exposed to 8 pulses (square waves) at 800V/cm during 1 msec (with 1 Hz repetition frequency) and left thereafter 15 min at room temperature in the same medium. They were then dispersed in drug-free DMEM for appropriate times.

Incubation: Cells were incubated in DMEM containing increasing concentrations of GEN during appropriate times.

Quantification of apoptosis and necrosis: The percentage of apoptotic cells was determined by enumeration in the optic microscope after nuclear staining with DAPI. Necrosis was assessed by measuring the amount of lactate deshydrogenase (LDH) released in the medium.

Measurement of GEN content: GEN content was assessed by a disc-plate microbiological technique using *Bacillus subtilis* (ATCC 6633) as test organism.

Detection of Bax: This was performed by western blot analysis.

Detection of ubiquitinated Bax: Bax protein was immunoprecipitated with anti-Bax antibody overnight at 4°C. Protein-A agarose beads were then added and incubated at 4°C for 2 hours. Immunoprecipitated Bax was then analysed by western blot using a primary antibody directed against ubiquitinated proteins.

CONCLUSIONS

- GEN induces apoptosis at much lower extracellular concentrations (about 100 times lower; 30µM) in electroporated cells compared to cells incubated with this antibiotic.

- Induction of apoptosis by GEN (after electroporation as well as after incubation with GEN) involves the pro-apoptotic Bax protein. This increase of Bax is associated with an increase in its ubiquitinated forms, suggesting a decrease of its degradation by the proteasome.

- Lysosomal sequestration could actually not trigger cell toxicity, but rather protect cells from the apoptogenic effect of GEN. If delivered into the cytosol, GEN would precipitate cell death by apoptosis (or necrosis at large concentrations).

REFERENCES

- Gilbert DN (2005) Aminoglycosides, in *Principles and Practice of Infectious Diseases* (Mandell GL, Bennet J and Dolin R eds) pp 328-356, Elsevier/Churchill Livingstone, Philadelphia.
- El Mouedden M *et al*., 2000a. *Antimicrob Agents Chemother* **44**: 665-675.
- El Mouedden M *et al*., 2000b *Toxicol Sci* **56**: 229-239.
- Servais, H *et al*., 2005 *Toxicol.Appl.Pharmacol.*, **206**: 321-333.
- Tulkens, P.M., 1986 *Am J Med* **80**:105-114.