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Subcellular mechanisms involved in apoptosis induced by aminoglycoside antibiotics: Insights on p53, proteasome and endoplasmic reticulum

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ABSTRACT

Gentamicin, an aminoglycoside used to treat severe bacterial infections, may cause acute renal failure. In the renal cell line LLC-PK1, gentamicin accumulates in lysosomes, induces alterations of their permeability, and triggers the mitochondrial pathway of apoptosis via activation of caspase-9 and -3 and changes in Bcl-2 family proteins. Early ROS production in lysosomes has been associated with gentamicin induced lysosomal membrane permeabilization. In order to better understand the multiple interconnected pathways of gentamicin-induced apoptosis and ensuing renal cell toxicity, we investigated the effect of gentamicin on p53 and p21 levels. We also studied the potential effect of gentamicin on proteasome by measuring the chymotrypsin-, trypsin- and caspase-like activities, and on endoplasmic reticulum by determining phopho-eIF2α, caspase-12 activation and GRP78 and 94. We observed an increase in p53 levels, which was dependent on ROS production. Accumulation of p53 resulted in accumulation of p21 and of phospho-eIF2α. These effects could be related to an impairment of proteasome as we demonstrated an inhibition of trypsin-and caspase-like activities. Moderate endoplasmic reticulum stress could also participate to cellular toxicity induced by gentamicin, with activation of caspase-12 without change in GRP74 and GRP98. All together, these data provide new mechanistic insights into the apoptosis induced by aminoglycoside antibiotics on renal cell lines.

1. Introduction

Gentamicin is a commonly used aminoglycoside antibiotic for the treatment of serious and life-threatening infections caused by Gram-negative aerobes. Although displaying desirable properties such as rapid and high bactericidal activity, low cost and controlled levels of resistance, nephrotoxicity induced by aminoglycosides remain a serious limiting factor in their daily use. The mechanisms of aminoglycoside-induced nephrotoxicity have been extensively studied for many years (Mingeot-Leclercq and Tulkens, 1999; Quiros et al., 2011; Lopez-Novoa et al., 2011), but remain incompletely deciphered. Unraveling these mechanisms at the cellular level might open new avenues for decreasing their toxicity.

The observation that aminoglycosides induce apoptosis in vivo at therapeutically-relevant doses (El Mouedden et al., 2000a) was an important step in our appreciation of the importance of the early stages in cell dysfunction leading to nephrotoxicity. Apoptosis, detected in proximal tubules of rats treated with low therapeutic doses of gentamicin, can be reproduced in the porcine proximal tubular cell line LLC-PK1 (El Mouedden et al., 2000b), where gentamicin accumulates in lysosomes following adsorptive and/or receptor-mediated endocytosis (Sastrasinh et al., 1982; Moestrup et al., 1995). Within these subcellular organites, vital confocal imaging revealed that gentamicin induces ROS production (Denamur et al., 2011). ROS production appears prior to, and at lower drug concentrations than what is required for lysosomal permeabilization, which is observed only 6 h after incubation of cells with gentamicin (Servais et al., 2006; Servais et al., 2005). ROS antioxidants or scavengers, such as catalase or *N*-acetylcysteine, largely prevent these events (Denamur et al., 2011). Deferoxamine, an iron chelator, which is endocytosed and accumulates in lysosomes, also largely reduces gentamicin-induced ROS production as well as apoptosis (Denamur et al., 2011). After lysosomal membrane permeabilisation, the intrinsic pathway of apoptosis is then activated with disruption of mitochondrial membrane potential, release of cytochrome *c* and activation of caspase-3 (Servais et al., 2005). Bcl-2 and Bax are involved in this process since Bcl-2 overexpression (through transfection) prevents apoptosis-induced by gentamicin (El Mouedden et al., 2000b) and significant increases of the cellular levels of Bax and ubiquinitated Bax (without changes in Bax mRNA) (Servais et al., 2005; Servais et al., 2006) were observed.

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The link between lysosomal membrane permeabilization and activation of the mitochondrial intrinsic apoptotic pathway has been the object of much attention. In the past few years, lysosomes have been identified as important actors of cell death induction (see (Kirkegaard and Jaattela, 2009; Villamil Giraldo et al., 2014) for review), mainly via the release of lysosomal proteases such as cathepsins. Beside a potential role of the release of proteases, gentamicin-induced apoptosis probably involves other ways of interaction between lysosomes and mitochondria. Indeed, electroporation of cells in the presence of very low concentrations of gentamicin (allowing the drug to directly reach the cytoplasm and avoiding the need of lysosomal membrane permeabilization) induces a marked increase of Bax and ubiquinitated Bax as well as apoptosis (Servais et al., 2006). This suggests that gentamicin, released from lysosomes to cytosol after lysosomal membrane permeabilization or moving by retrograde trafficking through the Golgi apparatus and the endoplasmic reticulum (Sandoval and Molitoris, 2004), could play an important role in the apoptotic process. Direct interactions of gentamicin with mitochondria has been hypothesized (Mather and Rottenberg, 2001), but other organelles as proteasome or endoplasmic reticulum could also play a critical role, as suggested in a variety of models ranging from in vitro to in vivo experiments (Servais et al., 2006; Peyrou and Cribb, 2007; Peyrou et al., 2007; Horibe et al., 2004).

A possible inhibition of the ubiquitin-proteasome pathway by gentamicin has been suggested based on our previous results. We indeed observed an increase of the cytosolic protein Bax together with its ubiquitinated form, upon incubation of cells with gentamicin while competitive RT-PCR failed to reveal significant changes in the cell content of Bax mRNA (Servais et al., 2006). Moreover, since H_2O_2 has been linked to 26S proteasome disassembly (Wang et al., 2010), gentamicin-induced ROS production (Denamur et al., 2011) could modulate proteasome activities. Such effect could trigger alterations of endoplasmic reticulum since proteasome is involved in part of the endoplasmic reticulum-associated machinery for protein degradation that removes unfolded and misfolded proteins from endoplasmic reticulum (Oyadomari et al., 2006). In turn, this could induce eIF2α phosphorylation as a cellular stress response against proteasome inhibition (Jiang and Wek, 2005; Baird and Wek, 2012) and accumulation of p53, a transcription factor often involved in apoptosis induced by nephrotoxicants (Molitoris et al., 2009) and normally kept at low concentration by the proteasome (Zhang et al., 2011; Pant and Lozano, 2014). An increase of p53 levels induces expression of its downstream genes involved in diverse cellular stress responses including cell cycle arrest via the cell cycle inhibitor p21 (An et al., 2000), DNA damage response (Meek, 2015) or apoptosis (Dashzeveg and Yoshida, 2015; Meek, 2015). Another critical actor for cell cycle arrest, and apoptosis are the ceramides which may interplay (Sawada et al., 2001; Kim et al., 2002; El Assaad et al., 2003) or not (Yang and Duerksen-Hughes, 2001; Kim et al., 2000) with p53.

Our study was designed to further decipher the cellular and molecular mechanisms involved in gentamicin-induced apoptosis. We first considered a possible role of p53 as well as sphingomyelinase pathway in apoptosis induced by gentamicin. The importance of ROS for changes in p53 was investigated. Second, we studied the inhibition of proteasome activity induced by gentamicin by following the chymotrypsin-, trypsin- and caspase-like activities of proteasome. Then, we investigated the potential effect of gentamicin on endoplasmic reticulum by looking for phosphorylation of eIF2α, caspase-12 activation and up-regulation of the chaperones GRP78 and GRP94. Collectively, all these results have been integrated for giving new insights about the molecular and cellular mechanisms involved in aminoglycoside antibiotics induced apoptosis.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and trypsin-EDTA were purchased from Life Technologies, Paisley, UK. Gentamicin sulfate (Gentalline®) was from Schering-Plough (Merck, Whitehouse Station, NJ, USA). Proteasome substrates Suc-LLVY-amc, Ac-RLR-amc and Ac-nLPnLD-amc were purchased from Bachem (Bachem AG, Bubendorf, Switzerland). Tunicamycin, cisplatin, epoxomicin, nocodazole, ribonuclease A, propidium iodide, *N*-acetylcystein and glucosamine hydrochloride were from Sigma Aldrich (St-Louis, MO, USA). 4′, 6′-diamidine-2′-phenylindole (DAPI) was from Roche (Basel, Switzerland).

2.2. Cells and treatments

All experiments were performed with LLC-PK1 (Lilly Laboratories Culture-Pig Kidney Type 1) cells obtained from American Tissue Culture Collection (LGC Promochem, Teddington, UK) as ATCC CL-101. Cultures were grown in Dubelcco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in an atmosphere of 95% air - 5% CO_2. Cells were subcultured twice a week and used when reaching approximately 80% of confluence. All solutions for incubation were adjusted to pH 7.4 prior being added to the culture medium or to lysis buffers.

2.3. Short interference RNA and transfection

p53 protein expression was knocked down using p53 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Transient transfections were performed when the cells had reached 60% confluence in a 6 well-plate using Lipofectamine® RNAiMAX Reagent (Invitrogen; Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Briefly, cells were first washed with phosphate-buffered saline (PBS) and cultured with fresh Opti-MEM Medium (Invitrogen, Carlsbad, CA, USA). A mix of transfection reagent and 60 pmol of p53 siRNA was then added to the wells. Mock groups were treated with the transfection reagent only. Twenty four hours after transfection, cells were treated, or not, with gentamicin 3 mM for 24 h. To assess gene silencing, p53 protein levels were evaluated by Western Blot and cell apoptosis was assessed by DAPI labeling.

2.4. Western blot analysis

p21 and p27, phosphorylated eIF2α, ER chaperones GRP78 and 94, and caspase-12 were analyzed by western blot analysis. After incubation for 24 h with gentamicin, tunicamycin (as positive control of eIF2α phosphorylation and ER stress (Peyrou and Cribb, 2007)), cisplatin (as positive control of caspase 12 activation and p21 overexpression (Liu and Baliga, 2005)) or epoxomicin (as positive control of inhibition of chymotrypsin- and caspase-like activities of proteasome (Giguere and Schnellmann, 2008), supernatant was kept. Cells were washed with PBS and therafter detached by trypsinization, pelleted by centrifugation at 290*g* for 7 min and washed two times with gentle resuspension and repelleting in ice-cold PBS. Cells were then resuspended in RIPA buffer (Tris-HCl 25 mM, pH 7.6; NaCl 150 mM, NP-40 1%; SDS 0.1%; sodium deoxycholate 1%) supplemented with protease and phosphatase inhibitor cocktail, lysed by sonication, and centrifuged for 15 min at 14,000 g and 4 °C. The protein content of cellular lysates was assessed using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Western blots were performed using the NuPAGE electrophoresis system (Invitrogen, Paisley, UK). Appropriate quantities of proteins were mixed to 4× NuPAGE LDS sample buffer and 10× NuPAGE reducing agent, then heated at 70 °C for 10 min. Samples were loaded on acrylamide gels (NuPAGE 1% Bis-Tris Gel, Invitrogen). After migration, proteins were electro-transferred on a PVDF membrane (0.45 μM, Pierce), which was blocked by a 1 h incubation with bovine serum albumin (BSA) 5% in Tris-buffered saline (TBS; 20 mM Tris-HCl, 500 mM NaCl pH 7.5). Membranes were then incubated for 2 h at room temperature with the primary antibodies anti-ubiquitin (sc-47,721, Santa Cruz Biotechnologies, Santa Cruz), anti-p21 (sc-397, Santa Cruz Biotechnologies), anti-GRP78 (76-E6) (sc-13,539, Santa Cruz Biotechnologies),

anti-GRP94 (SPA-850F, Stressgen, Enzo Life Sciences, Farmingdale, NY, USA) and anti-caspase-12 (2202, Cell Signaling Technology) or overnight at 4 °C with anti-eIF2α (2103, Cell Signaling Technology) or anti-phosphorylated eIF2α antibodies (3597, Cell Signaling Technology, Danvers, MA, USA), to detect ubiquitin, p21, GRP78, GRP94, caspase-12, and eiF2α and its phosphorylated form, respectively. Then, membranes were exposed to appropriate horseradish peroxidase-coupled secondary antibodies for 1 h. Blots were revealed by chemiluminescence (SuperSignal West Pico, Pierce). Membranes were then washed with Restore Western Blot Stripping Buffer (Pierce) for 30 min, and anti-actin polyclonal antibodies (sc-1616, Santa Cruz Biotechnologies) were used as loading control.

Given the failure to detect p53 protein by this procedure, the protocol was adapted for p53 western blot as followed. Cells were resuspended in a lysis buffer (Tris-HCl 50 mM, pH 7.6; DTT 2 mM; EDTA 1 mM; EGTA 1 mM; β-glycerophosphate 1 mM; NaF 10 mM; NP-40 0.5%, glycerol 20%; p-toluenesulfonyl fluoride 1 mM; benzamidin 5 mM; sodium orthovanadate 1 mM, leupeptin 5 μg/ml; antipain 5 μg/ml), kept for 20 min on ice and centrifuged for 10 min at 18,000 g and 4 °C. The protein content of cellular lysates was assessed using the Bradford method (Pierce, Rockford, IL). Before migration, samples were boiled (100 °C) for 5 min in Laemmli buffer containing SDS 8%, Tris 125 mM (pH 6.8), sucrose 40% (w/v), bromophenol blue 0.03% (w/v) and DTT 40 mM. Migration of proteins (50 μg) was made on 12% acrylamide gels at 200 V for 1 h. Semi-dry transfer on PVDF membrane (1 h 15 min, 100 mA/gel) was followed by blocking and marking steps as described above. Anti-p53 (DO-1) antibody (sc-126, Santa Cruz Biotechnologies) was used to detect p53. For investigating the potential role of ROS in p53 expression, cells were preincubated for 3 h with medium containing *N*-acetylcysteine (1 mM) which is kept during incubation with gentamicin.

2.5. Proteasome activity assay

Chymotrypsin-, trypsin-, and caspase-like activities of proteasome were evaluated by generation of the fluorescent species amino-methyl-coumarin (amc) from peptides substrates of each proteasome hydrolytic activity (Kisselev and Goldberg, 2005). Chymotrypsin-like activity was assessed with Suc-LLVY-amc, trypsin-like activity with Ac-RLR-AMC and caspase-like activity with Ac-nLPnLD-amc.

Cells were scrapped in extraction buffer containing Tris-HCl 10 mM pH 7.5, 1 mM EDTA, 2 mM ATP, 5 mM DTT, 20% (V/V) glycerol. Samples were sonicated for 10 s and centrifuged for 15 min at 15,000 g and 4 °C. Supernatants were collected and protein contents measured using the Bradford's method. Assays were performed in polypropylene black 96-well plates, to avoid proteasome adsorption. Adequate quantity of proteins (25 μg for chymotrypsin-like, 10 μg for trypsin-like, and 100 μg for caspase-like activity assay) were incubated for 1 h at 37 °C in extraction buffer containing the indicated concentrations of gentamicin or epoxomicin (a proteasome inhibitor used as positive control (Giguere and Schnellmann, 2008)). Fifty microliter of reaction buffer (Tris-HCl 50 mM pH 7.4, EDTA 0.5 mM) containing suitable substrate concentrations (100 μM for Suc-LLVY-amc, 200 μM for Ac-RLR-amc and 100 μM for Ac-nLPnLD-amc) were then added in the wells, and the fluorescence was read immediately and after 4 h incubation at 37 °C with a FluoroCount Microplate Fluorometer (Packard Instruments Company, Downers Grove, IL, USA) with excitation wavelength set at 380 nm and readings made at 460 nm. We checked that neither gentamicin nor epoxomicin interfered with AMC fluorescence.

2.6. Counting of apoptotic cells

Apoptotic nuclear fragmentation, revealed by DNA staining with 4′,6′-diamidine-2′-phenylindole (DAPI) (Servais et al., 2006), was identified by random counting of 500 cells per condition. Clusters of apoptotic bodies were given a single count. Data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

2.7. Measurement of intracellular ceramide levels

The intracellular levels of ceramides after incubation of LLC-PK1 with gentamicin (1 and 2 mM) for 15 min up to 24 h were measured as described previously (Fillet et al., 2003) using liquid chromatography-electrospray ionization tandem-mass spectrometry (LC-ESI-MS/MS). Ceramides were separated by liquid chromatography on a C18 column and eluted using a solvent gradient made of a mixture of water, acetonitrile and 2-propanol. MS detection was carried out using a Ultima triple quadrupole instrument (Waters, Manchester, UK) configured with a electrospray ionization source used in positive ion mode. Collision-induced fragmentations conducted on ceramides produced a well characteristic product ion at *m/z* 264, making multiple reactions monitoring (MRM) well suited for various ceramides quantitative measurements (Fillet et al., 2002).

For LC-ESI-MS/MS quantitative purpose, standard solutions were prepared by dissolving ceramides (Acros Organics, Gell, Belgium) in a 99.8/0.2 (v/v) mixture of ethanol/formic acid to reach a concentration of 1 μg/ml. For extraction of cellular lipids, cells were rinsed twice with ice-cold PBS, then scraped in PBS and centrifuged at 800*g*. The resulting pellet was homogenized in distilled water by sonication. An aliquot of the cell homogenate was reserved for determination of protein levels. Ten nanogram of C12-ceramide (as an internal standard) was added to samples of cell lysates containing 300 μg of protein. Lipids were extracted using Folch's partition with a mixture of chloroform and methanol (2/1, v/v). Samples were then centrifuged at 1500*g* and washed with chloroform, methanol and water (5/48/47, v/v/v). The organic phase was evaporated to near dryness under a gentle stream of dry nitrogen. The samples were reconstituted by vortexing with 100 μl of a mixture of ethanol/formic acid (99.8/0.2) until they were completely dissolved. To avoid any loss of lipids, the entire procedure was performed in siliconized glassware.

The analysis showed that C16-, C18-, C20-, C22- and C24-ceramides were present in our cell model. The levels of each ceramide species were expressed as their relative abundance compared with ceramide level in untreated cells. All determinations have been done on cells incubated with gentamicin in conditions where apoptosis was described.

2.8. Acidic and neutral sphingomyelinase assays

Apart the production of ceramide by de novo synthesis through the sequential activation of serine-palmitoyl transferase, ceramide synthetase, and dehydro-ceramide desaturase (Hannun and Luberto, 2000; Zeidan and Hannun, 2010), ceramides can also arise from the hydrolysis of sphingomyelin by the activation of the two major known types of sphingomyelinases, the acidic sphingmomyelinase with an optimum pH close to 4.8 (Goni and Alonso, 2002) and the neutral sphingomyelinase which exhibits a pH optimum around 7.5 and is a membrane-bound and Mg^{2+}-dependent (Kitatani et al., 2008) enzyme. Briefly, the activities of neutral- and acidic sphingomyelinases were determined as follows. After culture, cell pellets were suspended in distilled water and sonicated (3 × 15 s). Cell lysates were incubated at 37 °C for the indicated periods with sphingomyelin and [*N*-methyl^{14}-C]-sphingomyelin. For the determination of the activity of acidic sphingomyelinase (EC 3.1.4.12), the reaction was performed in acetate buffer 200 mM, pH 5.5 in presence of EDTA (4 mM), taurocholate de sodium (1%), and Triton X-100 (1%). For the measure of the activity of neutral sphingomyelinase, Tris buffer 100 mM, pH 7.5 containing dithiothreitol (10 mM), Triton X-100 (1%), and $MgCl_2$ (10 mM) was used. After incubation, serum bovine albumin and TCA were added. The mixture was placed at 0 °C for 30 min before centrifugation. The radioactivity associated to the supernatant was counted.

2.9. Gentamicin dosage

Gentamicin concentrations were measured by microbiological assay. *Bacillus subtilis* ATCC 6633 was used as the test organism (lowest limit of detection, 1 μg/ml; linearity [$R^2 = 0.934$], concentrations up to 256 μg/ml) and antibiotic medium #11 was adjusted at pH 8 (Carryn et al., 2002).

2.10. Statistical analysis

All statistical analyses were performed with the GraphPad Prism 6.0 software and GraphPad InStat 3.06 (GraphPad software, San Diego, CA, USA). The comparison of 3 or more groups of data was performed using the one- or two-way ANOVA followed by the Tukey's post hoc test to compare values to control, and with Sidak's post hoc test to compare all values.

3. Results

3.1. p53 pathway is involved in gentamicin-induced apoptosis

3.1.1. Gentamicin increased p53 levels. p53 is known to play a critical role in renal cell apoptosis (Jiang et al., 2004) including when induced by known nephrotoxicants such as cisplatin (Sanchez-Perez et al., 2010). To determine if gentamicin-induced apoptosis was also dependent on p53, we first measured the effect of gentamicin on p53 levels (Fig. 1).

After incubation for 8 h and 24 h with 2 and 3 mM of gentamicin, an increase of p53 was observed reaching around 1.7 and 3.7 fold increase respectively, as compared to controls. The increase was similar after 8 and 24 h of incubation. No statistically significant increase was observed when cells were incubated with gentamicin 1 mM. For investigating the potential downstream effect of DNA damage, we also measured the cellular levels of phosphoSer15-p53. No statistically-significant change was observed whatever the concentration of gentamicin and the time of incubation used (data not shown).

3.1.2. Role of p53 in apoptosis induced by gentamicin: effect of p53 inhibitor and of p53 siRNA. To confirm the role of p53 in apoptosis induced by gentamicin, cells were incubated with pifithrin- α, an inhibitor of the transcriptional pathway of p53 (Yano et al., 2007; Zeng et al., 2016) described for protecting against genotoxic agents, including cisplatin (Jiang et al., 2004) or UV-induced damages (Komarov et al., 1999). The percentage of apoptotic cells after incubation with 1–3 mM gentamicin for 24 h was determined by DAPI staining (Fig. 2).

We observed a decrease of apoptosis induced by gentamicin by >50% after 24 h incubation. For longer time of incubation (48 h), the same observation was done with again a partial protective effect afforded by pifithrin-α. After incubation with 3 mM gentamicin for 48 h, gentamicin induced 17.15% apoptosis in absence of pifithrin-α and 9.6% apoptosis in presence of this inhibitor of transcriptional pathway of p53. The cellular gentamicin content after incubation with pifithrin-α was assessed by a disc-plate microbiological technique using *Bacillus subtilis* (ATCC 6633) as previously described (Tulkens and Trouet, 1978), and no statistically significant change in gentamicin accumulation was observed (data not shown).

To confirm the involvement of p53 protein in gentamicin-induced apoptosis, we also performed p53 siRNA-mediated gene silencing experiments. First, we evaluated the effects of the p53-targeting siRNA on the expression of endogenous p53 protein in LLC-PK1 cells. We assessed the expression of p53 by western blotting. p53 siRNA induced a reduction in the expression of endogenous p53 in comparison with the mock (Fig. 3, Top). We then investigated whether p53 protein knockdown decreased the gentamicin-induced apoptosis using DAPI labeling (Fig. 3, Below). p53 siRNA transfection reduced gentamicin-induced nuclear fragmentation, both after 24 and 48 h of incubation. This result is in accordance with those of the experiments using the p53 inhibitor pifithrin-α.

Thus, in this first set of experiments, we demonstrated an increase of p53 levels induced by gentamicin and partial protection against apoptosis induced by gentamicin afforded by the p53 inhibitor, pifithrin-α, and by p53 siRNA suggesting implication of both p53 transcriptional-dependent and -independent pathways in apoptosis-induced by gentamicin.

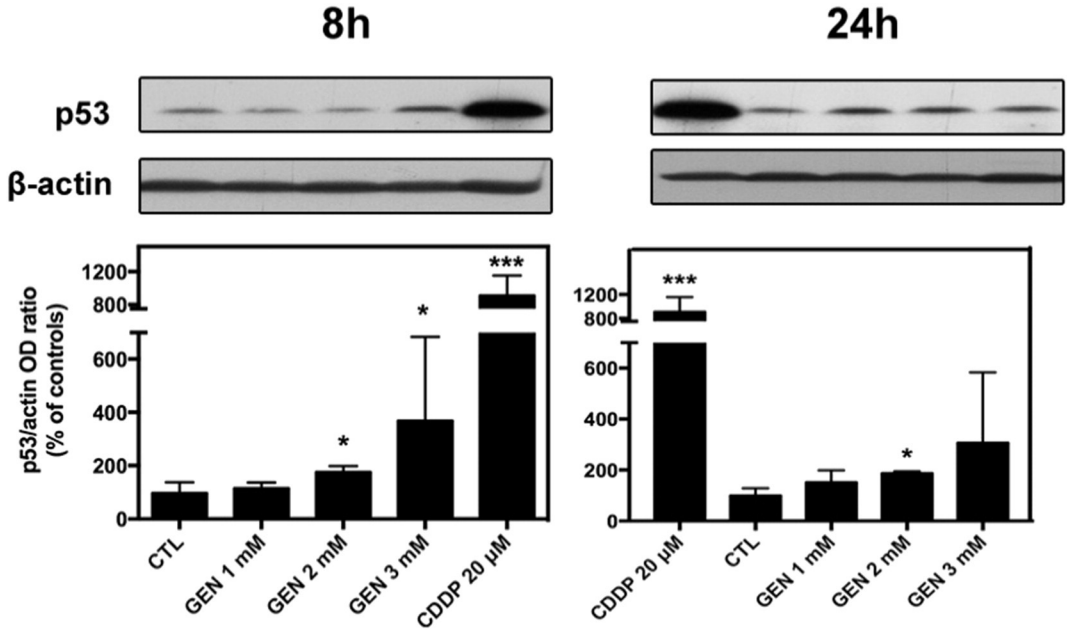

Fig. 1. Effect of gentamicin on p53 protein levels. Cells were incubated for 8 h (left) or 24 h (right) without (controls, CTL) or with gentamicin (GEN 1–3 mM). Densitometric values of p53 protein are normalized to β-actin. The blot presented is representative from 3 independent experiments. Data are given as means ± SD (*$p < 0.05$; ***$p < 0.001$). Cisplatin (CDDP), known to induce apoptosis in LLC-PK1 cells via p53 (Sanchez-Perez et al., 2010), was used as positive control.

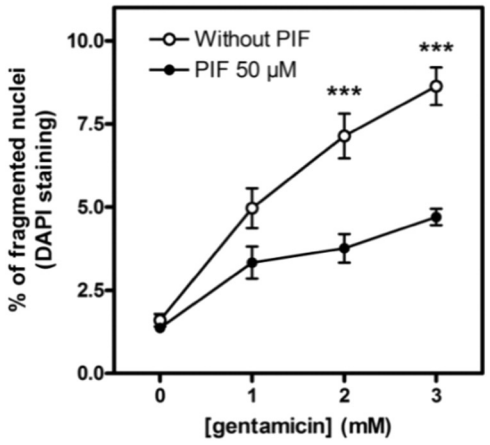

Fig. 2. Prevention by the p53 inhibitor pifithrin-α of gentamicin-induced apoptosis. Cells were pre-incubated or not for 1 h with 50 μM pifithrin-α and then incubated with gentamicin (1–3 mM) for 24 h, in continued presence of pifithrin-α. Data are given as means ± SD ($n = 6$ from 2 separate experiments). Statistical analysis: Two-way analysis of variances, with Bonferroni post-tests to compare points treated with the same concentration of gentamicin together (***$p < 0.001$).

3.1.3. ROS is involved in p53 increase induced by gentamicin. Because we had demonstrated an increase of reactive oxygen species (ROS) after incubation of LLC-PK1 with gentamicin (Denamur et al., 2011) and since ROS production is involved in p53 activation in renal cells (Jiang et al., 2007; Ju et al., 2014), we next investigated the potential role of ROS in the increase of p53 level induced by gentamicin (Fig. 4).

Cells were pre-incubated with *N*-acetylcysteine, an antioxidant known to inhibit ROS-dependent apoptosis (Curtin et al., 2002) including apoptosis induced by gentamicin (Denamur et al., 2011). Pre-incubation of cells with *N*–acetylcysteine induced a marked decrease of the p53 accumulation observed after incubation for 24 h with 2 or 3 mM of gentamicin. *N*-acetylcysteine also decreased p53 levels in control cells or in cells incubated with 1 mM gentamicin but the effect was not statistically significant.

These data suggest that ROS exert an important role in the increase of p53 levels induced by gentamicin.

3.1.4. Increase of p53 induced by gentamicin leads to p21 increase. We examined whether overexpression of p53 induced by gentamicin could allow it to accumulate in the nucleus and to transcriptionally regulate genes to control the cell's fate. For instance, p53 can lead to an increase of cellular levels of cyclin-dependant kinases inhibitors like p21 (Lu and Hunter, 2010), which are substrate for proteasomal degradation. We therefore monitored the potential effect of gentamicin on p21 protein levels (Fig. 5).

We showed a marked and statistically significant increase (up to 180%) in p21 protein levels after treatment with 2 and 3 mM gentamicin (Fig. 5).

To determine the implication of p53 activation in the increase in p21 levels, we measured the p21 cellular levels in cells exposed to gentamicin in the presence of the p53 transcription inhibitor pifithrin-α. Fig. 6 shows that the increase in cellular levels of p21 induced by gentamicin was considerably reduced when pifithrin-α was present, suggesting that p21 accumulation is related to gentamicin-induced p53 activation.

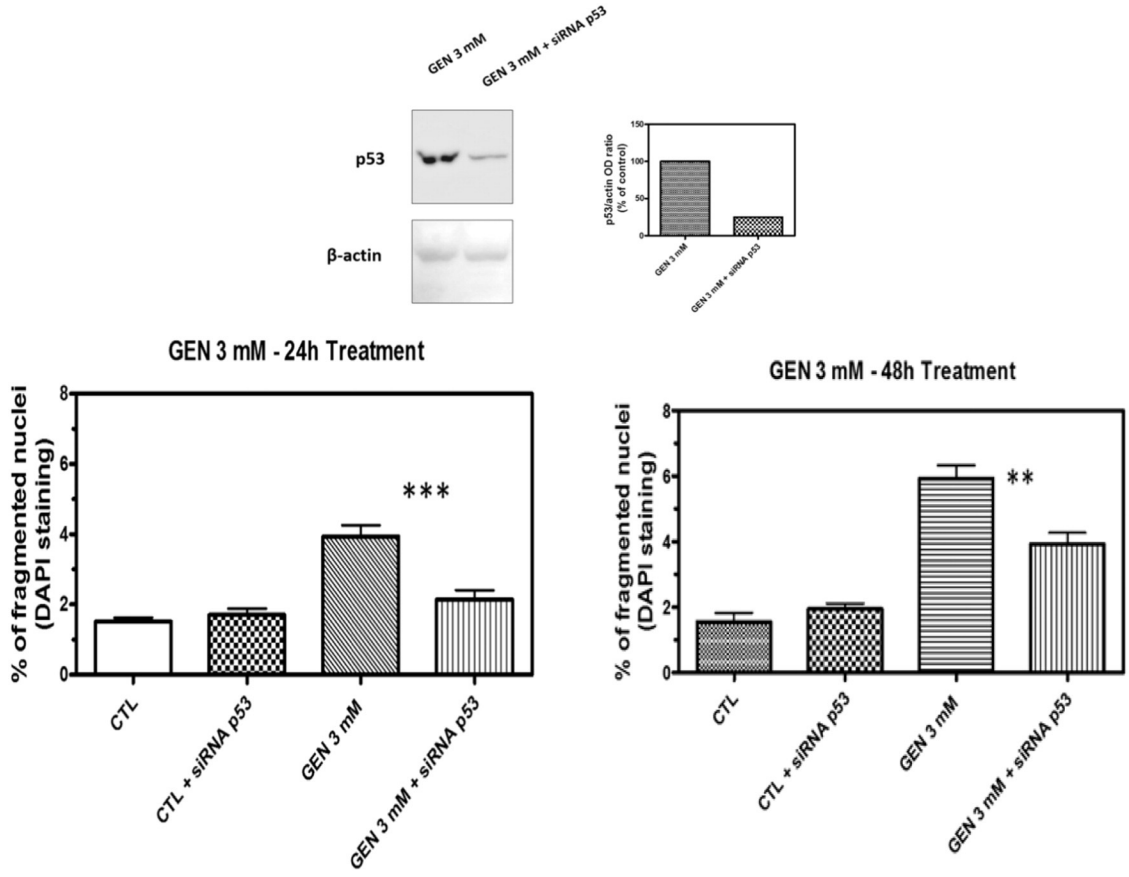

Fig. 3. (Top) Reduction of p53 expression by p53-targeting small interfering (si)RNA (p53 siRNA) in LLC-PK1 incubated with gentamicin 3 mM for 24 h. The p53 protein levels were assessed by western blotting analysis. β-actin levels were also assessed and served as a loading control. A representative blot is shown from three independent experiments with identical results. (Below) Prevention by the p53 siRNA of gentamicin-induced apoptosis. Transfected cells were incubated with gentamicin (3 mM) for 24 h or 48 h. Data are given as means ± SD (n = 6 from 2 separate experiments). Statistical analysis: Two-way analysis of variances, with Bonferroni post-tests to compare points treated with the same concentration of gentamicin together (**$p < 0.01$; ***$p < 0.001$).

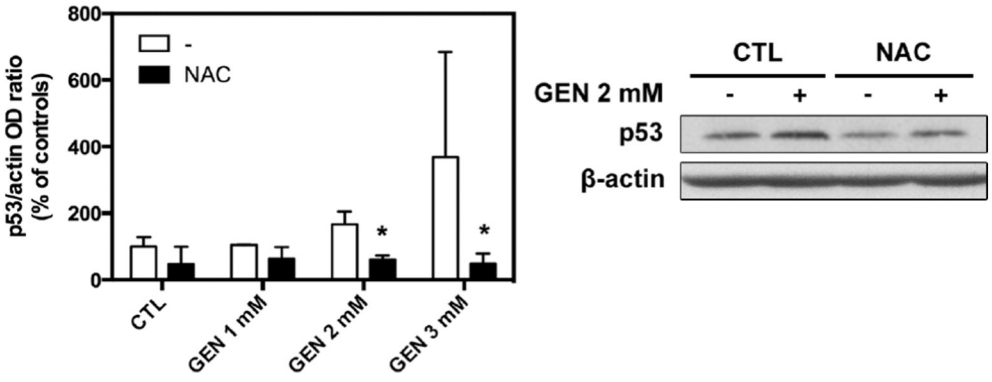

Fig. 4. Effect of N-acetylcysteine on gentamicin induced increase of p53 protein levels. Cells were incubated for 24 h without (controls, CTL) or with gentamicin (GEN 1–3 mM) after preincubation or not with *N*-acetylcysteine (1 mM). Densitometric values are normalized to β-actin (* $p < 0.05$). The blot presented is representative from 3 independent experiments performed after incubation with gentamicin 2 mM.

3.2. Sphingomyelinase pathway is not involved in gentamicin-induced apoptosis

3.2.1. Gentamicin decreased ceramides levels by inhibiting the activity of acidic sphingomyelinase. The partial protection afforded by the p53 inhibitor, pifithrin-α (Fig. 2), as well as by p53 siRNA (Fig. 3) against apoptosis induced by gentamicin suggests that other signaling pathways are probably involved. Ceramide accumulation has been observed in response to various extracellular stimuli including p53 upregulation (Heffernan-Stroud and Obeid, 2011; Kim et al., 2002; El Assaad et al., 2003), and cause apoptosis and/or cell cycle arrest (Dbaibo et al., 1998; Fillet et al., 2002; Fillet et al., 2003). To investigate the potential role of ceramides in apoptosis induced by gentamicin, we determined the effect of gentamicin on the amounts of endogenous ceramides in LLC-PK1. We incubated LLC-PK1 cells with gentamicin at extracellular concentrations of 1 or 2 mM and for periods of time ranging from 15 min to 24 h before measurement of C-14 to C-24 ceramides contents by LC-ESI-MS/MS. No major change was observed when cells were incubated with gentamicin 2 mM for 5 min up to 3 h (Fig. 7). Thereafter, we observed a marked decrease of ceramide levels in cells incubated with gentamicin as compared to control cells (Fig. 7). Similar results were found after incubation with 1 mM of gentamicin (data not shown).

To decipher the mechanism involved in the decrease of ceramide levels in cells incubated with gentamicin, and since previous studies have shown a decrease of lysosomal sphingomyelinase in presence of gentamicin (Laurent et al., 1982), we measured the activities of neutral and acidic sphingomylinases in LLC-PK1 cells incubated with gentamicin. Results of a typical experiment in which LLC-PK1 were cultivated over a period of 24 h in a control medium or in presence of gentamicin 2 mM are shown in Fig. 8.

The activity of acidic sphingomyelinase in gentamicin treated cells dropped to significantly lower values as compared to controls, for pH lower than 6.5. Similar results were obtained with gentamicin 1 mM (data not shown). The measurement of neutral sphingomyelinase showed no detectable changes (data not shown).

Decrease of ceramides contents resulting from inhibition of acidic sphingomyelinase suggests that sphingomyelinase pathway is not involved in apoptosis induced by gentamicin.

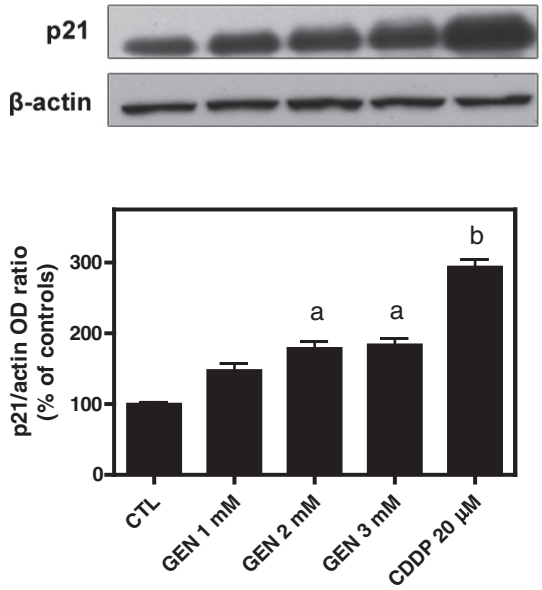

Fig. 5. Effect of gentamicin on p21 protein levels. Cells were incubated for 24 h without (controls, CTL) or with gentamicin (GEN 1–3 mM) or cisplatin (CDDP 20 μM). Densitometric values are normalized to β-actin. The blot presented is representative from 3 independent experiments, data are means ± SD ($n = 3$) (bars with different letters are significantly different from each other; *$p < 0.05$).

3.3. Involvement of proteasome and endoplasmic reticulum in apoptosis induced by gentamicin

3.3.1. Gentamicin increased the ubiquitin conjugates.. Increase of p53 and p21 levels after gentamicin incubation, two proteins normally kept at low cellular concentration by a rapid degradation by the proteasome and involved in cell apoptosis as well as accumulation of ubiquitinated protein Bax (Servais et al., 2006) pushed us to investigate the global impact of gentamicin on cellular accumulation of ubiquitinated proteins.

We used ubiquitin immunoblotting to assess the effects of gentamicin on accumulation of ubiquitin-conjugates (Fig. 9). LLC-PK1 cells were incubated for 24 h with the antibiotic or with epoxomicin, a highly selective and potent inhibitor of the proteasome (Giguere and Schnellmann, 2008) used as positive control (EPX, 20 μM). A concentration-dependant increase of ubiquitinated proteins in the cells incubated with gentamicin that was statistically significant for an extracellular concentration of 3 mM.

3.3.2. Gentamicin inhibited the trypsine-like activity of proteasome. To further assess the potential effects of gentamicin on proteasome, we assayed the activities of the three types of active sites of proteasome (chymotrypsin-like, trypsin-like and caspase-like) using lysates of LLC-PK1 cells incubated with gentamicin (Fig. 10). These lysates were collected and crude extracts incubated for 1 h at 37 °C with increasing concentrations of gentamicin (0 up to 30 mM) before adding specific

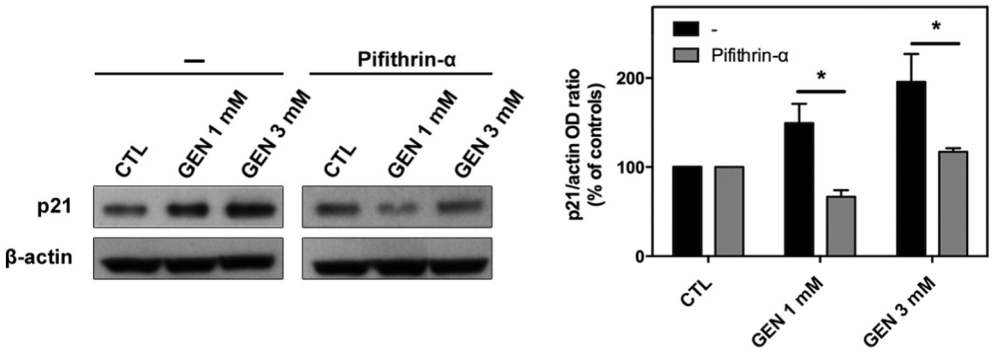

Fig. 6. Effect of pifithrin-α on gentamicin-induced accumulation of cellular p21 proteins. Cells were pre-incubated or not for 1 h with 50 μM pifithrin-α and then incubated with gentamicin (1 and 3 mM) for 24 h, in continued presence of pifithrin-α. Data are given as means ± SD ($n = 3$; $^*p < 0.05$).

fluorogenic substrates of each catalytic activity. The proteasome inhibitor epoxomicin was used as positive control.

While chymotrypsin-like activity was completely inhibited by epoxomicin since the fluorescence level detected with this inhibitor is quite the same as the fluorescence emitted by the substrate alone (SA, Fig. 10a), incubation with gentamicin induced an increase of chymotrypsin-like activity at 5 mM, followed by a slight decrease of activity up to 30 mM.

In contrast, gentamicin induced an important decrease in trypsin-like activity, from the first concentration tested (5 mM) and which becomes complete (same fluorescence level as the substrate alone) from 15 mM. Note that trypsin-like activity was not inhibited by epoxomicin (Fig. 10b), as described previously (Naujokat et al., 2007).

Caspase-like activity, after undergoing an increase of activity at 5 and 10 mM, was inhibited by gentamicin at higher concentrations (Fig. 10c). As for chymotrypsin-like activity, epoxomicin induced an important inhibition of this proteasomal activity.

3.3.3. Gentamicin increased the phospho-eIF2α protein levels. A well-known consequence of proteasome inhibition is the activation of GCN2 kinase and the resulting phosphorylation of the translation initiation factor eIF2α, which leads to apoptosis (Jiang and Wek, 2005). We measured the cellular levels of eIF2α and of its phosphorylated form

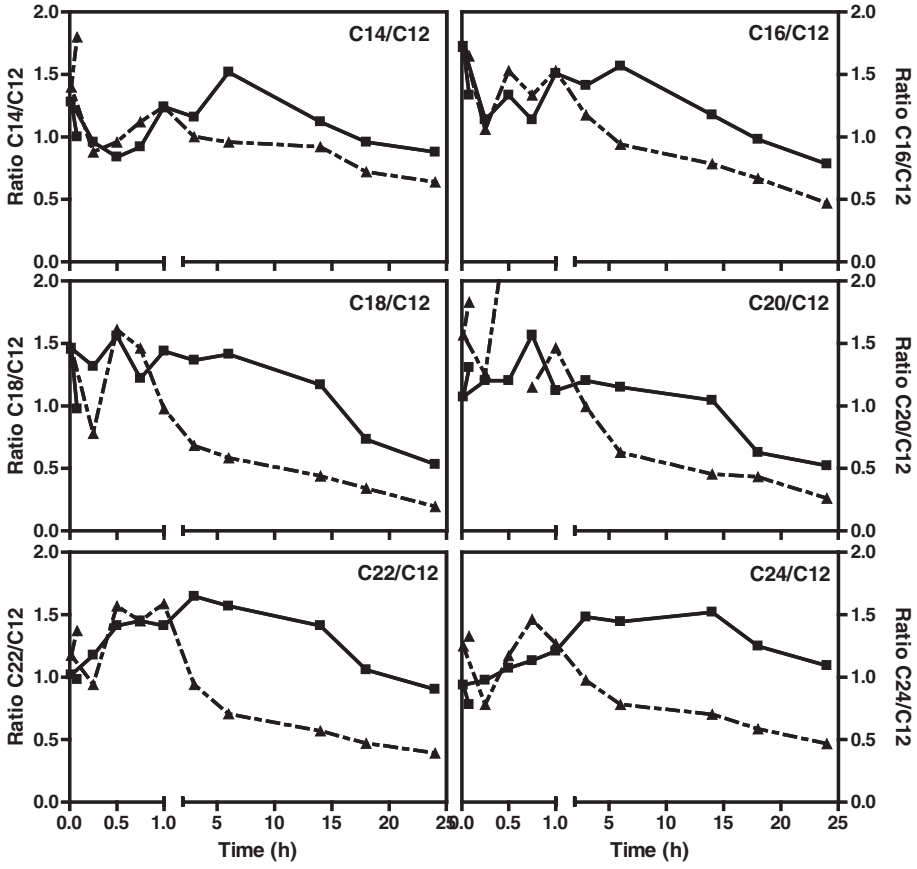

Fig. 7. Ceramide levels in LLC-PK1 incubated with gentamicin. Cells were treated with 2 mM gentamicin for various periods of time from 15 min to 24 h before assessment of C14–24-ceramide levels by LC-ESI-MS/MS. The results are expressed in function of an internal standard (C-12 ceramide). Results obtained for controls and cells incubated with 2 mM of gentamicin are represented as continuous (squares) and dotted lines (triangles), respectively. $n = 3$; 2 independent experiments.

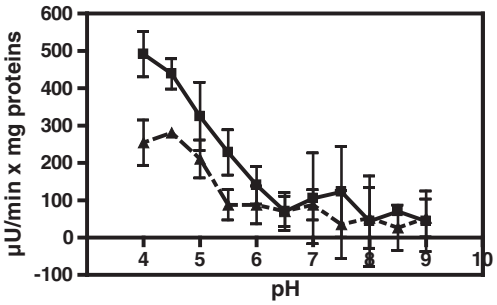

Fig. 8. Effect of gentamicin on the activity of acidic sphingomyelinase at pH ranging from 4 to 9. The cells were incubated for 24 h in absence (full lines; squares) and presence of 2 mM gentamicin (dotted lines; triangles) $n = 3$.

to test for this hypothesis in our model and the results are illustrated in Fig. 11.

Despite the absence of statistical significance, these experiments showed an increase in the cellular levels of eIF2α to up to 200% after incubation with gentamicin 3 mM (Fig. 11a). Regarding the cellular levels of phosphorylated eIF2α, these increased in a dose-dependent fashion with gentamicin reaching 315% for cells incubated with 3 mM of gentamicin and all differences vs control were statistically significant (Fig. 11b).

3.3.4. Gentamicin induced activation of caspase-12 without change in ER chaperones GRP78 and GRP94. In addition to the link between inhibition of the proteasome and phosphorylation of eIF2, the latter is also connected to ER stress. The hypothesis of an ER implication in gentamicin-induced apoptosis would be consistent with the binding of ER chaperones proteins by gentamicin (Horibe et al., 2004) and the observation of markers of ER stress in rats treated for 7 days with 100 mg gentamicin (Peyrou et al., 2007). To investigate this hypothesis, we evaluated caspase-12 activation by immunoblotting (Fig. 12).

As shown in Fig. 12, treatment of cells with gentamicin induced an activation of caspase-12 by cleavage of the pro-form (detected at 55 kDa) into cleaved active caspase-12 (42 kDa), the cellular levels of which increased after gentamicin treatment. The small subunit is detected at 20 kDa. Bands detected at 85 and 110 kDa could correspond to dimers of (pro) caspase-12, which also correspond to activation of caspase-12 (Yoneda et al., 2001). Gentamicin induced a significant increase of caspase-12 (42 kDa) fragment. There was no statistically significant difference in the effect induced by 1 mM of gentamicin as compared to that observed for cells incubated with 3 mM of gentamicin. An increase caspase-12 (20 kDa) was also observed.

As cleavage of caspases is a common indicator of their activation, and since caspase-12 can be specifically activated by apoptotic signals with an ER stress component (Nakagawa et al., 2000), we wanted to investigate if gentamicin could induce ER stress in LLC-PK1 cells. Specifically, we determined the potential role of ER chaperones by incubating cells with or without gentamicin (1–3 mM) for 24 h and measuring the expression levels of two ER chaperones, GRP78 and GRP94 (Fig. 13, a–b). Tunicamycin (5 µl/ml), a specific inhibitor of *N*-linked glycosylation which prevents post-translational maturation of proteins and is known as ER stress inducer in LLC-PK1 cells (Peyrou and Cribb, 2007) was used as positive control for ER stress markers induction.

While tunicamycin induced a marked increase in GRP94 and GRP78 cellular levels, treatment with gentamicin did not induce any change in the expression of these chaperones in our conditions.

4. Discussion

After endocytosis by proximal tubular cells, gentamicin mainly accumulates in lysosomes inducing lysosomal membrane permeabilisation (Denamur et al., 2011). At therapeutically-relevant doses, gentamicin induces mitochondrial pathway of apoptosis (Servais et al., 2006; Servais et al., 2005). The present work moves us one step ahead in the understanding of the cellular mechanisms underlying the apoptosis induced in cells by gentamicin by focusing on p53 and sphingomyelinase pathways as well as on the effect of gentamicin on proteasome and endoplasmic reticulum.

On LLC-PK1 proximal tubular cells, we demonstrate that p53 is a key cornerstone in apoptosis induced by gentamicin as suggested by the increase of its level upon gentamicin treatment and by the partial protective effect afforded by pifithrin-α or by transfection with p53 siRNA. Upon stress signals, p53 undergoes a series of post translational modifications as phosphorylation at Ser15. Even any effect was observed towards this specific phosphorylation, other post translational modifications could be involved in p53 activation induced by gentamicin. First, Ser-46 phosphorylation is required for p53-dependent transcriptional activation of the apoptotic gene p53AIP1. Second, in addition to phosphorylation, mediating p53 stabilization, the acetylation of p53 has also been shown to be a key signal, promoting its activation upon DNA damage (Kruse and Gu, 2009; Tang et al., 2008).

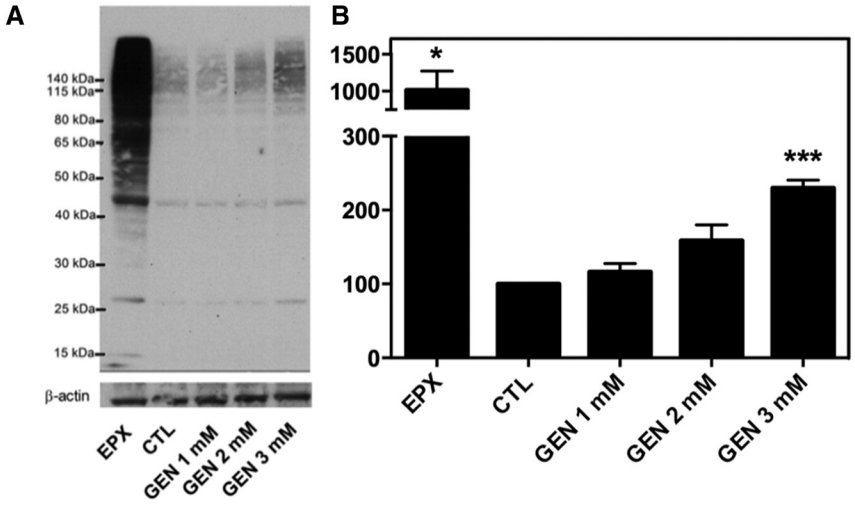

Fig. 9. Effect of gentamicin on ubiquitin conjugates. Ubiquitin immunoblot of lysates of LLC-PK1 cells incubated for 24 h with or without (control, CTL) gentamicin (GEN 1–3 mM) or epoxomicin (EPX, 20 µM). Densitometric values detected for ubiquitin was normalized to β-actin and results are expressed in % of ratio obtained for control cells. Data are means ± SD ($n = 3$; $^{*}p < 0.05$, $^{***}p < 0.001$).

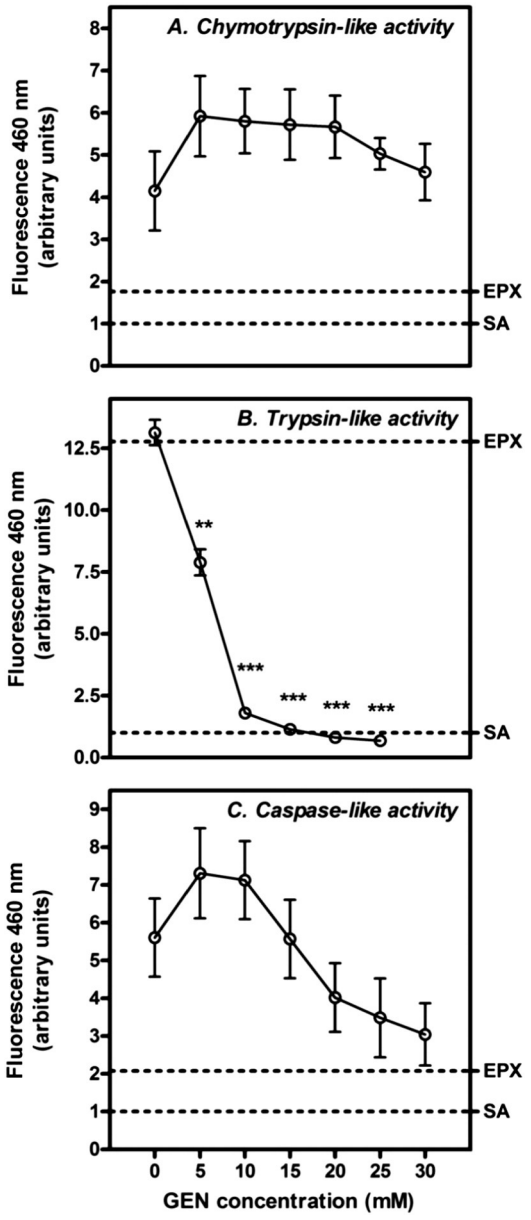

Fig. 10. Effect of gentamicin on proteasome catalytic activities, determined by measuring fluorescence produced by fluorogenic specific substrates of the three proteasomal activities. Results are expressed in arbitrary units, fluorescence of the substrate alone being considered as having a value of 1. Cellular lysates were incubated at 37 °C for 1 h with (or without) gentamicin (GEN) at indicated concentrations. Epoxomicin (EPX) 20 μM was used as positive control. Chymotrypsin-like activity (A), trypsin-like activity (B) and peptidyl-glutamyl peptide hydrolyzing or caspase-like activity (C) were determined with Suc-LLVY-amc, Ac-RLR-amc and Ac-nLPnLD-amc respectively. Fluorescence was read at the end of 4 h incubation at 37 °C after addition of the substrate. On each graph, fluorescence level of the substrate alone (SA, substrate not incubated with cell lysates) and of lysates incubated with epoxomicin (EPX) are represented by a dotted-line. Data are means ± SD ($n = 6$ from 3 separate experiments; where not visible, error bars are included in the symbol). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test to compare each point to control value (without gentamicin) (**$p < 0.01$, ***$p < 0.001$, where not indicated, differences are not significant).

Especially, acetylation at lysine 164 could play a critical role for blocking repression of p53, mediated by Mdm2- and Mdmx, by preventing their recruitment to target promoters (Kruse and Gu, 2009; Tang et al., 2008).

The partial protective effect afforded by pifithrin-α and by siRNA p53 suggest a potential activation of both p53 transcriptional-dependent- and independent pathways, as also demonstrated for toxicities induced by cisplatin (Rebillard et al., 2008) or manganese (Wan et al., 2014) or in radio-resistant head and neck squamous carcinoma cells (Bionda et al., 2007). The potential role of p53 transcriptional pathway in gentamicin-induced apoptosis is in agreement with what was observed by microarray experiments on rats treated with gentamicin (Ozaki et al., 2010). Activation of the transcription factor NFκB, for which p65 subunit could activate the p53 promoter (Wu and Lozano, 1994), is consistent with the increase of NFκB activation demonstrated after incubation of renal tubular cells with gentamicin (Chen et al., 2011; Juan et al., 2007). JNK pathway activation (Yang et al., 2004) could be also involved even though this pathway is dependent upon the cell strains. Indeed, no modification of JNK or pJNK expression levels was observed in proximal tubular cells after 24 h of incubation with gentamicin 3 mM despite an increase of p53 expression (Chen et al., 2011), although JNK implication has been demonstrated after gentamicin treatment of mesangial cells (Martinez-Salgado et al., 2005) and increase of Jun. transcription was evidenced by microarray studies (Ozaki et al., 2010). Even most p53 functions are mediated by transcriptional activation of its target genes in response to various stress signals, cytoplasmic p53 was also shown to directly bind to proteins like Bax to trigger mitochondrial outer membrane permeabilization (MOMP) and apoptosis (Green and Kroemer, 2009; Nikoletopoulou et al., 2013). This could be critical for apoptosis induced by gentamicin since Bcl-2 and Bax are involved in this process. Indeed, Bcl-2 overexpression (through transfection) prevents apoptosis-induced by gentamicin (El Mouedden et al., 2000b) and significant increases of the cellular levels of Bax and ubiquinitated Bax (without changes in Bax mRNA) (Servais et al., 2005; Servais et al., 2006) were observed. In addition, sphingomyelinase pathway could also be important. Sphingomyelinases are responsible for the conversion of sphingomyelin to ceramides which have been proposed to play a key role in apoptosis and in cellular responses to stresses (Mullen and Obeid, 2012) including activation of proapoptotic receptors (CD95/Fas and TNFR). However, we observed a marked decrease of ceramides which we related to the inhibition of acidic sphingomyelinase activity, in agreement with previous studies (Laurent et al., 1982). Sphingomyelinase pathway would be therefore not be involved in apoptosis induced by gentamicin and could even afford partial protection against gentamicin-induced apoptosis.

One potential mechanism explaining the increase of p53 levels induced by gentamicin is ROS production. By measuring fluorescence of 2′,7′-dichlorofluoresceine production (DCF) resulting from H_2DCF conversion and by vital confocal imaging, we showed that gentamicin-induced ROS production occurs in lysosomes. Moreover, gentamicin-induced ROS production as well as apoptosis was largely prevented by deferoxamine, an iron chelator, which is endocytosed and accumulates in lysosomes. We also reported that gentamicin induced ROS production prior to, and at lower drug concentrations than required for apoptosis (Denamur et al., 2011). The effect afforded by *N*-acetylcysteine on p53 levels is consistent with this hypothesis. The role of lysosome for ROS production is is in accordance with numerous publications revealing that autophagosomes and/or lysosomes are the major sites for basal ROS generation in addition to mitochondria (Kubota et al., 2010; Hu et al., 2016; Walker and Shah, 1987). Moreover, an interconnected pathway could be involved since the release of mitochondrial ROS leads to subsequent lysosomal membrane permeabilization.

This role of ROS could be critical since, as suggested in literature, ROS could also interact with the functions of the proteasome by inhibiting its ability to breakdown proteins (Meek, 2015). This could explain (i) the increased cell content in ubiquitinated Bax in cells exposed to gentamicin (Servais et al., 2006; Servais et al., 2005), (ii) the increase in total ubiquitinated proteins shown here after cell incubation with gentamicin and (iii) the inhibition of trypsin- and caspase-like activities by gentamicin in cellular lysates (this study). While the role of these two activities is limited in yeast protein, where chymotrypsin-like is considered as rate-limiting in protein breakdown (Heinemeyer et al., 1997), all three types of active sites contribute to protein breakdown in

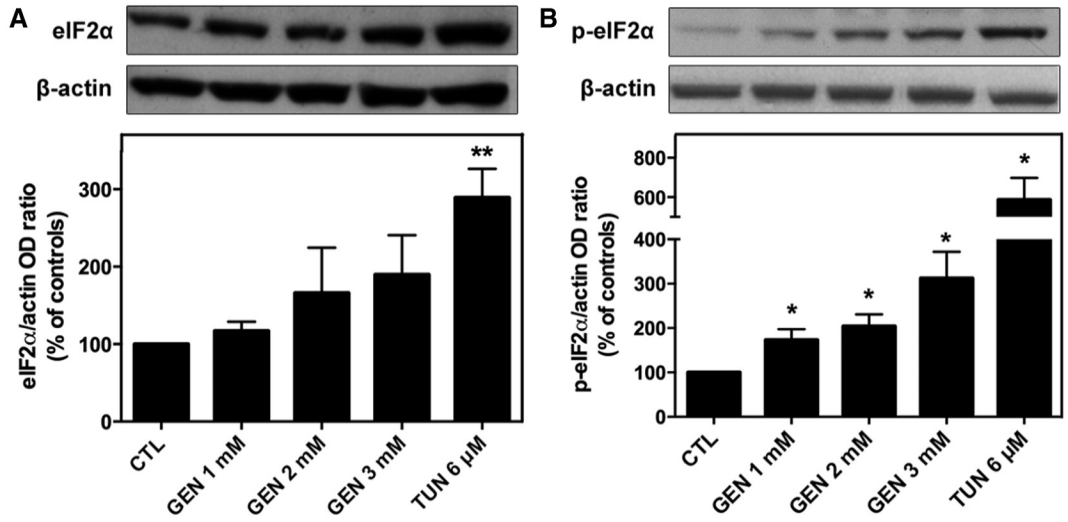

Fig. 11. Effect of gentamicin on eIF2α and p-eIF2α protein levels. Cells were incubated for 24 h without (controls, CTL) or with gentamicin (GEN 1–3 mM) or tunicamycin (TUN 6 μM). eIF2α (panel A) and its phosphorylated form (panel B) bands were detected at 38 kDa; densitometric values are normalized to β-actin. The blot presented is representative from 3 independent experiments, data are means ± SD ($n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$).

mammalian cells, even though the respective importance of each catalytic site varies widely with the substrate. So, simultaneous inhibition of trypsin- and caspase-like activities reduces the degradation of model proteins by pure 26S proteasome between 30 and 56% depending on the substrate used (Kisselev et al., 2006). Although the effect of gentamicin on proteasome could result from direct interaction of the antibiotic with the proteasome, such as observed in vitro (Horibe et al., 2004), it is likely that ROS production induced by gentamicin accumulated in lysosomes could also alter the proteasomal activity since ROS are able to disassembly 26S proteasome (Wang et al., 2010). ROS production in itself and the redox oxidized proteins signaling pathways could activate kinase cascades and gene transcription aimed at rescuing oxidized proteins, which might disrupt important cellular processes including proteasome-mediated protein degradation (Demasi et al., 2015; Hohn et al., 2014; Lee et al., 2001). Induction of ROS by gentamicin could therefore be responsible for further lysosomal membrane permeabilization, and decreased catalytic activity of the proteasome. The question of the dose of gentamicin needed for inhibiting proteasome activities has also to be questioned but gentamicin could bind to intracellular sites (membranes or even proteins, making interpretations of the concentrations data quite difficult to analyze.

The increase in p53 cellular levels we observed can fully explain the increase of p21, another target of degradation by the proteasome. p21 is known for its role in cell cycle inhibition and apoptosis modulation. We showed here an increased expression of p21 protein levels that most probably results from p53 transcription factor activation since it is completely inhibited by co-treatment with pifithrin-α. The role of p21 in apoptosis is dependent upon its localization and p21 is supposed to be protective since bortezomib-induced apoptosis is markedly enhanced in the p21 knock-out cells (Yu et al., 2003). Besides on their effect in apoptosis, the expression levels of the p21 family CDK inhibitors play an important role in the regulation of cyclin-dependant kinases activity, which are responsible for cell cycle progression. However, since human adult renal proximal tubular cells present a low rate of proliferation (Nadasdy et al., 1994), the roles of p21 as cyclin-dependent

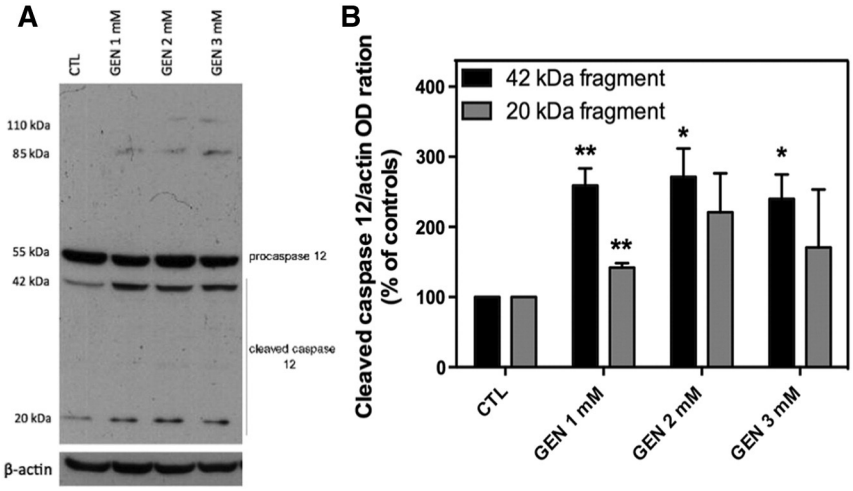

Fig. 12. Effect of gentamicin on caspase 12 activation. Cells were incubated for 24 h without (controls, CTL) or with gentamicin (GEN 1–3 mM). Pro-caspase12 band is detected at 55 kDa, and cleaved caspase-12 at 42 kDa (large subunit) and 20 kDa (small subunit). Bands appearing at 110 and 85 kDa could correspond to dimers of (pro) caspase-12. Densitometric values are normalized to β-actin. The blot presented is representative from 3 independent experiments, data are means ± SD ($n = 3$), statistical analysis compares values to correspondent controls ($^{*}p < 0.05$, $^{**}p < 0.01$).

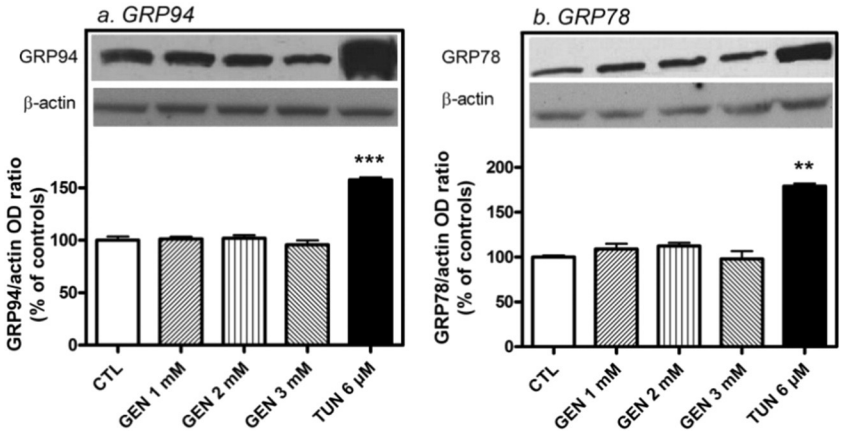

Fig. 13. Effect of gentamicin on cellular levels of GRP94 (a), GRP78 (b). Cells were incubated for 24 h with or without (control, CTL) gentamicin (GEN) or tunicamycin (TUN) at indicated concentrations. Densitometric values are normalized with β-actin, results are presented as percentage of OD ratio obtained for control cells. Values are means ± SD ($n = 3$, from 3 separate experiments; $^{**}p < 0.01$, $^{***}p < 0.01$). GRP94 and GRP78 were detected at 94 and 78 kDa respectively.

kinases inhibitor is probably not crucial in the process of toxicity induced by gentamicin in patients.

In addition to increase in p53 cellular levels induced by gentamicin, we also evidence here an increase in eIF2α phosphorylation. This could indirectly result from proteasome inhibition through phosphorylation by eIF2α kinases such as GCN2 (Jiang and Wek, 2005; Yu et al., 2003; Zhang et al., 2011) or following endoplasmic reticulum stress induction (Teske et al., 2011; Chauhan et al., 2008). Such signs of endoplasmic reticulum stress induction after treatment of rats with gentamicin have been previously described (Peyrou et al., 2007). In the present work, however, we show that activation of caspase-12 occurs independently of chaperones upregulation as already been described for cisplatin, without real explanation of this phenomenon (Peyrou and Cribb, 2007). Studies showing chaperones induction and caspase-12 activation upon gentamicin treatment of animals have been conducted with very high doses of the drug (160 mg/kg), while apoptosis is observed in vivo at much lower and more therapeutically significant dosages (typically 10 mg/kg; (Mingeot-Leclercq and Tulkens, 1999; El Mouedden et al., 2000a).

A summary of the most likely current view of cellular mechanisms involved in gentamicin toxicity, as derived from the results of this study and our previous observations is illustrated in Fig. 14. The new data are that p53 is a key element in the signaling pathway leading to apoptosis following its increased expression mediated by ROS. Since

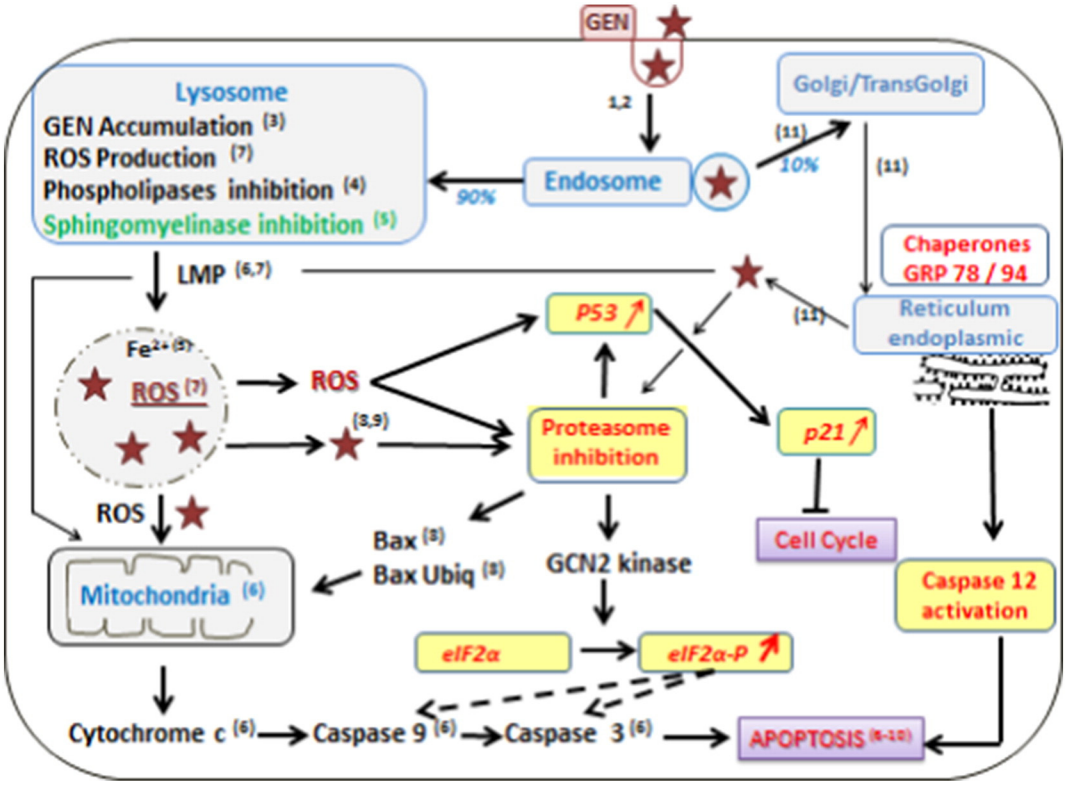

Fig. 14. Proposed signaling pathway triggered by gentamicin. Observations from current study which result to- or protect against- apoptosis (in red and green, respectively). The upper number referred to results from previous studies from our group: [1]Sastrasinh et al. (1982), [2]Moestrup et al. (1995), [3]Tulkens and Trouet (1978), [4]Mingeot-Leclercq et al. (1988), [5]Laurent et al. (1982), [6]Servais et al. (2005), [7]Denamur et al. (2011), [8]Servais et al. (2006), [9]Denamur et al. (2008), [10]El Mouedden et al. (2000a; 2000b), [11]Sandoval and Molitoris (2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the protective effect against apoptosis exerted by the p53 transcription inhibitor pifithrin-α and by p53 siRNA is only partial, the process could implicate p53 transcription-dependent and –independent pathways. We also demonstrated an increase in p21 protein levels directly related with p53 increase. A complex interplay between subcellular organites is probably involved since we showed an inhibition of trypsin- and caspase-like activities of proteasome in addition with an effect of gentamicin on reticulum endoplasmic stress as demonstrated by the increase of phosphorylation of eIF2α, and caspase 12-cleavage without chaperones accumulation. The role played by p53 and endoplasmic reticulum in pathways leading to apoptosis induced gentamicin might be extended to other nephrotoxicants such as cisplatin (dos Santos et al., 2012) and cyclosporine A (Xiao et al., 2013).

In a nutshell, incubation of renal cells with gentamicin induced (i) activation of p53 pathway (as demonstrated by measuring the expression of p53 and the effect induced by an inhibitor of transcriptional effect of p53 [pifithrin-α] or p53 siRNA approach), (ii) inhibition of proteasomal activity and (iii) response of endoplasmic reticulum, which in turn resulted in apoptosis. How these pathways are interconnected is not entirely solved yet but autophagy could be a key process. Indeed, data from literature suggested that (i) perturbation of either endoplasmic reticulum homeostasis or endoplasmic reticulum functions increases autophagy and apoptotic cell death (Nikoletopoulou et al., 2013), (ii) endoplasmic reticulum-induced autophagy has an important role in disposing of unwanted polyubiquinitated protein aggregates, thus protecting against cell death and (iii) there is a link between p53 and autophagy, by inhibition of mTor via activation of AMP kinase (Feng et al., 2005).

This work could help in deciphering the cellular and molecular mechanisms involved in aminoglycoside-induced nephrotoxicity and designing new approaches to improve the treatment of critically-ill patients. The results could also help in deciphering the most interesting options to consider in the research of sensitive and reliable biomarkers to detect early toxicity induced by toxicants such as aminoglycosides.

Transparency document

The Transparency document associated to this article can be found, in the online version.

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References

An, W.G., Hwang, S.G., Trepel, J.B., Blagosklonny, M.V., 2000. Protease inhibitor-induced apoptosis: accumulation of wt p53, p21WAF1/CIP1, and induction of apoptosis are independent markers of proteasome inhibition. Leukemia 14, 1276–1283.

Baird, T.D., Wek, R.C., 2012. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv. Nutr. 3, 307–321.

Bionda, C., Hadchity, E., Alphonse, G., Chapet, O., Rousson, R., Rodriguez-Lafrasse, C., Ardail, D., 2007. Radioresistance of human carcinoma cells is correlated to a defect in raft membrane clustering. Free Radic. Biol. Med. 43, 681–694.

Carryn, S., Van Bambeke, F., Mingeot-Leclercq, M.P., Tulkens, P.M., 2002. Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. Antimicrob. Agents Chemother. 46, 2095–2103.

Chauhan, D., Singh, A., Brahmandam, M., Podar, K., Hideshima, T., Richardson, P., Munshi, N., Palladino, M.A., Anderson, K.C., 2008. Combination of proteasome inhibitors bortezomib and NPI-0052 trigger in vivo synergistic cytotoxicity in multiple myeloma. Blood 111, 1654–1664.

Chen, Y.C., Chen, C.H., Hsu, Y.H., Chen, T.H., Sue, Y.M., Cheng, C.Y., Chen, T.W., 2011. Leptin reduces gentamicin-induced apoptosis in rat renal tubular cells via the PI3K-Akt signaling pathway. Eur. J. Pharmacol. 658, 213–218.

Curtin, J.F., Donovan, M., Cotter, T.G., 2002. Regulation and measurement of oxidative stress in apoptosis. J. Immunol. Methods 265, 49–72.

Dashzeveg, N., Yoshida, K., 2015. Cell death decision by p53 via control of the mitochondrial membrane. Cancer Lett. 367, 108–112.

Dbaibo, G.S., Pushkareva, M.Y., Rachid, R.A., Alter, N., Smyth, M.J., Obeid, L.M., Hannun, Y.A., 1998. p53-dependent ceramide response to genotoxic stress. J. Clin. Invest. 102, 329–339.

Demasi, M., Simoes, V., Bonatto, D., 2015. Cross-talk between redox regulation and the ubiquitin-proteasome system in mammalian cell differentiation. Biochim. Biophys. Acta 1850, 1594–1606.

Denamur, S., Tyteca, D., Marchand-Brynaert, J., Van Bambeke, F., Tulkens, P.M., Courtoy, P.J., Mingeot-Leclercq, M.P., 2011. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. Free Radic. Biol. Med. 51, 1656–1665.

Denamur, S., Van Bambeke, F., Mingeot-Leclercq, M.P., Tulkens, P.M., 2008. Apoptosis induced by aminoglycosides in LLC-PK1 cells: comparative study of neomycin, gentamicin, amikacin, and isepamicin using electroporation. Antimicrob. Agents Chemother. 52, 2236–2238.

dos Santos, N.A., Carvalho Rodrigues, M.A., Martins, N.M., dos Santos, A.C., 2012. Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update. Arch. Toxicol. 86, 1233–1250.

El Assaad, W., Kozhaya, L., Araysi, S., Panjarian, S., Bitar, F.F., Baz, E., El Sabban, M.E., Dbaibo, G.S., 2003. Ceramide and glutathione define two independently regulated pathways of cell death initiated by p53 in Molt-4 leukaemia cells. Biochem. J. 376, 725–732.

El Mouedden, M., Laurent, G., Mingeot-Leclercq, M.P., Tulkens, P.M., 2000b. Gentamicin-induced apoptosis in renal cell lines and embryonic rat fibroblasts. Toxicol. Sci. 56, 229–239.

El Mouedden, M., Laurent, G., Mingeot-Leclercq, M.P., Taper, H.S., Cumps, J., Tulkens, P.M., 2000a. Apoptosis in renal proximal tubules of rats treated with low doses of aminoglycosides. Antimicrob. Agents Chemother. 44, 665–675.

Feng, Z., Zhang, H., Levine, A.J., Jin, S., 2005. The coordinate regulation of the p53 and mTOR pathways in cells. Proc. Natl. Acad. Sci. U. S. A. 102, 8204–8209.

Fillet, M., Bentires-Alj, M., Deregowski, V., Greimers, R., Gielen, J., Piette, J., Bours, V., Merville, M.P., 2003. Mechanisms involved in exogenous C2- and C6-ceramide-induced cancer cell toxicity. Biochem. Pharmacol. 65, 1633–1642.

Fillet, M., Van Heugen, J.C., Servais, A.C., De Graeve, J., Crommen, J., 2002. Separation, identification and quantitation of ceramides in human cancer cells by liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chromatogr. A 949, 225–233.

Giguere, C.J., Schnellmann, R.G., 2008. Limitations of SLLVY-AMC in calpain and proteasome measurements. Biochem. Biophys. Res. Commun. 371, 578–581.

Goni, F.M., Alonso, A., 2002. Sphingomyelinases: enzymology and membrane activity. FEBS Lett. 531, 38–46.

Green, D.R., Kroemer, G., 2009. Cytoplasmic functions of the tumour suppressor p53. Nature 458, 1127–1130.

Hannun, Y.A., Luberto, C., 2000. Ceramide in the eukaryotic stress response. Trends Cell Biol. 10, 73–80.

Heffernan-Stroud, L.A., Obeid, L.M., 2011. p53 and regulation of bioactive sphingolipids. Adv. Enzym. Regul. 51, 219–228.

Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., Wolf, D.H., 1997. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. J. Biol. Chem. 272, 25200–25209.

Hohn, A., Jung, T., Grune, T., 2014. Pathophysiological importance of aggregated damaged proteins. Free Radic. Biol. Med. 71, 70–89.

Horibe, T., Matsui, H., Tanaka, M., Nagai, H., Yamaguchi, Y., Kato, K., Kikuchi, M., 2004. Gentamicin binds to the lectin site of calreticulin and inhibits its chaperone activity. Biochem. Biophys. Res. Commun. 323, 281–287.

Hu, J., Kholmukhamedov, A., Lindsey, C.C., Beeson, C.C., Jaeschke, H., Lemasters, J.J., 2016. Translocation of iron from lysosomes to mitochondria during acetaminophen-induced hepatocellular injury: Protection by starch-desferal and minocycline. Free Radic. Biol. Med. 97, 418–426 http://dx.10.1016/j.freeradbiomed.2016.06.024. Epub 2016 Jun 23.

Jiang, H.Y., Wek, R.C., 2005. Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. J. Biol. Chem. 280, 14189–14202.

Jiang, M., Wei, Q., Pabla, N., Dong, G., Wang, C.Y., Yang, T., Smith, S.B., Dong, Z., 2007. Effects of hydroxyl radical scavenging on cisplatin-induced p53 activation, tubular cell apoptosis and nephrotoxicity. Biochem. Pharmacol. 73, 1499–1510.

Jiang, M., Yi, X., Hsu, S., Wang, C.Y., Dong, Z., 2004. Role of p53 in cisplatin-induced tubular cell apoptosis: dependence on p53 transcriptional activity. Am. J. Physiol. Ren. Physiol. 287, F1140–F1147.

Ju, S.M., Pae, H.O., Kim, W.S., Kang, D.G., Lee, H.S., Jeon, B.H., 2014. Role of reactive oxygen species in p53 activation during cisplatin-induced apoptosis of rat mesangial cells. Eur. Rev. Med. Pharmacol. Sci. 18, 1135–1141.

Juan, S.H., Chen, C.H., Hsu, Y.H., Hou, C.C., Chen, T.H., Lin, H., Chu, Y.L., Sue, Y.M., 2007. Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin. Nephrol. Dial. Transplant. 22, 732–739.

Kim, S.S., Chae, H.S., Bach, J.H., Lee, M.W., Kim, K.Y., Lee, W.B., Jung, Y.M., Bonventre, J.V., Suh, Y.H., 2002. P53 mediates ceramide-induced apoptosis in SKN-SH cells. Oncogene 21, 2020–2028.

Kim, W.H., Kang, K.H., Kim, M.Y., Choi, K.H., 2000. Induction of p53-independent p21 during ceramide-induced G1 arrest in human hepatocarcinoma cells. Biochem. Cell Biol. 78, 127–135.

Kirkegaard, T., Jaattela, M., 2009. Lysosomal involvement in cell death and cancer. Biochim. Biophys. Acta 1793, 746–754.

Kisselev, A.F., Goldberg, A.L., 2005. Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. Methods Enzymol. 398, 364–378.

Kisselev, A.F., Callard, A., Goldberg, A.L., 2006. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. J. Biol. Chem. 281, 8582–8590.

Kitatani, K., Idkowiak-Baldys, J., Hannun, Y.A., 2008. The sphingolipid salvage pathway in ceramide metabolism and signaling. Cell. Signal. 20, 1010–1018.
Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., Gudkov, A.V., 1999. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 285, 1733–1737.
Kruse, J.P., Gu, W., 2009. Modes of p53 regulation. Cell 137, 609–622.
Kubota, C., Torii, S., Hou, N., Saito, N., Yoshimoto, Y., Imai, H., Takeuchi, T., 2010. Constitutive reactive oxygen species generation from autophagosome/lysosome in neuronal oxidative toxicity. J. Biol. Chem. 285 (1), 667–674 http://dx.10.1074/jbc.M109.053058, Epub 2009 Oct 22, PMID: 19850931.
Laurent, G., Carlier, M.B., Rollman, B., Van Hoof, F., Tulkens, P., 1982. Mechanism of aminoglycoside-induced lysosomal phospholipidosis: in vitro and in vivo studies with gentamicin and amikacin. Biochem. Pharmacol. 31, 3861–3870.
Lee, M.H., Hyun, D.H., Jenner, P., Halliwell, B., 2001. Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. J. Neurochem. 78, 32–41.
Liu, H., Baliga, R., 2005. Endoplasmic reticulum stress-associated caspase 12 mediates cisplatin-induced LLC-PK1 cell apoptosis. J. Am. Soc. Nephrol. 16, 1985–1992.
Lopez-Novoa, J.M., Quiros, Y., Vicente, L., Morales, A.I., Lopez-Hernandez, F.J., 2011. New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. Kidney Int. 79, 33–45.
Lu, Z., Hunter, T., 2010. Ubiquitylation and proteasomal degradation of the p21(Cip1), p27(Kip1) and p57(Kip2) CDK inhibitors. Cell Cycle 9, 2342–2352.
Martinez-Salgado, C., Rodriguez-Barbero, A., Eleno, N., Lopez-Novoa, J.M., 2005. Gentamicin induces Jun-AP1 expression and JNK activation in renal glomeruli and cultured mesangial cells. Life Sci. 77, 2285–2298.
Mather, M., Rottenberg, H., 2001. Polycations induce the release of soluble intermembrane mitochondrial proteins. Biochim. Biophys. Acta 1503, 357–368.
Meek, D.W., 2015. Regulation of the p53 response and its relationship to cancer. Biochem. J. 469, 325–346.
Mingeot-Leclercq, M.P., Tulkens, P.M., 1999. Aminoglycosides: nephrotoxicity. Antimicrob. Agents Chemother. 43, 1003–1012.
Mingeot-Leclercq, M.P., Laurent, G., Tulkens, P.M., 1988. Biochemical mechanism of aminoglycoside-induced inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases. Biochem. Pharmacol. 37, 591–599.
Moestrup, S.K., Cui, S., Vorum, H., Bregengard, C., Bjorn, S.E., Norris, K., Gliemann, J., Christensen, E.I., 1995. Evidence that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs. J. Clin. Invest. 96, 1404–1413.
Molitoris, B.A., Dagher, P.C., Sandoval, R.M., Campos, S.B., Ashush, H., Fridman, E., Brafman, A., Faerman, A., Atkinson, S.J., Thompson, J.D., Kalinski, H., Skaliter, R., Erlich, S., Feinstein, E., 2009. siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. J. Am. Soc. Nephrol. 20, 1754–1764.
Mullen, T.D., Obeid, L.M., 2012. Ceramide and apoptosis: exploring the enigmatic connections between sphingolipid metabolism and programmed cell death. Anti Cancer Agents Med. Chem. 12, 340–363.
Nadasdy, T., Laszik, Z., Blick, K.E., Johnson, L.D., Silva, F.G., 1994. Proliferative activity of intrinsic cell populations in the normal human kidney. J. Am. Soc. Nephrol. 4, 2032–2039.
Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J., 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403, 98–103.
Naujokat, C., Berges, C., Hoh, A., Wieczorek, H., Fuchs, D., Ovens, J., Miltz, M., Sadeghi, M., Opelz, G., Daniel, V., 2007. Proteasomal chymotrypsin-like peptidase activity is required for essential functions of human monocyte-derived dendritic cells. Immunology 120, 120–132.
Nikoletopoulou, V., Markaki, M., Palikaras, K., Tavernarakis, N., 2013. Crosstalk between apoptosis, necrosis and autophagy. Biochim. Biophys. Acta 1833, 3448–3459.
Oyadomari, S., Yun, C., Fisher, E.A., Kreglinger, N., Kreibich, G., Oyadomari, M., Harding, H.P., Goodman, A.G., Harant, H., Garrison, J.L., Taunton, J., Katze, M.G., Ron, D., 2006. Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. Cell 126, 727–739.
Ozaki, N., Matheis, K.A., Gamber, M., Feidl, T., Nolte, T., Kalkuhl, A., Deschl, U., 2010. Identification of genes involved in gentamicin-induced nephrotoxicity in rats–a toxicogenomic investigation. Exp. Toxicol. Pathol. 62, 555–566.
Pant, V., Lozano, G., 2014. Limiting the power of p53 through the ubiquitin proteasome pathway. Genes Dev. 28, 1739–1751.
Peyrou, M., Cribb, A.E., 2007. Effect of endoplasmic reticulum stress preconditioning on cytotoxicity of clinically relevant nephrotoxins in renal cell lines. Toxicol. In Vitro 21, 878–886.
Peyrou, M., Hanna, P.E., Cribb, A.E., 2007. Cisplatin, gentamicin, and p-aminophenol induce markers of endoplasmic reticulum stress in the rat kidneys. Toxicol. Sci. 99, 346–353.
Quiros, Y., Vicente-Vicente, L., Morales, A.I., Lopez-Novoa, J.M., Lopez-Hernandez, F.J., 2011. An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. Toxicol. Sci. 119, 245–256.
Rebillard, A., Rioux-Leclercq, N., Muller, C., Bellaud, P., Jouan, F., Meurette, O., Jouan, E., Vernhet, L., Le Quement, C., Carpinteiro, A., Schenck, M., Lagadic-Gossmann, D., Gulbins, E., Dimanche-Boitrel, M.T., 2008. Acid sphingomyelinase deficiency protects from cisplatin-induced gastrointestinal damage. Oncogene 27, 6590–6595.
Sanchez-Perez, Y., Morales-Barcenas, R., Garcia-Cuellar, C.M., Lopez-Marure, R., Calderon-Oliver, M., Pedraza-Chaverri, J., Chirino, Y.I., 2010. The alpha-mangostin prevention on cisplatin-induced apoptotic death in LLC-PK1 cells is associated to an inhibition of ROS production and p53 induction. Chem. Biol. Interact. 188, 144–150.
Sandoval, R.M., Molitoris, B.A., 2004. Gentamicin traffics retrograde through the secretory pathway and is released in the cytosol via the endoplasmic reticulum. Am. J. Physiol. Ren. Physiol. 286, F617–F624.
Sastrasinh, M., Knauss, T.C., Weinberg, J.M., Humes, H.D., 1982. Identification of the aminoglycoside binding site in rat renal brush border membranes. J. Pharmacol. Exp. Ther. 222, 350–358.
Sawada, M., Nakashima, S., Kiyono, T., Nakagawa, M., Yamada, J., Yamakawa, H., Banno, Y., Shinoda, J., Nishimura, Y., Nozawa, Y., Sakai, N., 2001. p53 regulates ceramide formation by neutral sphingomyelinase through reactive oxygen species in human glioma cells. Oncogene 20, 1368–1378.
Servais, H., Jossin, Y., Van Bambeke, F., Tulkens, P.M., Mingeot-Leclercq, M.P., 2006. Gentamicin causes apoptosis at low concentrations in renal LLC-PK1 cells subjected to electroporation. Antimicrob. Agents Chemother. 50, 1213–1221.
Servais, H., Van Der, S.P., Thirion, G., Van der, E.G., Van Bambeke, F., Tulkens, P.M., Mingeot-Leclercq, M.P., 2005. Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria. Toxicol. Appl. Pharmacol. 206, 321–333.
Tang, Y., Zhao, W., Chen, Y., Zhao, Y., Gu, W., 2008. Acetylation is indispensable for p53 activation. Cell 133, 612–626.
Teske, B.F., Wek, S.A., Bunpo, P., Cundiff, J.K., McClintick, J.N., Anthony, T.G., Wek, R.C., 2011. The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. Mol. Biol. Cell 22, 4390–4405.
Tulkens, P., Trouet, A., 1978. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. Biochem. Pharmacol. 27, 415–424.
Villamil Giraldo, A.M., Appelqvist, H., Ederth, T., Ollinger, K., 2014. Lysosomotropic agents: impact on lysosomal membrane permeabilization and cell death. Biochem. Soc. Trans. 42, 1460–1464.
Walker, P.D., Shah, S.V., 1987. Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. Am. J. Phys. 253, C495–C499.
Wan, C., Ma, X., Shi, S., Zhao, J., Nie, X., Han, J., Xiao, J., Wang, X., Jiang, S., Jiang, J., 2014. Pivotal roles of p53 transcription-dependent and -independent pathways in manganese-induced mitochondrial dysfunction and neuronal apoptosis. Toxicol. Appl. Pharmacol. 281, 294–302.
Wang, X., Yen, J., Kaiser, P., Huang, L., 2010. Regulation of the 26S proteasome complex during oxidative stress. Sci. Signal. 3, ra88.
Wu, H., Lozano, G., 1994. NF-kappa B activation of p53. A potential mechanism for suppressing cell growth in response to stress. J. Biol. Chem. 269, 20067–20074.
Xiao, Z., Shan, J., Li, C., Luo, L., Lu, J., Li, S., Long, D., Li, Y., 2013. Mechanisms of cyclosporine-induced renal cell apoptosis: a systematic review. Am. J. Nephrol. 37, 30–40.
Yang, J., Duerksen-Hughes, P.J., 2001. Activation of a p53-independent, sphingolipid-mediated cytolytic pathway in p53-negative mouse fibroblast cells treated with N-methyl-N-nitro-N-nitrosoguanidine. J. Biol. Chem. 276, 27129–27135.
Yang, Y., Ikezoe, T., Saito, T., Kobayashi, M., Koeffler, H.P., Taguchi, H., 2004. Proteasome inhibitor PS-341 induces growth arrest and apoptosis of non-small cell lung cancer cells via the JNK/c-Jun/AP-1 signaling. Cancer Sci. 95, 176–180.
Yano, T., Itoh, Y., Matsuo, M., Kawashiri, T., Egashira, N., Oishi, R., 2007. Involvement of both tumor necrosis factor-alpha-induced necrosis and p53-mediated caspase-dependent apoptosis in nephrotoxicity of cisplatin. Apoptosis 12, 1901–1909.
Yoneda, T., Imaizumi, K., Oono, K., Yui, D., Gomi, F., Katayama, T., Tohyama, M., 2001. Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. J. Biol. Chem. 276, 13935–13940.
Yu, J., Tiwari, S., Steiner, P., Zhang, L., 2003. Differential apoptotic response to the proteasome inhibitor Bortezomib [VELCADE, PS-341] in Bax-deficient and p21-deficient colon cancer cells. Cancer Biol. Ther. 2, 694–699.
Zeidan, Y.H., Hannun, Y.A., 2010. The acid sphingomyelinase/ceramide pathway: biomedical significance and mechanisms of regulation. Curr. Mol. Med. 10, 454–466.
Zeng, F., Yu, X., Sherry, J.P., Dixon, B., Duncker, B.P., Bols, N.C., 2016. The p53 inhibitor, pifithrin-alpha, disrupts microtubule organization, arrests growth, and induces polyploidy in the rainbow trout gill cell line, RTgill-W1. Comp Biochem. Physiol C. Toxicol. Pharmacol. 179, 1–10.
Zhang, L., Hu, J.J., Gong, F., 2011. MG132 inhibition of proteasome blocks apoptosis induced by severe DNA damage. Cell Cycle 10, 3515–3518.