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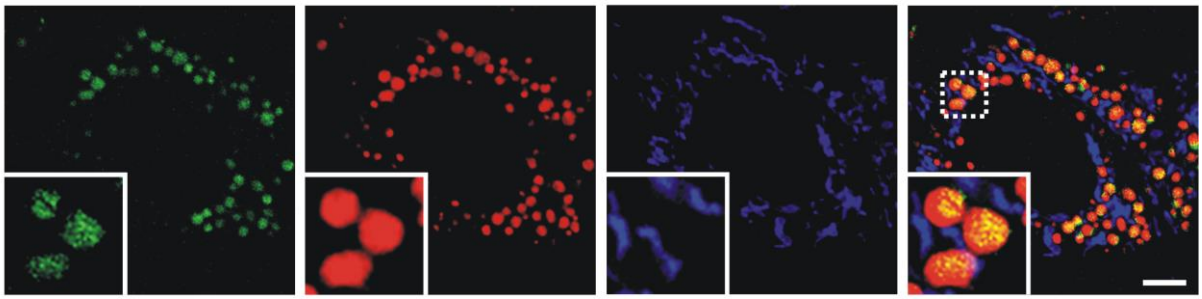
Intracellular mechanisms of apoptosis induced by aminoglycoside antibiotics



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Promoteur : Professeur Marie-Paule Mingeot-Leclercq
Co-promoteur : Professeur Paul M. Tulkens

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“Ne demande pas ton chemin à quelqu’un qui le connaît

car tu ne pourrais pas t’égarer »

Rabbi Nahman de Braslav

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ABBREVIATION LIST

AME : Aminoglycoside modifying enzyme
APAF-1 : Apoptotic peptidase activating factor-1
ASK1 : Apoptosis signal-regulating kinase 1
ATF4 : Activating transcription factor 4
Bax : Bcl-2 associated X protein
Bcl-2 : B-cell lymphoma-2
DNA : Desoxyribonucleic acid
DR5 : Death receptor 5
GCN2 : General control non-repressed 2
GFR : Glomerular filtration rate
GRP : Glucose-regulated protein
H_2DCFDA : 2',7'-dichloro-dihydro-fluorescein diacetate
HIV : Human immunodeficiency virus
HRI : Heme-regulated eIF2α kinase
Hsp : Heat shock protein
iNOS : inducible Nitric oxide synthase
LLC-PK1 : Lilly Laboratories Cells Pig Kidney Type I
LMP : Lysosomal membrane permeabilization
MAP kinase : Mitogen-activated protein kinase
MDCK : Madin-Darby canine kidney cells
Mdm2 : Murine double minute 2
MOMP : Mitochondrial outer membrane permeabilization
PDI : Protein disulfide isomerase
Pidd : p53 induced protein, with death domain
PKR : Double stranded RNA (ds-RNA)-activated protein kinase
Puma : p53-upregulated mediator of apoptosis
RNA : Ribonucleic acid
ROS : Reactive oxygen species
SAPK/JNK : Stress-activated protein kinase/c-Jun NH2-terminal kinase
shRNA : small hairpin ribonucleic acidsiRNA : small interfering ribonucleic acid
TUNEL : Terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick-end labeling
XIAP : X-linked inhibitor of apoptosis

CHAPTER I. Introduction

I.1. Concept of apoptosis

Apoptosis and cell survival promotion are inseparable signal transduction pathways that determine the fate of cells submitted to different stress such as DNA damage, oxidative stress or exposure to pharmacological agents. Studies aiming the understanding of the underlying mechanisms have pointed out, besides nucleus, initially considered as the primary executioner of apoptosis, an active role for other cellular organelles such as mitochondria, lysosomes, endoplasmic reticulum and proteasome. This work, based on the model of aminoglycoside gentamicin-induced apoptosis in renal proximal tubular cells, aimed to get a better understanding of the organelles cross-talks in the apoptotic cell death processes.

Apoptosis is described as an active and a genetically regulated form of cell death. It is an evolutionary conserved process used by an organism to selectively eliminate cells that are no longer needed, such as damaged or infected ones (Wyllie et al., 1980). The term “apoptosis” was introduced in 1972 by Kerr and colleagues to distinct from necrosis a morphologically and biochemically different type of cell death (Kerr et al., 1972). Apoptosis is characterized by a decrease of cellular volume and the maintenance of membrane integrity until the late stages of the process. Condensation of chromatin in one or more masses in the nucleus is one of the first detectable morphological features. It is followed by DNA cleavage at the internucleosomal linker region and production of DNA fragments that are multimers of about 180 bp (Wyllie et al., 1980). Other characteristics are cytoplasmic condensation and finally cellular fragmentation into membrane-bound fragments. These fragments are called apoptotic bodies and are taken up by other cells and degraded in phagosomes (Kerr et al., 1972).

Apoptosis is primarily a physiological phenomenon. It plays a crucial role during embryogenesis, and later in the maintenance of homeostasis, by clearance of old or damaged cells, and contributes to the maturation and function of the immune system (Lockshin and Zakeri, 2007; Krammer, 2000). Inappropriate apoptosis (either too little or too much) is involved in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Thompson, 1995; Elmore, 2007). Apoptosis can result from an exposition of the cell to a toxic substance or a drug, such as aminoglycosides (Servais et al., 2008). Cell death through apoptotic or necrotic pathway is depending on both the nature and the severity of the insults, evolving from apoptotic to necrotic form of cell death. Indeed, the same insult in a mild form can lead to apoptosis and when severe can lead to necrosis (Ueda et al., 2000; Yu et al., 2003b).

I.2. Why to be interested in aminoglycoside antibiotics in 2013?

Aminoglycosides antibiotics are important antibiotics to treat severe infections due to Gram-negative bacteria.

Aminoglycosides are based on an essential core, composed of a six-membered ring with amino-group substituents, named aminocyclitol. Two or more amino-containing or non-amino-containing sugars are linked to this ring by glycosidic bonds. The central aminocyclitol is 2-deoxystreptamine for all current aminoglycosides (except streptidine for streptomycin).

Aminoglycosides are highly soluble in water and insoluble in organic solvents. They have a molecular weight in the range of 445 to 600 daltons. Individual pKa values of the amino-groups range from 5.5 to 9 (Botto and Coxon, 1983). Hence, at pH 7.4 as well as in acidic environments, as is the case in lysosomes where they accumulated, aminoglycosides are positively charged.

Gentamicin, elaborated by *Micromonospora* spp., is frequently used in clinical practice. It is a mixture of three closely related components, C_1, C_{1a} and C_2, with glycosidic linkages at positions 4 and 6 (see Fig. 1). The three components, as well as gentamicin C_{2a} and C_{2b} differ in the degree of methylation in the 2-amino-hexoses ring. Gentamicin C_{1a} lacks the methyl group in 6' position of ring I, while gentamicin C_1, C_2 and C_{2a} have this methyl group in 6' position. Gentamicin C_1 and C_{2b} are also N-methylated in this position, while C_{1a}, C_2 and C_{2a} have free amines instead.

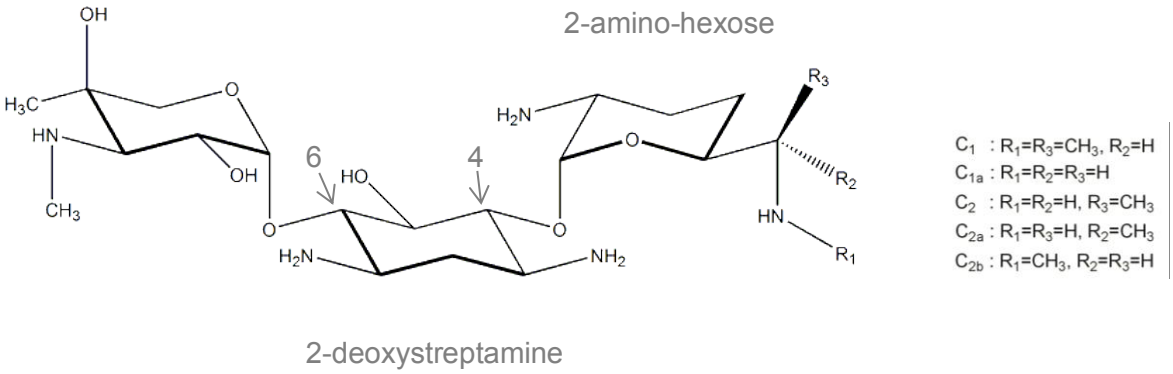


Fig.1. Chemical structure of gentamicin. The molecule is based on a 2-deoxystreptamine central aminocyclitol, with glycosidic linkages in positions 4 and 6. Gentamicin used for clinical practice is a mixture of the constituents C1, C1a and C2 which differ in the degree of methylation of the cycle 2-aminohexose.

The **current most common clinical indication** for aminoglycosides is in combination therapy with other antimicrobial agents for serious Gram-negative bacillary infections. Aminoglycosides are also important drugs in the treatment of mycobacterial infections and in infections caused by less common pathogens, such as *Yersinia pestis, Brucella* spp, and *Francisella tularensis.* In addition, they are used in combination with cell-wall-active antibiotics to provide synergistic bactericidal activity in treatment of serious Gram-positive coccal infections such as staphylococcal, enterococcal, and streptococcal endocarditis (Chen and Kaye, 2009).

Unfortunately, induction of oto- and nephrotoxicity (see further) has limited their use, particularly since other therapeutic alternatives (third and fourth generation cephalosporins, β-lactams and β-lactamase inhibitors combination, and fluoroquinolones) have been available. However, due to emergence of resistance to most of these new agents, associated to other advantages of aminoglycosides such as chemical stability, fast bactericidal effect, synergy with betalactamic antibiotics, low cost, better knowledge of toxicity mechanisms and adaptation of posology (once daily administration) leading to a better control of toxicity apparition, aminoglycosides have undergone a **resurgence in use** (Drusano et al., 2007) and still remain an important effective **therapeutic alternative against germs insensitive to other antibiotics** (Lopez-Novoa et al., 2011). Furthermore, other potential applications of aminoglycosides as **antivirals/HIV** and **for the treatment of genetic diseases** maintain aminoglycosides as molecules of great interest. Moreover, gentamicin is used as reference nephrotoxicant in new molecules toxicity studies, development, and identifying safety biomarker candidates (Houghton et al., 2010; Com et al., 2012)

First, importance of aminoglycosides in the treatment of bacterial infections can be explained by the fact that multidrug-resistant Gram-negative bacteria causing severe nosocomial infections are not sensitive to most currently approved antibiotics. One approach proposed for the **treatment of infectious diseases** is the development of new molecules by continuing to modify existing classes of previously successful antibiotics (Bush and Pucci, 2011). For aminoglycosides, at least one of these new molecules has advanced into human clinical trials. Plazomicin (ACHN-490), a next generation aminoglycoside under development at Achaogen, Inc., is a semi-synthetic compound derived from sisomicin (Armstrong and Miller, 2010). Structural modifications render this compound resistant to the effects of aminoglycoside modifying enzymes (AMEs) thus conferring activity against aminoglycoside-resistant bacterial isolates. This molecule is presently in clinical trials for treatment of pneumonia and complicated urinary tract infections (Galani et al., 2012).

A second interesting role of aminoglycosides is based on their **anti-viral properties** that give place to use aminoglycoside-based compounds in the treatment of HIV. Aminoglycosides and aminoglycoside derivatives/conjugates are able to target many steps in the HIV life cycle, and also have the ability to induce the production of retrocyclins, making the development of these compounds the goal of many research efforts for HIV treatment and prevention in the recent years (Houghton et al., 2010). Targets in the HIV viral life cycle include multiple receptors or intermediates involved in the entry of HIV-1 to the host cell such as the glycoprotein-120 (gp-120)-cluster of differentiation 4 (CD4) binding, gp120-CXC chemokine receptor 4 (CXCR4) binding, and gp41 hairpin formation. The later stages are affected through interaction with regulatory RNAs, namely the HIV-1 Tat-responsive element (TAR) and HIV-1 Rev responsive element (RRE) (Lapidot et al., 2008).

Aminoglycosides also target the HIV-dimerization initiation site (DIS), which is responsible for initiating genome dimerization by forming a loop-loop complex, a vital step in the replication cycle (Ennifar et al., 2007). Finally, another possible mechanism of action of aminoglycosides is their ability to cause RNA cleavage upon binding, which might cause enough distortion in the RNA backbone to make it susceptible to intramolecular transesterification (Belousoff et al., 2009).

Other viruses are also targets of aminoglycosides such as influenza A virus, the promoter (vRNA) of which can interact with aminoglycosides at submicromolar concentrations. It has been shown that the binding of neomycin will induce conformational change which would affect further transcription processes. With respect to this mechanism, aminoglycosides represent lead compounds for the discovery of antiviral therapeutics against influenza A virus (Kim et al., 2012).

Finally, aminoglycosides have an emerging role in **nonsense mutation suppression** as a therapeutic strategy for producing a full-length protein from disease-causing premature stop mutations in mammalian cells. Premature termination codons, often the result of base pair insertion, deletion or substitutions, generally lead to the production of truncated, nonfunctional proteins. In humans, premature termination codons have been linked to over 1800 genetic disorders and, consequently, the suppression of premature termination codons is an attractive strategy in the treatment of many genetic disorders.

Aminoglycoside antibiotics bind to the decoding site of ribosomal RNA of procaryotes and eukaryotes and bring about a conformational change that allows codon-anticodon pairing during translation. They reduce discrimination between cognate and near-cognate tRNA, permitting an amino-acid to be inserted at the stop codon. The net effect is continuation of translation through the natural stop codon. Figure 2 shows an example of gentamicin-

induced read-through in a premature stop codon in the voltage-dependent potassium channel Kv1.5 translation.

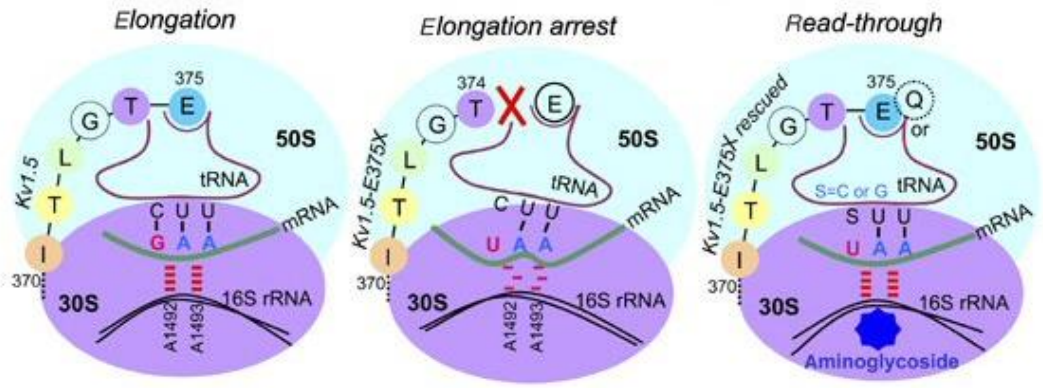


Fig.2. Aminoglycoside-induced read-through of the premature E375X stop codon in voltage-dependent potassium channel Kv1.5. Normally, tRNA carrying glutamic acid (E) matches the mRNA codon to process Kv1.5 polypeptide elongation. Matching of the mRNA codon to the proper tRNA anticodon results in conformational alignment of A1492 and A1493 in the ribosomal decoding center (red dashes) and polypeptide chain elongation. E375X (UAA codon in mRNA) mutation prevents codon–anticodon pairing and excludes the possibility of the A1492 and A1493 alignment in the ribosomal decoding center (red dashes) terminating protein translation. Aminoglycoside binding to 16S rRNA induces conformational alignment in the ribosomal decoding center despite codon/anticodon mismatch. In the presence of aminoglycosides, the UAA codon may be paired with CUU or GUU tRNA anticodon promoting polypeptide chain elongation with glutamate or glutamine. Reproduced from (Zingman et al., 2007) .

This concept was first investigated in cystic fibrosis after report of expression of full-length cystic fibrosis transmembrane conductance regulator protein and the restoration of its cyclic AMP-activated chloride channel activity in HeLa cells harboring premature stop codon mutations following treatment with gentamicin (Howard et al., 1996). The findings on cystic fibrosis with aminoglycosides provided a template for other genetic studies which follow, as for Duchenne muscular dystrophy, caused by mutations in the corresponding gene whose large size predisposes to an increased incidence of mutations. With regard to the potential for aminoglycosides read-through, it is estimated that 13-15% are nonsense mutations that create a premature termination codon in the reading frame (Malik et al., 2010). Although clinical efficiency for gentamicin in treatment of Duchenne muscular dystrophy have not yet been demonstrated, Nudelman and colleagues developed three different novel aminoglycosides, NB54, NB74, and NB84 with interesting properties as superior efficiency in suppression of diseases causing non-sense mutations and lower cell-toxicity and ototoxicity compared with gentamicin (Nudelman et al., 2010; Nudelman et al., 2009).

I.3. The nephrotoxicity of aminoglycosides, a major drawback.

To take benefits of aminoglycoside antibiotics, it is important to control their toxicity and therefore to understand the mechanisms involved in their side effects.

Aminoglycosides are known to induce oto- and nephrotoxicity; in the context of this work, we will focus only on nephrotoxicity.

Aminoglycoside nephrotoxicity remains a common and potentially serious clinical problem. In humans treated at therapeutic doses, aminoglycosides induce conspicuous and characteristic changes in lysosomes of proximal tubular cells consistent with the accumulation of polar lipids (myeloid bodies) (de Broe et al., 1984). These changes are preceeded and accompanied by signs of tubular dysfunctions or alterations (release of brush border and lysosomal enzymes; decreased reabsorption of filtered proteins; wasting K^+, Mg^{2+}, Ca^{2+}, and glucose; phospholipiduria; and cast excretion). The occurrence of these signs may be followed by the development of overt renal failure characterized by a nonoliguric and even polyuric fall in creatinine clearance, with a slow rise in serum creatinine and a hypoosmolar urinary output developing after several days of treatment. Progression to oliguric or anuric renal failure is infrequent, and recovery upon drug discontinuation is often observed (Mingeot-Leclercq and Tulkens, 1999).

Despite the accurate control and follow-up exercised on patients, incidence of aminoglycoside nephrotoxicity reaches 10-25% of the treatments (Lopez-Novoa et al., 2011) and is associated with increased risk of clinical mortality (Selby et al., 2009). The incidence of renal damage varies largely depending on the target population. Main risk factors for developing aminoglycosides nephrotoxicity have been identified as older age, reduced renal function, duration of the treatment, hepatic dysfunction or interactions with other drugs (Moore et al., 1984). Therefore, patients in intensive care units, for whom aminoglycoside treatment can be necessary, are particularly susceptible to develop acute kidney injury, which is associated with a high rate of mortality (Oliveira et al., 2009). Moreover, besides the acute and short-term problem generated by acute kidney injury, some long term effects on renal function are also suggested (Ponte et al., 2008).

Gentamicin nephrotoxicity probably results from a combination of tubular, glomerular, and vascular effects, with an important component of tubular injury (Lopez-Novoa et al., 2011). The tubular toxicity of gentamicin involves the death of tubular epithelial cells, mainly within the proximal segment, and the non-lethal functional alteration of key cellular components involved in water and solute transport. The contribution of glomerular to the reduction of glomerular filtration rate is also suggested through a process that involves mesangial

contraction (Martinez-Salgado et al., 2007). Reduction of renal blood flow by gentamicin causes a decrease in glomerular filtration and sensitizes tubule cells to cell death by reduction of oxygen and ATP availability. The majority of data concerning glomerular effects of gentamicin was obtained in rats treated with high doses of gentamicin (> 80 mg/kg/day; for review, see (Lopez-Novoa et al., 2011). In this work, we focused on proximal tubular effects of gentamicin at low, therapeutically relevant doses.

I.4. Cellular mechanisms involved in apoptosis induced by aminoglycoside antibiotics and pending questions

Mechanisms underlying aminoglycosides-induced nephrotoxicity have been extensively studied for the last decades (Quiros et al., 2011), but remain unfortunately incompletely understood. Gentamicin accumulates in the renal cortex and induces renal morphological changes very similar in humans and experimental animals (Luft et al., 1977). Although toxicity has been characterized in humans (de Broe et al., 1984), available data on the mechanisms responsible for aminoglycoside antibiotics nephrotoxicity has mostly been obtained both in animal and cellular experimental models (Williams, 1989; Servais et al., 2005; Servais et al., 2006; Negrette-Guzman et al., 2013; Houghton et al., 1978; El Mouedden et al., 2000a; Chen et al., 2011).

Observation that aminoglycosides induce apoptosis in rats treated with therapeutically-relevant doses (El Mouedden et al., 2000a) has shed new lights on the mechanisms of the early stages of nephrotoxicity. Rats show a clearly detectable apoptotic reaction in the proximal tubules after 4 days of treatment, which becomes conspicuous after 10 days. This apoptosis has been shown to be dose-dependent, occurring in absence of necrosis, and is nonlinearly correlated with the proliferative response. No simple correlation with induction of phospholipidosis (see further) can be drawn.

Two renal cell lines (LLC-PK1 and MDCK, proximal and distal tubular cells respectively) have been used to further characterize and quantify this apoptotic process by electron microscopy, terminal deoxynucleotidyl tranferase-mediated dUTP-biotin nick-end labeling of fragmented DNA (TUNEL) and DNA size analysis (oligonucleosomal laddering). Apoptosis can also be reproduced in non-renal (embryonic fibroblasts) cell lines, suggesting that specific gentamicin nephrotoxicity after systemic administration is related to its capacity to accumulate in cortical proximal tubular renal cell (El Mouedden et al., 2000b).

It is now clear that the major role in apoptosis is played by cysteine proteases from the **caspase family**, and that their activation, which can be achieved in several ways, is a

critical step in apoptosis induction (Pop and Salvesen, 2009). The main pathways of apoptosis induction are the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (see Fig.3). Both pathways have been described to be activated by gentamicin (Servais et al., 2005; Juan et al., 2007), and can converge to mitochondria which can be considered as the central organelle in apoptosis. Mitochondrial outer membrane permeabilization (MOMP) allows the release to the cytosol of cytochrome c, which will form with the apoptotic peptidase activating factor-1 (APAF-1) a multimeric complex called apoptosome. Apoptosome recruits and activates pro-caspase 9. Activated caspase 9 activates in turn effector caspases 3 or 7 that lead to apoptosis progression. The main guardians of mitochondrial integrity are the **proteins from Bcl-2 family** (Kroemer et al., 2007; Danial and Korsmeyer, 2004). Whereas proapoptotic proteins such as Bax and Bak are responsible for pore formation, the role of the antiapoptotic family members such as Bcl-2, Bcl-X_L and Mcl-1 is to prevent the proapoptotic function of Bax and Bak. Finally, proapoptotic proteins from the BH-3 only family serve as cell death sensors and can shift the balance toward apoptosis (Youle and Strasser, 2008).

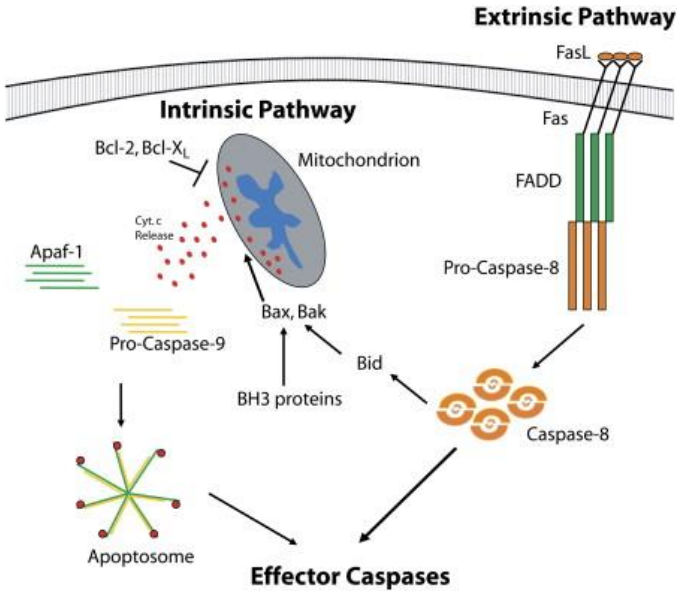


Fig.3. Caspase activation via the intrinsic and extrinsic pathways of apoptosis. Intrinsic pathway of apoptosis involves mitochondrial outer membrane permeabilization by pro-apoptotic Bcl-2 proteins Bak and Bax. This can be prevented by anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-x_L. The membrane permeabilization allows the release from cytochrome c to the cytosol and the formation of a complex named apoptosome involving Apaf-1, cytochrome c and the pro-caspase 9. Activation of caspase 9 in the apoptosome induces activation of effector caspases. Extrinsic pathway is initiated by the ligation of death recpetors with their cognate ligands, leading to the recruitment of adaptator molecules such as Fas-associated death domain protein (FADD), and then caspase 8. This results in the dimerization and activation of caspase 8, which can then directly cleave and activate effector caspases 3 and 7, leading to apoptosis. Crosstalk between the extrinsic and intrinsic pathway occurs through the activation of the BH3-only protein Bid by caspase 8, which is in turn able to activate Bax and Bak. Reproduced from (Schafer and Kornbluth, 2006)

Although mechanisms of intracellular pathways that could be implicated in aminoglycoside-induced apoptosis have been studied (Servais et al., 2005), they are not fully

understood. The main objective of our work was to understand the mechanism involved in the lysosomal membrane permeabilization and to shed some light on the puzzle of the cross-talks between cellular organelles induced by gentamicin.

I.4.1. Cellular uptake

In proximal tubular cells, aminoglycosides can be taken up after glomerular filtration from both basolateral and luminal membrane (Pastoriza-Munoz et al., 1979; Ishikawa et al., 1985; Williams and Hottendorf, 1986), although binding and uptake by brush border membrane predominate (Collier et al., 1979). Concentration of aminoglycosides in renal cortex is several times higher than in plasma. Accumulation of gentamicin in proximal tubular cells was first attributed to adsorptive **endocytosis** by binding of the drug to **acidic phospholipids** (Sastrasinh et al., 1982), such as phosphatidylinositol of the brush-border membrane (Gabev et al., 1989; Schacht, 1979). But this could not easily explain the high specificity of uptake by kidney because acidic phospholipids are commonly distributed in plasma membrane of various tissues. It is the discovery of **megalin** and the subsequent observation that it mediated the uptake of polybasic drugs, including gentamicin (Moestrup et al., 1995; Nagai et al., 2001) that allowed to explain this phenomenon. Currently, both phospholipids adsorptive-mediated endocytosis and megalin receptor-mediated endocytosis are considered to be involved in gentamicin cellular uptake (Nagai et al., 2006).

Adsorptive endocytosis is defined as a low-affinity and high-capacity mechanism to internalize molecules, in comparison with high-affinity receptor-mediated endocytosis. Moreover, endocytosis of fluid and surface adsorbed proteins is clathrin-independent, whereas receptor mediated endocytosis initiated at coated pits is clathrin-mediated (Stromhaug et al., 1997). **Megalin**, also called LRP-2, is a brush border membrane receptor that belongs to the low-density lipoprotein (LDL) receptors family (Moestrup et al., 1995). This family includes structurally related endocytic receptors that include the low-density lipoprotein (LDL) receptor, apoE receptor 2, LDL-receptor-related protein (LRP) and the very low density lipoprotein (VLDL) receptor.

Originally identified as an autoimmune target antigen in Heymann nephritis, megalin is an important endocytic receptor for clearance of low molecular weight plasma proteins from the glomerular filtrate. It is abundant in the apical endocytic pathway of renal proximal tubule including microvilli and can be detected in podocytes in some species (Moestrup, 1994). Physiological ligands taken up by megalin includes insulin, transthyrethin and carriers for lipophilic vitamins (for review, see (Christensen and Birn, 2002). Megalin may also be responsible for the clearance of xenobiotic compounds from the primary urine. In particular,

polybasic substances such as aminoglycosides may interact with abundant negative charges on the extracellular receptor domain thereby gaining entrance to proximal tubular cells (Moestrup et al., 1995). While some experimental evidence clearly links megalin to aminoglycosides uptake (Moestrup et al., 1995; Nagai et al., 2001; Schmitz et al., 2002), the quantitative contribution of the receptor to aminoglycoside accumulation remains unclear. However, megalin, perhaps in cooperation with acidic phospholipids, is probably the main driver in the uptake of aminoglycosides in kidney (Nagai et al., 2006).

I.4.2. Lysosomal accumulation

Through **endocytosis**, aminoglycosides enter in cells within an endocytotic vesicle named endosome, which then fuses with the lysosomes (Just et al., 1977; Silverblatt and Kuehn, 1979), where the drug mostly accumulates, to reach concentrations evaluated at 30 mM and above (El Mouedden et al., 2000b).

Lysosomes have essential functions of degradation of endocytosed materials, and are also responsible for the normal turnover of organelles and long-lived proteins by autophagocytotic degradation. Lysosomes exist in all types of animal cells, except in erythrocytes which present a minimal turnover of their constituents. The lysosomal compartment consists of numerous acidic vesicles (pH ~ 4 to 5) that constantly fuse and divide. The acidic environment is maintained by ATP-dependent proton pumps present in the lysosomal membrane (Schwake et al., 2013). Lysosomal hydrolases are synthesized in the endoplasmic reticulum, are tagged with mannose-6-phosphate and directed to the *trans*-Golgi network with the help of mannose-6-phosphate receptors (Coutinho et al., 2012). The newly produced hydrolases are transported to slightly acidic (pH ~ 6) late endosomes, which arise from early endosomes containing endocytosed material. Lysosomal hydrolases are there freed from mannose-6-phosphate receptors and activated, while the receptors are re-circulated to the Golgi apparatus. The late endosomes then mature to lysosomes that lack mannose-6-phosphate receptors, are rich in acid hydrolases, have a pH of 4-5 and contain endocytic material to be degraded.

Lysosomal membrane is thick (7-10 nm) and possess a number of highly specialized proteins to allow the transport of products of lysosomal catabolism and certain cytosolic compounds which are destined to degradation. To withstand the luminal milieu with acidic pH and hydrolases, lysosomal membrane proteins are usually glycosylated probably forming a continuous glycoprotein layer at the luminal side of lysosomal membrane (Fukuda, 1991). The most abundant proteins of lysosomal membrane are the lysosomal associated membrane proteins LAMP-1 and LAMP-2 with more than 10 used glycosylation sites. The

thickness of lysosomal glycoprotein coat is estimated at 8 nm (Wilke et al., 2012). The specialized glycoprotein layer may be important to regulate the stability and integrity of lysosomes. It may indirectly modulate the fusion of lysosomes with phagosomes, autophagosomes ro with the plasma membrane during exocytosis (Schwake et al., 2013), short cytoplasmic domains bearing single (usually di-leucine) or double lysosome-targetting motives (such as cystinosin) and mediating interaction with regulatory machineries controlling fusion/fission events or signalling to the nucleus.

Lysosomes altered by the treatment with gentamicin do not fuse, or fuse less effectively, with incoming pinocytic vesicles. These lysosomes, therefore, appear somewhat excluded from the endocytic pathway in the cell (Giurgea-Marion et al., 1986).

Although endocytic uptake and trafficking were for a long time believed to direct the compound via the endocytic route exclusively to lysosomes, Sandoval and Molitoris have suggested that an alternative pathway transports about 10% of endocytosed aminoglycosides in a **retrograde manner through the Golgi complex and the endoplasmic reticulum** (ER), where they are probably transported to the cytoplasm (Sandoval and Molitoris, 2004).

I.4.3. Inhibition of lysosomal phospholipases and formation of myeloid bodies

In the lysosomes, gentamicin induces alteration of lipid metabolism resulting into phospholipidosis and lysosomal overload (de Broe et al., 1984). Multilamellar structures known as myeloid bodies are generated (Houghton et al., 1978), whose pathophysiological role remains uncertain.

Impairment by aminoglycosides of acid sphingomyelinase and other lysosomal phospholipases has been highlighted (Laurent et al., 1982; Carlier et al., 1983). Hydrolysis of phospholipids as phosphatidylcholine is critically dependent on the negative charges carried by the bilayer. Gentamicin, which is polycationic and fully protonated in lysosomes where it is exposed to a fairly acidic pH (4.5-5), impairs the activities of lysosomal phospholipases towards phosphatidylcholine by decreasing the available negative charges (Mingeot-Leclercq et al., 1988), and perhaps by masking the accessibility of the substrate to the enzyme catalytic site (Carrier et al., 1997). Dysfunction of sphingomyelinase and phospholipase A1 induces a lysosomal phospholipidosis *in vitro* in rat cultured fibroblasts and *in vivo* in renal cortex of rats, rabbits and human treated with gentamicin (Aubert-Tulkens et al., 1979; Libert et al., 1979). These morphological and biochemical lesions have also been reported in the kidney cortex at gentamicin close to therapeutic doses (10 mg/kg)

(Laurent et al., 1982; Giuliano et al., 1984). However, this dose of gentamicin, which is considered as very low compared to the dose generally used (100-160 mg/kg), does not cause a widespread tubular necrosis but mostly focal necrosis (Giurgea-Marion et al., 1986).

The accumulation of **myeloid bodies**, which essentially contain phospholipids and proteins probably entrapped in the bilayer but little cholesterol (Appelkvist et al., 1991), results from the impaired cytoplasmic degradation of sequestered organelles membranes due to aminoglycoside accumulation, and is responsible for the subsequent tubular necrosis (Luft et al., 1975). Other types of depot were also observed at high doses of drug, like debris of mitochondria or ill-defined electron dense material, suggesting a stimulation of autophagy (Vera-Roman et al., 1975).

I.4.4. Permeabilization of lysosomal membrane

A potential link between the lysosomal accumulation of gentamicin and apoptosis induced could be **lysosomal membrane permeabilization**. The latter has been suggested by change of the emission fluorescence of acridine orange after treatment of LLC-PK1 cells with gentamicin (Servais et al., 2005).

Acridine orange is a fluorescent organic weak base which diffuses into cells and accumulates into the lysosomes and related acidic vacuoles by proton-trapping (Olsson et al., 1989). This fluorescent dye emits a green fluorescence in monomer form when in low concentration in solution or intercalated to double-stranded DNA, and a red fluorescence when polymerized in concentrated solutions or after binding to single-strand DNA or RNA.

Although changes in acridine orange red emission are interpreted as indicating lysosomal permeabilization (Lin et al., 2010; Choi et al., 2002), reversion of acidotropic sequestration due to change of lysosomal pH remains an alternative explanation (Moriyama et al., 1982). The change in acridine orange fluorescence emission ratio, detected as from 2h after gentamicin treatment, can thus reflect either disruption of the lysosomal membrane, a marked change in lysosomal pH, or both. Lysosomal membrane permeabilization thus remained hypothetical, and its underlying mechanism unclear. Several data suggest a role of reactive oxygen species (ROS) in this process.

Lysosomal membrane permeabilization would be an attractive explanation for the link between aminoglycosides lysosomal accumulation and induction of apoptosis. Causing the release of gentamicin or of cysteine- and aspartyl-cathepsins, this permeabilization could cause apoptosis by either direct interaction of released molecules with mitochondria or

activation of cell death signaling pathways. Previous experiments show the ability of gentamicin to induce a fast and marked increase of the release of entrapped calcein from liposomes when their composition is similar to the one of lysosomal membranes and at acidic pH (5.4). The release of calcein was slower when the composition of liposomes mimicked that of inner and outer mitochondrial membrane (Servais et al., 2005).

A potential mechanism explaining lysosomal membrane permeabilization is the production of ROS. **Free radicals** are molecules carrying one or more unpaired electrons, thus particularly reactive. They are normally produced by enzymes such as xanthine oxidase, cyclooxygenase, the cytochrome P450s, and by the electron transport chain in mitochondria (Cadenas, 2004). Under normal conditions, reactive oxygen species (ROS) are rapidly detoxified by the cell. However, when rate of ROS production greatly increases, scavenging by the cellular defense mechanisms can be surpassed.

The first ROS to be formed from dioxygen is the superoxide radical, O_2^-. Superoxide is not very reactive by itself but is the precursor of very reactive compounds. In conditions of inflammation, superoxide reacts with iNOS-derived NO and produces peroxinitrite ($ONOO^-$), which can induce heavy nitrosative stress. Addition of a second electron to superoxide gives the peroxide ion O_2^{2-}, which is not a free radical by itself. However, at neutral pH values, O_2^{2-} becomes protonated to yield hydrogen peroxide, H_2O_2. H_2O_2 crosses cellular membranes mainly through aquaporins (Bienert et al., 2007) and undergoes cleavage when interacting with transition metals (known as Fenton reaction), there giving the hydroxyl radical, OH^-. This is one of the most reactive short-live and dangerous free radical species, and it reacts with a wide number of cellular constituents so as to cause enormous damages to biological molecules.

Implication of ROS production in apoptotic processes can be confirmed by the **protective effect afforded by antioxidant molecules** such as *N*-acetylcysteine (NAC), catalase (He et al., 2013; Sancho et al., 2003) and superoxide dismutase (SOD).

N-acetylcysteine (NAC) is metabolized into the intracellular antioxidant glutathione (GSH). Both NAC and GSH are capable of neutralizing ROS by conjugation with electrophiles. The sulfhydryl (SH) group (pKa 9.52) is responsible for a great part of the metabolic activity of NAC, while the acetyl-substituted amino group makes the molecule more stable against oxidation and metabolization. The pKa of carboxylic acid moiety is 3.25.

The exact mechanism for the NAC entry into cells is not completely understood. NAC bearing a five carbon backbone and a net negative charge, has been shown to be a substrate for the organic anion transporter 1 (OAT1) in the kidney, as well as the anion exchanger 1 (AE1) transporter in erythrocytes (Koh et al., 2002; Raftos et al., 2007). The AE1 transporter is also found in α-intercalated cells in the distal nephron of the kidney, where

it transports bicarbonate in exchange for chloride across the basolateral membrane. Whether NAC is a substrate for AE1 transporter in the kidney is unknown (Walsh et al., 2008; Pang et al., 2008). NAC may also diffuse across the cell membranes (Aoyama et al., 2006; Holdiness, 1991; Moldeus et al., 1986), and has been shown to be taken up by cultured hepatocytes (Banks and Stipanuk, 1994).

Once within kidney cell cytosol, NAC can be converted to cysteine by acylases. Acylases, primarily acylase I (N-acyl-L-amino acido amidohydrolase, EC 3.5.1.14), are cytosolic enzymes that catalyze deacetylation of *N*-acyl-L-amino acids such as NAC. Acylase I has been localized in the glomeruli, proximal and distal convoluted tubules (Uttamsingh et al., 2000; Yamauchi et al., 2002). The cysteine so realeased from NAC deacetylation can then be used for GSH synthesis by the cell.

As a source of SH group, NAC can stimulate GSH synthesis, enhance glutathione-S-transferase activity, promote detoxification, and act directly on reactive oxidant radicals. In cell culture expriments, NAC promotes the uptake of cysteine from the culture medium for cellular GSH biosynthesis. Since SH groups are essential for defense against reactive oxygen species, NAC is a powerful scavenger of hypochlorous acid, and is capable of reducing hydroxyl radicals and hydrogen peroxide (De Vries and De Flora, 1993).

Catalase (EC 1.11.1.6) is a source of protection against oxidative stress by scavenging hydrogen peroxide, decomposing H_2O_2 into H_2O and O_2. Catalase is an homotetramer (four equal subunits of 60kDa each). Each subunit contains iron bound to a protoheme IX group. Catalase is concentrated in peroxisomes of numerous tissues, and is present in important amounts in the kidney. However, addition of exogenous catalase is known to have protective effects against lipid peroxidation and oxidative stress-induced renal cells injury (Thamilselvan et al., 2000).

The optimum pH for catalase activity is 7, but no change in activity is detected in a range of pH from 5,1 to 8, and exogenous catalase, once internalized into lysosomes, is active therein (Muro et al., 2003).

Superoxide dismutases (SOD; EC 1.15.1.1) are a group of low-molecular weight metalloproteins present in all aerobic cells. They catalyze dismutation of superoxide anion into oxygen and hydrogen peroxide, and play a critical role in the defense of cells against the toxic effects of oxygen radicals.

A **potential source of ROS production in the cell is iron-catalyzed Fenton reactions** which result in the production of highly reactive hydroxyl radicals from H_2O_2 ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+}+OH^-+OH^{\bullet}$). These generated free radicals can induce the peroxidation of adjacent lipids and proteins and cause oxidative damage to DNA. To avoid this pro-oxidant

effect of iron, intracellular free iron levels are carefully controlled by proteins, from iron uptake (transferrin, transferrin receptor), to iron storage (ferritin) and iron export (ferroportin and ceruloplasmin) (Kruszewski, 2003). Intracellular iron levels have been described to increase in cell normal aging, senescence or in some age-related diseases such as Alzheimer's disease, Parkinson's disease or type II diabetes and macular degeneration (Lin et al., 2010). Although the mechanisms by which iron accumulates is not well understood, lysosomes, which are the main cellular organelles responsible for the normal turnover of organelles and long-lived proteins by autophagocytic degradation, and for degradation of extracellular endocytosed material (Klionsky and Emr, 2000), have been suggested to play an important role in the intracellular regulation and homeostasis of iron metabolism (Kurz et al., 2008).

The breakdown of iron-containing material such as metalloproteins induces accumulation of significant concentrations of **labile iron** within the lysosomes, where it participates to Fenton reactions and ROS production (Kurz et al., 2006). Since many autophagocytosed macromolecules contain iron (e.g., ferritin and mitochondrial electron-transport complexes), the lysosomal compartment become rich in this transition metal. (Kurz et al., 2008; Brun and Brunk, 1970; Persson et al., 2003; Zdolsek et al., 1993; Yu et al., 2003b). The iron content of lysosomes is thus variable and is dependent of the activity of lysosomes. Lysosomes that have recently been engaged in an autophagic degradation of iron-rich compounds should contain high concentrations of iron, while others which have been inactive for a while with respect to such autophagy may contain only negligible amounts of it (Nilsson et al., 1997; Kurz et al., 2007). These differences in lysosomal iron accumulation explain that a pronounced variation is found in the stability of individual lysosomes against oxidative stress in single cells, and also in the total lysosomal population amongst cells (Nilsson et al., 1997).

The importance of the intralysosomal pool of redox-active iron in cellular oxidant sensitivity of cultured cell lines has been assessed in J774 macrophages by Yu and colleagues, which have temporarily blocked lysosomal digestion to avoid iron accumulation, and demonstrated in these conditions a dramatic decrease in apoptotic death caused by subsequent exposure to H_2O_2 (Yu et al., 2003b). Similar experiments in human peripheral T cells yielded to the same conclusions (Ogawa et al., 2004). Delivery of iron to cells in culture, such as LLC-PK1 cells, is mediated by transferrin present in the serum added to culture medium; transferrin also contains iron in the form of ferric nitrate.

The favourable conditions for intralysosomal Fenton reactions are a low internal pH (~ 4 to 5) and the presence of reducing equivalents, such as cysteine, ascorbic acid or glutathione (Baird et al., 2006; Pisoni et al., 1990; Schafer and Buettner, 2000). At this pH, reducing agents easily reduces iron(III) to iron(II), allowing the Fenton reaction. The resultant

formation of hydroxyl radicals ($HO^{\bullet}$) could then damage and destabilize lysosomal membranes (Persson et al., 2001; Ollinger and Brunk, 1995; Brunk et al., 1995).

The release of redox-active iron from lysosomes may be a major intracellular source of free iron for the continued synthesis of new iron-containing proteins, however, the mechanisms of iron lysosomal export remain still unclear (Persson et al., 2001; Starke et al., 1985; Yu et al., 2003b).

In vitro observations demonstrated that gentamicin strongly binds to L-alpha-phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), a membrane lipid rich in arachidonic acid and that iron ions and gentamicin can simultaneously bond to phosphoinositides suggesting the existence of **ternary complexes among gentamicin, iron and phospholipids.** Peroxidation of PtdIns(4,5)P2 by iron significantly increases in the presence of gentamicin and is accompanied by arachidonic acid release. Arachidonic acid can also form with iron and gentamicin a ternary complex that reacts with lipid peroxides and molecular oxygen leading to the propagation of arachidonic acid peroxidation (Lesniak et al., 2005).

To evaluate the importance of intralysosomal iron in oxidant-dependent cellular damage, the iron chelator deferoxamine (DFO) has been largely used. Also known as desferrioxamine B, DFO is a bacterial siderophore used as iron chelator in routine clinical practice for more than 40 years for the treatment of chronic iron overload due to transfusion-dependent anemia and for treatment of acute iron intoxication (Kwiatkowski, 2011).

Being hydrophilic and with a molecular mass near 600 Daltons, DFO is taken up by the cells via endocytosis and localizes almost exclusively within the lysosomal compartment, where it seems to remain (Ollinger and Brunk, 1995; Laub et al., 1985; Cable and Lloyd, 1999). DFO binds all six co-ordination sites of iron making it inert (Graf et al., 1984), and protects cells against oxidant challenge (Yu et al., 2003b; Persson et al., 2003; Zdolsek et al., 1993; Ollinger and Brunk, 1995).

Whatever the role of the labile pool of iron present in lysosomes in apoptosis, one of the most important consequences of lysosomal membrane permeabilization should be the apoptosis induction. Lysosomal permeabilization, firstly considered as taking place in necrosis, was found to participate to apoptosis during mild oxidative stress through early translocation of the lysosomal proteases to the cytosol (Kagedal et al., 2001).

The first part of this work was dedicated to further test and to understand the mechanisms underlying the gentamicin-induced lysosomal membrane permeabilization and apoptosis; we investigated the hypothesis of ROS production

and iron implication during these events. Results are described in manuscript 1 (Denamur et al., 2011).

I.4.5. Release of lysosomal content

The next question is how lysosomes can step in induction of apoptosis. One hypothesis is that lysosomal membrane permeabilization would allow the release of lysosomal components, such as cathepsins whose role in apoptosis induction has been suggested, or gentamicin which is accumulated in huge concentrations within lysosomes.

I.4.5.1. Cathepsins

The term “cathepsin” stands for lysosomal proteolytic enzyme, and includes cysteine proteases (cathepsins B, C, H, L and S), aspartyl proteases (cathepsins D and E) and serine proteases (cathepsins A and G). Cathepsins D, B and L being probably the most important in apoptotic processes, we will focus this introduction on these enzymes.

In contrast to caspases that require activation to promote apoptosis, **cathepsins** are already active when released into the cytosol following lysosomal membrane permeabilization (Turk and Stoka, 2007). Once in the cytosol, cathepsins have to process other proteins in order to promote apoptotic signaling. Only a few substrates of cathepsins have been identified to date (Repnik et al., 2012), the major of them being the pro-apoptotic protein Bid, which was initially discovered as the caspase-8 substrate linking intrinsic and extrinsic apoptotic pathways of apoptosis. However, the processing of Bid by cathepsin D at neutral pH has not been observed *in vitro*, and additional studies are needed to clarify the role of cathepsins in Bid-mediated apoptosis (Cirman et al., 2004).

Cathepsin D is an aspartyl protease acting in proteolysis of endocytosed and autophagocytosed proteins in lysosomes. Cytosolic concentration of cathepsin D has shown a specific impact on apoptosis, and several arguments are consistent with an activity of lysosomal released cathepsin D in the cytosol. *In vitro* studies have shown that cathepsin D is stable in the pH range 1-9 and displays significant activity also above pH 6,5 (Moriyama and Takahashi, 1980; Bednarski and Lynch, 1996). Moreover, unlike cysteine proteases than can be inhibited when released into the cytosol, aspartyl proteases have no endogenous cytosolic inhibitor able to prevent extralysosomal proteolysis (Matus and Green, 1987). In the cytosol, cathepsin D triggers Bax-mediated cytochrome c release by proteolytic activation of Bid (Appelqvist et al., 2012), or direct caspase 8 cleavage (Conus et al., 2008). In T-cells, cathepsin D-mediated apoptosis involves the activation of Bax and the release of AIF and cystatin c (Bidere et al., 2003; Johansson et al., 2003).

The cysteine proteases **cathepsin B and L** can not only cleave the pro-apoptotic protein p22 Bid to generate active p15 t-Bid (Blomgran et al., 2007), but also degrade the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-X_L and Mcl-1 (Droga-Mazovec et al., 2008). Once released, cathepsins B and L are quickly inactivated at neutral pH in the cytosol due to irreversible unfolding. Yet, they can survive long enough to cleave several cellular substrates, and their cytosolic lifetime can be prolonged due to the lowering of pH in the proximity of the lysosomal leakage by the escaping protons. Another substrate described for degradation by cysteine cathepsins is the X-chromosome-linked inhibitor of apoptosis (XIAP), suggesting that mitochondria can also control apoptosis downstream of mitochondrial outer membrane permeabilization, and that other members of IAPs can be cathepsins target (Droga-Mazovec et al., 2008).

Currently, the release of cathepsins from lysosomes after gentamicin treatment has not been investigated. This would be an interesting study to perform in the next future (see discussion part).

I.4.5.2. Gentamicin

The hypothesis of a role of the release of gentamicin from lysosomes into the cytosol is supported by the results of Servais and colleagues who have shown the induction of apoptosis in LLC-PK1 cells electroporated with very low doses gentamicin (Servais et al., 2006). To test if these results could be applied to other aminoglycoside antibiotics, and would be correlated with the toxicity of different molecules, we incubated and electroporated cells with four aminoglycosides of different nephrotoxic potential: gentamicin, neomycin B, amikacin and isepamicin.

Gentamicin serves as reference for the evaluation of the nephrotoxic potential of newer molecules (Parker et al., 1982). Three other molecules were included in our study : neomycin B, which was considered as the most nephrotoxic aminoglycoside in rat model (Parker et al., 1982), and amikacin and isepamicin, both presenting a lower potential for nephrotoxicity (Matsumoto et al., 1985).

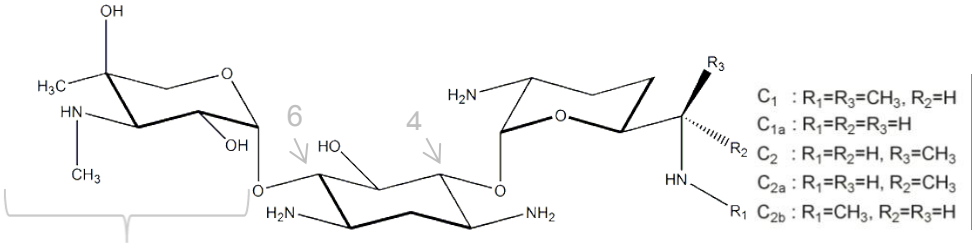
Regarding the structure of the four selected compounds (see Fig.4.), gentamicin, amikacin and isepamicin are 4,6-di-O-glycosyl, whereas neomycin B is 4,5-di-O-glycosyl derivative of the diaminocyclitol, 2-deoxystreptamine. Moreover, there are differences between the kanamycins (parent compound of amikacin) and gentamicins (which involve gentamicin C_{1a}, C_1, C_2, C_{2b} and also gentamicin B) since the amino sugar attached to the 6 position of the 2-deoxystreptamine unit is a 3-deoxy-α-D-glucose for kanamycins, and a 3-methylamino-3-deoxy-4-C-methyl-β-L-arabinose in the gentamicins.

Regarding the 2-deoxystreptamine of 4,6-disubstituted compounds, derivatives like amikacin and isepamicin, that belongs to kanamycin A and gentamicin B respectively show a peculiar

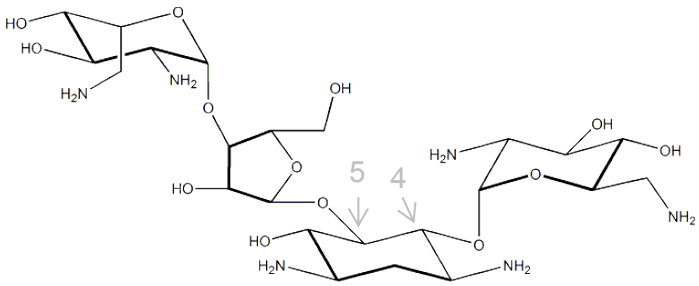
side chain (aminohydroxylacids) in position N-1. Both antibiotics are less toxic (Matsumoto et al., 1985) and more active than their parents compounds (Moellering, 1982). It was proposed that in aqueous solution, amikacin and isepamicin might adopt a conformation in which the aminohydroxypropyl (butyl) chain is bent so that its 4'''-terminal amino groups comes in close contact with the C1 and thus mimics the N1 aminogroup in kanamycin A or gentamicin B. The similarity of structure is probably still good enough for the antibiotic to keep most of its antimicrobial activity but not for recognition by aminoglycoside-modifying enzymes.

To address the potential role of cytosolic aminoglycoside for apoptosis, we compared the ability of gentamicin, neomycin B, amikacin and isepamicin, characterized by differences in their nephrotoxic potential, to induce apoptosis in LLC-PK1 cellular model. Results are described in manuscript 2 (Denamur et al., 2008).

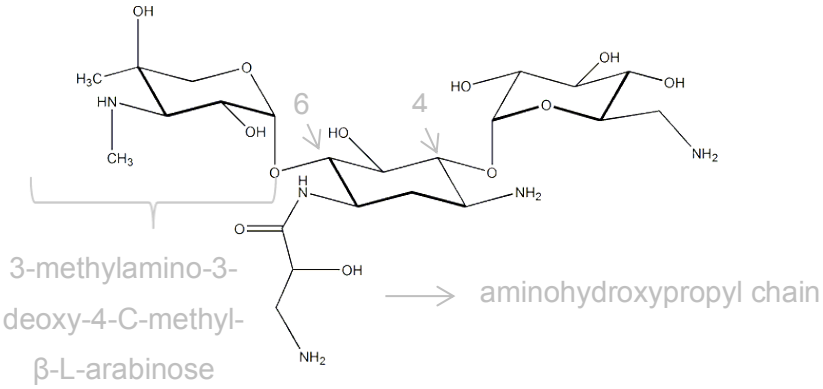
gentamicin



neomycin B



isepamicin



amikacin

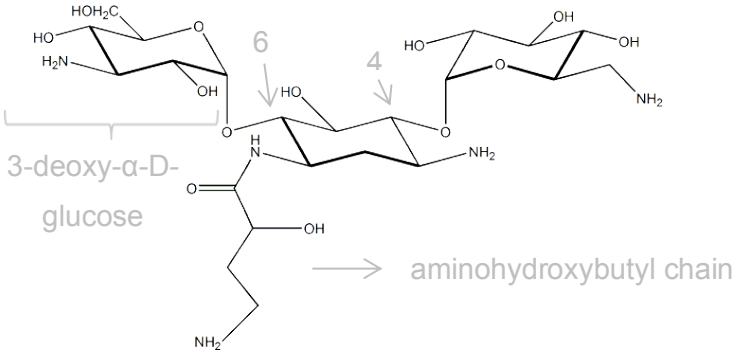


Fig.4. Chemical structures of gentamicin, neomycin B, isepamicin and amikacin.

1.4.6. Possible consequences of lysosomal membrane permeabilization

The next question is how lysosomal membrane permeabilization can be linked to mitochondrial pathway of apoptosis activated by gentamicin treatment. In this work, we considered several possibilities including p53 signalling pathway activation,cyclin-dependent kinase inhibitors accumulation, induction of endoplasmic reticulum stress and proteasome inhibition.

I.4.6.1. p53 signalling pathway activation

p53 signaling pathway activation can be a consequence of lysosomal membrane permeabilization. Indeed, p53-dependent protein Noxa is induced by lysosomal membrane permeabilization after treatment of mouse embryonic fibroblasts with H_2O_2, and is required for resulting mitochondrial outer membrane permeabilization (Eno et al., 2013). p53 is normally kept at low levels and its activity is enhanced in response to DNA damage through several mechanisms, including transcriptional upregulation and posttranslational modification (Meek, 2009). The nuclear import of p53 is an active mechanism dependent on the interaction of p53 with microtubule network and dynein (Giannakakou et al., 2000).

The **p53** transcription factor regulates the synthesis of mRNAs encoding proteins involved in diverse cellular stress responses such as cell-cycle arrest, apoptosis, autophagy and senescence. In unstressed cells, p53 is maintained at low levels by the action of Mdm2, an oncogenic E3 ligase. This mechanism implicates an autoregulatory circuit between the E3-ligase, Mdm2, and p53. Activation of p53, which localizes to the nucleus, induces Mdm2 expression. Mdm2 in turn, associates with p53 causing (i) its mono-ubiquitination and nuclear export (Geyer et al., 2000), inhibiting p53 transcriptional activity, or (ii) poly-ubiquitination, and degradation by the proteasome complex (Wu et al., 1993; Li et al., 2003). Although some degradation of p53 can occur in the nucleus, efficient degradation requires the nuclear export of the protein (Yu et al., 2000). How Mdm2 activity is regulated to effect mono- versus poly-ubiquitination of p53 is not well understood. Another demonstrated of repression of p53 activity by Mdm2 is the disruption of the ability of p53 to function as a transcription factor by binding and blocking its transactivation and DNA-bonding domains (Chen et al., 1993; Yu et al., 2006). Note that although Mdm2 is an important regulator of p53 ubiquitination, degradation and translocation, around twenty other ubiquitin E3-ligases have been identified as regulators of p53 activity (see (Jain and Barton, 2010) for review).

Upon activation by diverse stress stimluli, p53 activates transcription of hundreds of protein-coding target genes via direct binding to nearby p53 responsive elements (p53REs) and recruitment of transcriptional co-activators. p53 induces the expression of following proteins that target both the intrinsic and extrinsic pathways of apoptosis (see Fig.5) :

- **Puma** (p53-upregulated mediator of apoptosis, BBC3) is a key mediator of p53-induced apoptosis residing in the intrinsic pathway. Puma activates the pore-forming protein BAX/BAK via direct binding and/or inhibition of pro-survival BCL2 family members. Indeed, Puma allows release of cytosolic p53 held inactive by Bcl-x_L. Accumulation of cytosolic p53 and monoubiquitination then lead to Bax and Bak oligomerization (Nakano and Vousden, 2001; Yu et al., 2001).
- **Noxa** is also a p53 transcriptional target. It is a pro-apoptotic BH3-only member of the BCL-2 family of proteins that is upregulated at a transcriptional level by the nuclear protein p53 in response to cellular stresses such as DNA damage or growth factor deprivation. Noxa is able to interact with anti-apoptotic members of the BCL-2 family and causes the release of cytochrome c from mitochondria, and induction of apoptosis (Shibue et al., 2003).
- Transactivation of **death receptors FAS, DR5, and DR4** by p53 induces extrinsic apoptotic pathway (Muller et al., 1998; Wu et al., 1997; Liu et al., 2004). Moreover, p53 can also induce the relocalization of death receptors from the Golgi to the cell surface (Bennett et al., 1998).
- **p21** (CDKN1A) is a well-characterized p53 target gene and key mediator of p53-dependant cell-cycle arrest. p21 works as a cyclin-dependent kinase (CDK) inhibitor, associating with and inhibiting various cyclin-CDK complexes (el Deiry et al., 1994; Harper et al., 1995).

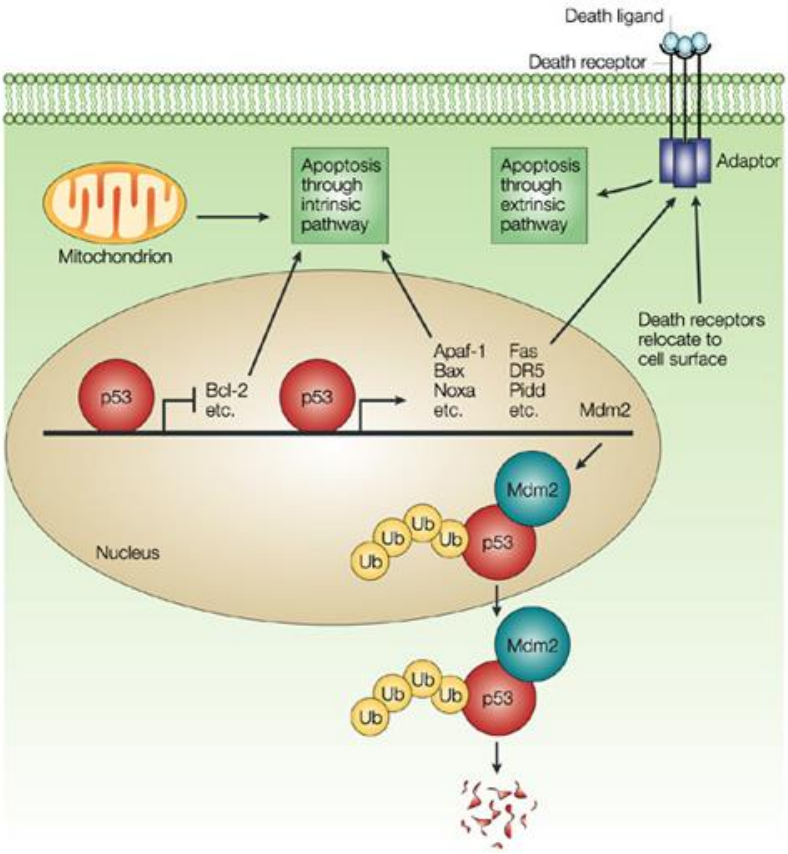


Fig. 5. Regulation of apoptosis by p53. Once activated, p53 induces the expression of proteins that target both intrinsic and extrinsic pathways of apoptosis. Further activities of p53 independent of transcriptional regulation have been proposed. They include the ability of p53 to drive relocalization of death receptors from the Golgi to the cell surface and to directly associate with mitochondria. Regulation of p53 is mainly provided by Mdm2, which itself is a transcriptional target of p53. Mdm2 binds to p53 and targets p53 for ubiquitin-proteasome dependent degradation.Ubiquitination (Ub) of p53 by Mdm2 probably also enhances the export of p53 into the cytoplasm, where degradation takes place. Reproduced from (Jesenberger and Jentsch, 2002).

I.4.6.2. Implication of cyclin-dependent kinase (CDK) inhibitors

As written above, p53 induces upregulation of the cyclin-dependent kinase inhibitor p21, and can interfere with the cell-cycle progression and apoptosis.

Eukaryotic cell cycle is regulated by the progressive activation and inactivation of a family of cyclin-dependent protein kinases (CDKs). The individual activation of Cdks is regulated in part by the timing of expression of their cognate cyclins together with both activating and inhibitory phosphorylation, but this regulation has also been shown to depend on presence of the inhibitors such as p21 and p27.

Biological functions of p21 are numerous and complex (see Fig.6). The first role discovered for p21 is **regulation of cell cycle**. p21 is a negative regulator that maintains cells in $\mathbf{G_0}$ when the conditions for cell cycle progression are not optimal (Li et al., 1994b). In

G_1 phase, association of p21 with both cyclin D-CDK4/6, which inhibits pRb phosphorylation, and cyclin E-CDK2 induces cell cycle arrest in G_1. p21 also associates with and inhibits E2F, normally responsible for transactivation of genes necessary for progression across the G_1/S transition and the S phase, leading to cell cycle arrest and cellular senescence (Afshari et al., 1996). In **S phase**, association of p21 with PCNA inhibits DNA polymerase δ and replication factor C activation. During **S and G_2 phase**, p21 inhibits both cyclin B1-Cdk1 and cyclin A Cdk1/2 complexes and blocks the G_2/M checkpoint (Li et al., 1994a). However, p21 also stabilizes and promotes active cyclin-Cdk complex formation on a dose-dependent manner, and under non-stressed conditions, a minimal amount of p21 is required for normal cell cycle progression (LaBaer et al., 1997).

The second important role of p21 is its implication in **cell death regulation.** Depending on the cell type and stress conditions, p21 may have either pro- or anti-apoptotic functions. Cytoplasmic p21 inhibits apoptosis in response to multiple pro-apoptotic stress signals. The underlying mechanisms namely involve inhibition of apoptosis signal-regulating kinase 1 (ASK1) and its substrate, the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in the MAP kinase cascade and inhibition of caspase 3 (Huang et al., 2003; Dotto, 2000; Suzuki et al., 2000). Upon DNA damage, caspase-3 mediated cleavage of p21 protein converts cancer cells from growth arrest to apoptosis. In contrast, p21 also **promotes apoptosis** in some cases, such as in the cell death response to cisplatin in ovarian carcinoma cell lines (Lincet et al., 2000), although mechanisms implicated remain less understood (Gartel, 2005).

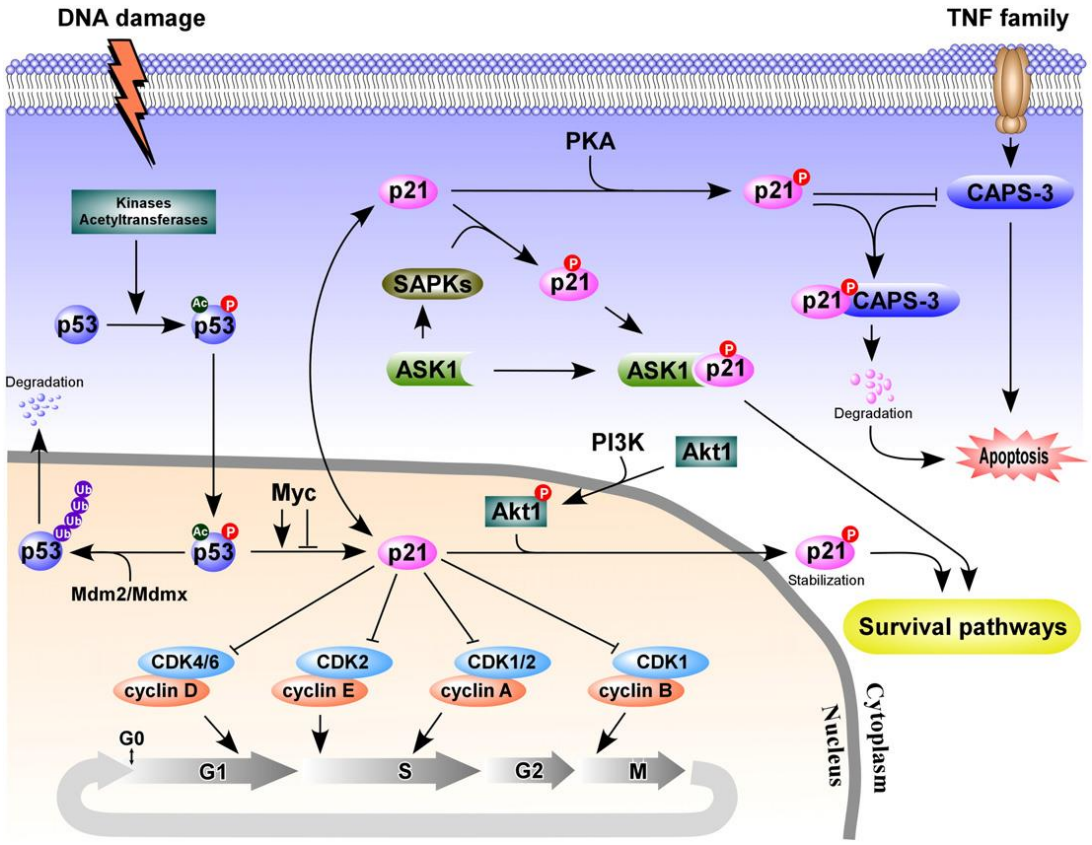


Fig.6. Biological functions of p21 in cell cycle and cell survival and death. Under stress conditions, p21 is expression is increased through p53-dependent pathways. Incresed p21 interacts with and inhibits cyclin/CDKs activity. Akt1 phosphorylates and then stabilizes p21 protein for cell survival. p21 inhibits apoptosis by interacting with pro-apoptotic molecules such as caspase 3 (CAPS-3) and apoptosis signal-regulating kinase 1 (ASK1). Reproduced from (Jung et al., 2010).

Regulation of p21 can occur at transcriptional, post-transcriptional and post-translational levels.

p21 is **transcriptionally** upregulated by **p53** by direct binding of p53 on p53-responsive elements on p21 promoter (Parker et al., 1995). In addition to p53, several transcription factors also activate p21 expression in a p53-independent manner thanks to the presence of DNA-binding elements in the proximal p21 promoter allowing regulation by butyrate, phorbol myristate acetate (PMA), TGFβ and the Rb protein (Gartel and Tyner, 1999). In contrast, p21 expression can also be transcriptionnaly repressed by transcription factors such as c-Myc via sequestering of DNA-binding elements of p21 promoter (Gartel et al., 2001). However, c-Myc also activates p21 transcription through $p19^{ARF}$ in a p53-dependent manner (Felsher et al., 2000).

p21 regulation can also occur at **post-transcriptional level**, namely by stabilization of p21 mRNA by RNA-binding proteins. So, RNPC1, a target of p53, is found to regulate p21 expression via stabilization of p21 mRNA in response to DNA damage (Shu et al., 2006).

Post-translational regulation of p21 includes phosphorylation and ubiquitin-dependent and -independent degradation of p21 (Sheaff et al., 2000). Several serine and threonine residues in **p21 are phosphorylated** by various protein kinases such as Akt1/PKB, PKA, PKC (for review, see (Jung et al., 2010). Depending on the site of phosphorylation and on the context, p21 phosphorylation can result in protein translocation from the nucleus to the cytoplasm, protein stabilization or decrease of its half-life time. For example, JNK and p38 phosphorylate p21 increasing its stabilization (Kim et al., 2002).

Proteasomal degradation plays a critical role in p21 regulation, exerting by this way a control on cell cycle progression (see Fig.7). Several cell-cycle related ubiquitin E3 ligases target p21 for degradation at different stages of the cell cycle. During G1 and S phase of cell cycle, p21 interacts with and is phosphorylated by cyclinE/CDK2 complex (Bornstein et al., 2003; Bendjennat et al., 2003). Phosphorylated p21 is ubiquitinated by different E3 ligases and is degraded by proteasome thereby resulting on cell cycle progression. A portion of phosphorylated p21 is exported to the cytoplasm where it is also degraded by proteasome. This cytoplasmic export is required for degradation induced by H_2O_2 or ERK2 (Hwang et al., 2007; Hwang et al., 2009). During G2 phase, ubiquitination depends on another type of E3 ligase, and does not need phosphorylation. Degradation in G2 phase occurs only in the nucleus (Amador et al., 2007).

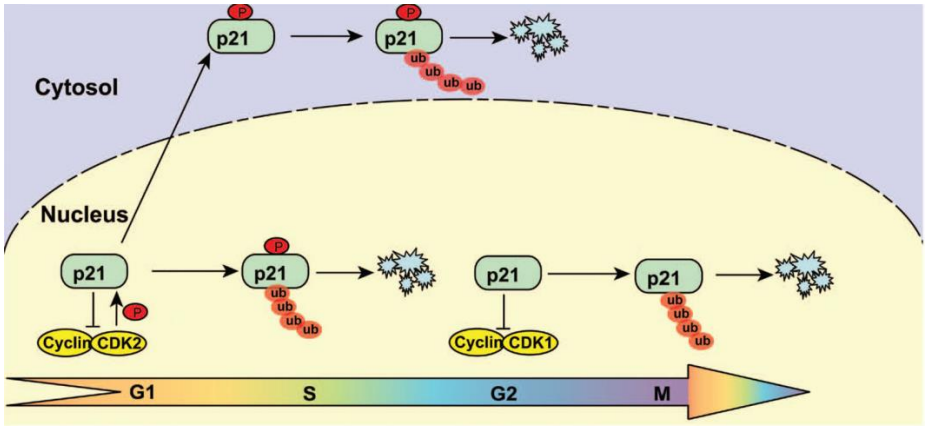


Fig. 7. Phosphorylation and ubiquitin-dependent degradation of p21. p21 is ubiquitilated and degraded in the nucleus in late G1, S and G2 phases. In late G1 and in S phases, a portion of p21 is phosphorylated and translocated into the cytosol where it is ubiquitylated and degraded by proteasomes. Adapted from (Lu and Hunter, 2010).

Another well-known cyclin-dependant kinase inhibitor is **p27**, which acts in G_0 and G_1-phase (Sutterluty et al., 1999), mainly targeting E-type cyclin/Cdk2 complexes. In addition to its role of negative regulator of protein kinases Cdk2/cyclin E and Cdk2/cyclin A, p27 is also an important factor for cellular differentiation of somatic cells (Pagano et al., 1995). The role of p27 in apoptosis induction is controversial. Indeed, p27 has been demonstrated to induce apoptosis via caspase 3 activation (Chen et al., 2009), but its stabilization in response to cellular stress induces autophagy offers protection against apoptosis (Liang et al., 2007).

Unlike p21, p27 mRNA levels are constant throughout the cell cycle and levels of p27 proteins are regulated by translation control (Hengst and Reed, 1996; Millard et al., 1997) and ubiquitin-mediated proteolysis (Pagano et al., 1995). p27 levels and stability are high in quiescent cells and fall during G_1 to reach a minimal level in S phase. Regulation of degradation by ubiquitin-proteasome pathway occurs via the ubiquitin ligase Skp2, and p27 accumulation and cell cycle arrest has been evidenced after inhibition of proteasome (Chen and Lin, 2004; Naujokat and Hoffmann, 2002), although an alternative degradation way has been described by proteolytic processing (Shirane et al., 1999).

I.4.6.3. Induction of endoplasmic reticulum stress

Several data suggest the involvement of ER in gentamicin-induced nephrotoxicity. Gentamicin can bind the ER chaperones proteins calreticulin and disulfide isomerase, inhibiting their chaperone activity (Horibe et al., 2004; Horibe et al., 2002). In renal cortex from rats treated with high doses of gentamicin, an increase of the ER-stress activated transcription factor XBP-1 has been evidenced after 7 days of treatment, accompanied by increase in GRP78 and GRP94 chaperone proteins and GRP78 mRNA expression (Peyrou et al., 2007). An increased cleavage of ER-specific caspase 12 has also been evidenced after 7 days of treatment (Peyrou et al., 2007).

Endoplasmic reticulum is the first compartment of the secretory pathway of secreted and plasma membrane proteins. Proteins or membrane-protein domains enter the ER through the translocon as unfolded polypeptide chains and fold within the lumen of this organelle (Wickner and Schekman, 2005). Oxidizing environment of the ER facilitates the formation of disulfide bonds in maturing proteins which are optimized by interactive exchange via the protein disulfide isomerase (PDI), stabilizing the proteins' structure. Protein folding is submitted to a quality control that ensures the correct assembly and functionality of proteins prior to delivery to their ultimate destination (Bernales et al., 2006).

When the capacity of ER to fold properly proteins is compromised or overwhelmed, ER stress lead to activation of an intracellular ER-to-nucleus pathway, termed the **unfolded protein response (UPR)**, that occurs through three mechanisms : (i) reduced translation of

misfolded proteins (Koumenis et al., 2002), (ii) enhanced translation of ER chaperones, such as GRP78 and GRP94 (Schroder and Kaufman, 2005), and (iii) ER-associated degradation (ERAD), which degrades misfolded proteins accumulated in the ER by returning these polypeptides to the cytosol to be degraded by the 26S proteasome (Ahner and Brodsky, 2004).

To date, three main pathways of the UPR have been identified (see Fig.8). Each pathway is composed of an unfolded protein sensor in the ER membrane, the transmembrane signaling protein Ire1α, PERK or ATF6. Ire1 and PERK are normally kept in an inactive form through an association of their N-terminal domain with the chaperone protein BiP (immunoglobulin heavy chain-binding protein, also named GRP78). BiP can also bind incorrectly assembled multimeric proteins, proteins with incorrect disulfide bonds, mutant proteins and aberrantly glycosylated proteins and under stress conditions dissociates from PERK, Ire1α and ATF6. PERK and Ire1 then undergo homo-oligomerization, stimulating trans-autophosphorylation within serine/threonine kinase domain.

PERK phosphorylates **the translation initiation factor eIF2α**, reducing global mRNA translation and preventing the continual accumulation in the ER during the stress, while favoring the translation of selected mRNAs, such as activating transcription factor-4 (*ATF-4)* mRNA. ATF-4 activates the transcription of UPR target genes encoding factors involved in restoring ER homeostasis via aminoacids biosynthesis, antioxidative stress response, apoptosis and autophagy. eIF2α phosphorylation can also occur independently of ER stress, as the alpha subunit of polypeptide chain initiation factor eIF2 can be phosphorylated by a number of related protein kinases which are activated in response to cellular stresses. Besides PERK, among eIF2α kinases are found GCN2, which is activated by amino-acid deprivation, UV irradiation or proteasomal inhibition; HRI, expressed mainly in erythroid tissues and PKR, central to an antiviral pathway (Jiang and Wek, 2005). Physiological conditions which result in eIF2 alpha phosphorylation include virus infection, heat shock, iron deficiency, nutrient deprivation, changes in cytosolic calcium, ER accumulation of unfolded or denatured proteins and the induction of apoptosis.

The phosphorylation of eIF2α is known to induce a global decrease in protein synthesis, accompanied by an increase of the translation of selected mRNA such as ATF4, which will in turn induce the transcription of proteins that modulate redox status and cell metabolism, and can induce apoptosis via CHOP/GADD153 (Jiang and Wek, 2005). CHOP/GADD153 is a 29kDa protein whose expression is mainly regulated at the transcriptional level. Overexpression of CHOP/GADD153 and microinjection of CHOP/GADD153 protein have been reported to lead to the apoptosis through the decrease in Bcl-2 proteins and the

induction of Bax translocation from the cytosol to mitochondria (Matsumoto et al., 1996; McCullough et al., 2001).

Ire1α contains a C-terminal endonuclease domain that excises a short sequence from the mRNA of the X-Box binding protein (XBP-1), generating an active transcription factor that stimulates transcription of ER chaperone genes, such as GRP94 and GRP78/BiP. In renal cortex from rats treated with gentamicin, an increase of the transcription factor XBP-1 has been evidenced after 7 days of treatment, accompanied by increase in GRP78 and GRP94 proteins and GRP78 mRNA expression (Peyrou et al., 2007). None of these signs were detected after 2 days of treatment.

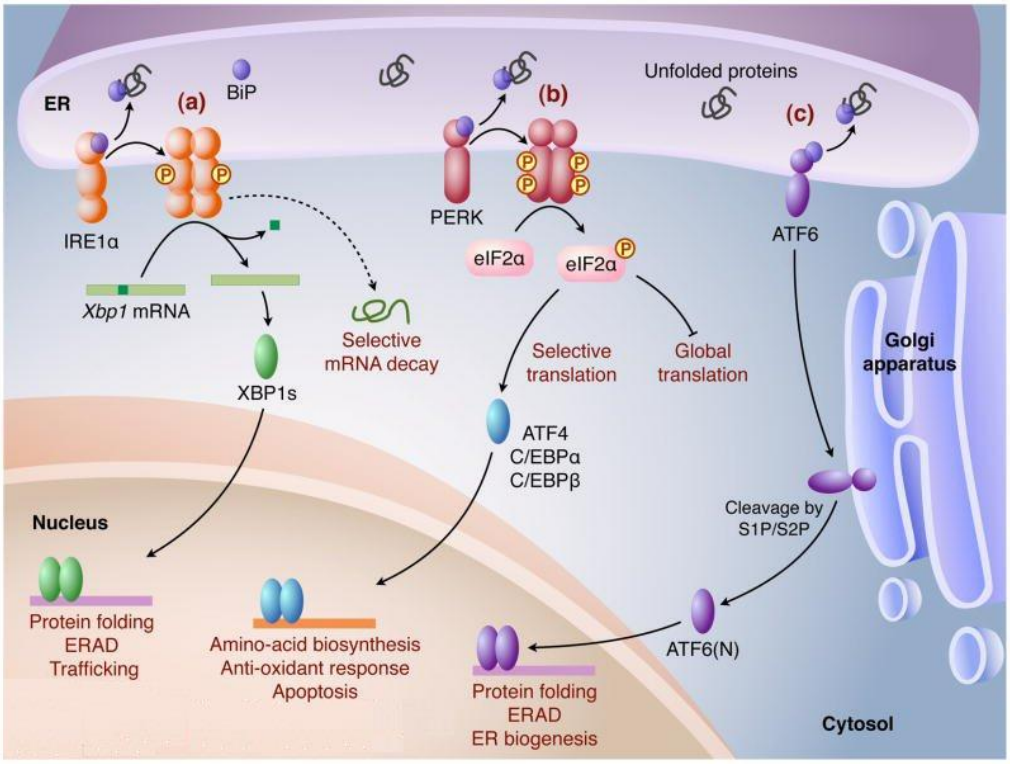


Fig.8. Intracellular signaling of the unfolded protein response (UPR). Accumulation of unfolded or misfolded proteins induces dissociation of the chaperone protein BiP and subsequently allows for activation of three ER-membrane sensors: (a) Ire1α, (b) PERK, and (c) ATF6. Ire1α undergoes dimerization and trans-autophosphorylation, activating its cytosolic endonuclease activity. IRE1α then removes a 26-base intron from *Xbp1* mRNA to generate a potent transcription factor XBP1s (XBP1 spliced) that translocates into the nucleus and regulates a diverse array of genes. In addition, activated IRE1α may selectively degrade certain mRNAs. (b) Activated PERK phosphorylates the translation initiation factor eIF2α to attenuate global translation, but also preferentially upregulates the translation of selected mRNAs including ATF4, C/EBPα, and C/EBPβ. ATF4 activates the expression of UPR target genes involved in amino acid biosynthesis, the anti-oxidant response, and apoptosis, whereas C/EBPα and C/EBPβ activates genes regulating glucose production and lipogenesis in the liver. (c) Activated ATF6 translocates to the Golgi, where it is cleaved by the proteases S1P/S2P, yielding the mature transcription factor ATF6(N), which activates the transcription of UPR target genes. Reproduced from (Sha et al., 2011).

Upregulation of ER chaperones is also mediated by another pathway involving ATF6 whose proteolytic cleavage after translocation to Golgi apparatus releases its active basic leucine zipper (bZIP) transcription factor domain which activates the transcription of UPR target genes (Breckenridge et al., 2003).

If the UPR is not able to reestablish a homeostatic balance or remain induced for a prolonged time, the cell undergoes apoptosis (Patil and Walter, 2001). Experimentally, ER stress followed by apoptosis is induced by pharmacological agents that inhibit N-linked glycosylation (tunicamycin), block ER to Golgi transport (brefeldin A), impair disulfide bond formation (dithiothreitol), or disrupt ER Ca^{2+} stores (thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps).

The link between ER stress and **apoptosis** is attributed to **caspase-12 activation** as caspase 12-null mice and cells are partially resistant to apoptosis induced by ER stress but not by other apoptotic stimuli (Nakagawa et al., 2000). However, the exact mechanism of caspase 12 activation remains unclear, but likely involve calpain (a calcium-dependant protease) -dependant removal of the prodomain and selfcleavage between the p20 and p10 subunits (Nakagawa et al., 2000; Fujita et al., 2002). Another pathway suggested is a link between caspase 12 activation and Ire1 signalling. The cytosolic tail of Ire1 can recruit TRAF2 and, when overexpressed, TRAF2 can interact with caspase 12 and induces its oligomerization and cleavage (Yoneda et al., 2001; Wang et al., 1998). An increased cleavage of caspase 12 has been evidenced after 7 days of treatment of rats with gentamicin at high doses (100 mg/kg/day) (Peyrou et al., 2007).

I.4.6.4. Inhibition of catalytic activity of proteasome

Gentamicin electroporation experiments have shown that low concentrations of cytosolic gentamicin are able to trigger apoptosis and to cause accumulation of the pro-apoptotic protein Bax, in particular its ubiquitinated form normally degraded by the proteasome (Servais et al., 2006). This suggests that gentamicin could decrease proteasome activity. To date, no study has been carried out to evaluate a potential inhibition of proteasomal activity by gentamicin, an issue on which we focused in this work.

As shown in Fig. 9, the **26S proteasome** is a multisubunit protease complex of 2500 kDa found in the cytoplasm and nucleus of mammalian cells, which consists of a 20S proteolytic core and one or two 19S regulatory complexes. The 20S proteolytic core is a hollow cylindrical particle formed by axial stacking of four heptameric rings : two identical inner β-rings, each form by 7 β-subunits $\beta_{1\text{-}7}$, and two identical outer α-rings each formed

also by seven α-subunits, α_{1-7}. Each β-ring has three different types of proteolytic activities: a chymotrypsin-like activity at β_5-subunit; a trypsin-like activity at β_2-subunit; and a caspase-like (also termed peptidyl-glutamyl peptide hydrolyzing or PGPH) activity at β_1-subunit. These activities cleave polypeptides on the COOH-terminal side of hydrophobic, basic and acidic amino acid residues, respectively (Voges et al., 1999). The chymotrypsin-like site is often considered the most important in protein breakdown, but inhibition of the two others activities has also been demonstrated to be important to reduce breakdown of proteins by 50% (Kisselev and Goldberg, 2005).

The 20S proteasome alone is able to degrade small peptides and unfolded proteins. The 20S particle with attached two 19S complexes forms 26S proteasome responsible for recognition and degradation of proteins tagged with polyubiquitin chains. The 19S regulatory particle regulates the proteolytic function of the protease core and can be divided into base and lid subcomplexes. The base has six different ATPase subunits, Rpt 1-6, and a few non-ATPase subunits such as Rpn1, Rpn2 and Rpn13. The lid contains more than ten other Rpn subunits.

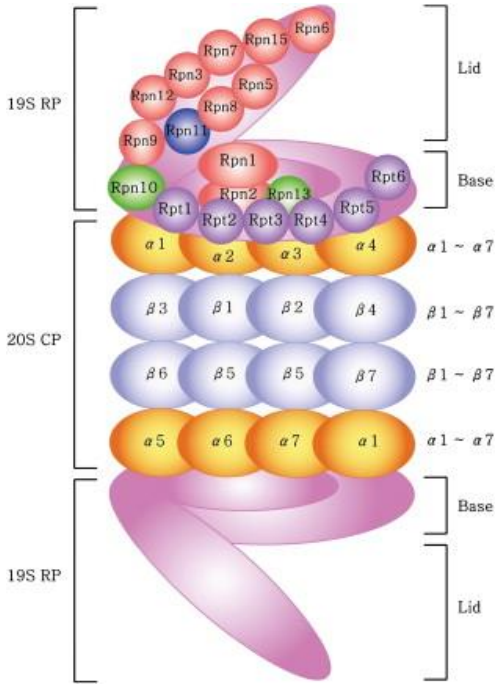


Fig. 9. Schematic diagram of the 26S proteasome. The proteasome is composed of one core particle (CP) and two regulatory particles (RP), which lie at both ends of the core particle. The core and regulatory particles are called the 20S and 19S proteasome, respectively. The 19S RP is further divided into two sub-complexes, the base and the lid. The base sub-complex is composed of six AAA-type ATPases (Rpt1 ~ 6) and three non-ATPase subunits (Rpn1, Rpn2 and Rpn13). The lid contains several Rpn family proteins, and Rpn15 (also known as Sem1) was recently identified. Reproduced from (Kwak et al., 2011)

Monitoring proteasome activity requires one to assay of all three catalytic activities in order to evaluate the state of the proteasome in the cells (Kisselev and Goldberg, 2005). Although multiple forms of proteasome exist within cells (e.g. 20S and 26S complexes (Yang et al., 1995), only the 26S proteasome has been proven to play a significant role in intracellular protein degradation.

Both natural and synthetic **inhibitors of proteasome** have been identified and developed, in order to better understand the proteasome by perturbing its function in a highly controlled manner. The most widely studied inhibitors are : (a) epoxomicin (see below); (b) lactacystin, a *Streptomyces* metabolite, which is metabolized to lactacystin β-lactone, the active proteasome inhibitor (Imajoh-Ohmi et al., 1995; Dick et al., 1997); (c) peptide aldehydes, such as carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG-132) and others (Banerjee and Liefshitz, 2001), and (d) boronic acid peptides (Teicher et al., 1999).

In this work, we used epoxomicin as a positive control of proteasome inhibition in our studies of gentamicin effect on proteasomal activity. Epoxomicin is a modified peptide that contain an α', β'-epoxyketone group at its C terminus such that when bound in the active site, two electrophilic carbon atoms are found in close proximity to the proteasome's nucleophilic threonine. Unlike other electrophilic peptide proteasome inhibitors that specifically target the hydroxyl nucleophile, epoxomicin reacts covalently with both the hydroxyl and the free amino groups of the N-terminal threonine, to produce a highly stable six-member ring (see Fig. 10). This unusual mode of inhibition provides an explanation for the extreme potency and selectivity of epoxomicin. No other cellular targets of epoxomicin are known, reinforcing its utility as a tool for studies of proteasome function (Bogyo and Wang, 2002).

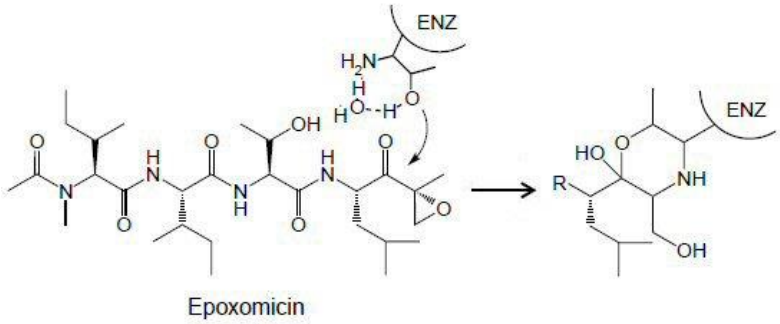


Fig. 10. Inhibition of proteasome by epoxomicin. Covalent binding of epoxomicin on hydroxyl and free amino groups of N-terminal threonine of proteasome results in the formation of a stable six-membered ring adduct. Reproduced from (Bogyo and Wang, 2002).

Intracellular protein degradation by the **ubiquitin-proteasome pathway** is a process that includes labelling of proteins targeted for degradation by covalent linkage to polyubiquitin chain, via a three-step cascade mechanism implicating ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-protein ligase E3 (Glickman and Ciechanover, 2002). Polyubiquitinated substrates are recognized by the 26S proteasome and degraded into short peptides and amino-acids by an ATP-dependent mechanism.

In eukaryotic cells, proteasome degradation pathway plays an important role in the degradation of most short-lived proteins that are critical in the regulation of many cellular processes such as cell cycle (Verma et al., 1997; Bech-Otschir et al., 2001), apoptosis (Guo and Peng, 2012), transcriptional regulation (Karin and Ben Neriah, 2000) and degradation of misfolded and damaged proteins (Hiller et al., 1996). This pathway has also a role in immune surveillance by generating antigenic peptides of foreign proteins to be presented by major histocompatibility complex (MHC) class I molecules to the T-cells of the immune system (Kloetzel, 2001; Rock et al., 1994).

Inhibition of proteasomal degradation pathways results in accumulation of unwanted proteins and cell death. As *in vitro* studies have demonstrated that inhibition of the proteasome activity induces apoptosis in cancer cells, the potential utilization of proteasome inhibitors in cancer therapy was suggested (Teicher et al., 1999; Shah et al., 2001) and **bortezomib** (PS-341 or Velcade®) was the first proteasome inhibitor approved by the FDA for treatment of relapsed/refractory multiple myeloma (Field-Smith et al., 2006). The induction of accumulation of ubiquitinated Bax by electroporated gentamicin (Servais et al., 2006) led to the suggestion that gentamicin impacts on ubiquitin-proteasome degradation pathway.

Pleiotropic **effects of inhibition of proteasome** have been evidenced, including p53-mediated apoptosis, targeting cell cycle regulatory proteins (An et al., 2000), inhibition of NF-κB, activation of heat shock proteins, activation of unfolded proteins response (UPR) pathway, DNA repair mechanisms, and activation of extrinsic and intrinsic apoptotic pathways, namely via activation of c-Jun amino-terminal kinase (JNK) (Chauhan et al., 2003). Inhibitors of proteasome also decrease the levels of several antiapoptotic proteins and triggers a dual apoptotic pathway of mitochondrial cytochrome c release and caspase 9 activation, as well as activation of Jun kinase and a Fas/caspase 8-dependent apoptotic pathway (Mitsiades et al., 2002). Pro-apoptotic protein Bax plays a critical role in apoptosis induced by proteasome inhibitors in colon cancer (Yu et al., 2003a). Moreover, stabilization of the Bax protein by bortezomib, an inhibitor of proteasome, has been associated to cell

death, and could play an important role in resistance to proteasome inhibitors in breast cancer cells (Xu et al., 2008).

In some cases, apoptosis induced by the proteasome inhibitor bortezomib was thought to be mediated by inhibition of the nuclear factor κB (**NF-κB**), which is mainly known as an inhibitor of apoptosis by promoting anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (Chen et al., 2000; Zong et al., 1999). Bortezomib blocks the degradation of its inhibitory cytoplasmic protein IκB; as a consequence, stabilization of IκB blocks NF-κB activation, and increases cell susceptibility to apoptosis (Hideshima et al., 2002). On the other hand, NFκB has been shown to be required for p53 induction of apoptosis (Ryan et al., 2000), and its activity increases after incubation of renal tubular cells with gentamicin (Chen et al., 2011).

Microarray studies have highlighted that a number of genes, such as **heat shock proteins Hsp70 and Hsp90**, can be activated at the transcriptional level by the proteasome inhibitor bortezomib or MG132 (Mitsiades et al., 2002; Lee and Goldberg, 1998), linking proteasome inhibition and induction of endoplasmic reticulum stress and ER-induced apoptosis (Khan et al., 2012). HSP proteins consist of several families including HSP70, HSP90 and the small HSPs (sHSPs). The HSP70 family includes stress-inducible Hsp70 and a resident endoplasmic reticulum (ER) family member called immunoglobulin-binding protein (BiP, also called regulatory-protein 78 or glucose-regulated protein 78 (GRP78)). Both HSP70 members act as molecular chaperones by binding to nascent or denaturated proteins and giving them a folding competent state. The HSP90 family consists of cytoplasmic/nuclear Hsp90 and the ER glucose-regulated protein 94 (GRP94). Hsp90 assists in protein folding and stabilization while GRP94 plays critical roles in folding client protein and secretory pathway in the ER. The enhanced-expression of stress-inducible HSP chaperone is regulated by the unfolded protein response (UPR) of the ER (Heikkila, 2010; Ohtsuka et al., 2005).

Proteasome is also closely linked to endoplasmic reticulum, and is part of the ER-associated machinery for protein degradation (ERAD) that removes unfolded and misfolded proteins from ER (Oyadomari et al., 2006). Inhibition of proteasome has been linked to ER stress and ER-induced apoptosis induction (Park et al., 2011; Fribley et al., 2004).

I.5. Aims of the study

In the context of resurgence in use of aminoglycosides as much for their effectiveness against bacterial strains resistant to other classes of antibiotics, as well as their activities in the treatment of genetic diseases or viral affections, the nephrotoxicity induced by these antibiotics remains so far a barrier against their use. In this work, we aimed to get a better understanding of cellular mechanisms underlying gentamicin-induced apoptosis.

In the first part of this work, we studied the consequences of gentamicin accumulation in lysosomes after its endocytosis by LLC-PK1 cells and we investigated lysosomal membrane permeabilization and the underlying mechanism by particularly focusing on the implication of ROS. *These results gave rise to the manuscript* ***:***

"Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic".

Denamur S, Tyteca D*, Marchand-Brynaert J, Van Bambeke F, Tulkens PM, Courtoy PJ**, Mingeot-Leclercq MP***. ***Free Radical Biology and Medicine (2011) 51 : 1656 – 1665.***

After demonstrating lysosomal membrane permeabilization, we focused our interest on the role that could play cytosolic aminoglycosides in the apoptotic process. To this aim, we compared the percentages of apoptosis induced by aminoglycosides of different nephrotoxic potential after electroporation in cells, by-passing the endocytic route, or after classical incubation. *These results were published in the manuscript:*

"Apoptosis induced by aminoglycosides in LLC-PK1 cells: comparative study of neomycin, gentamicin, amikacin and isepamicin using electroporation."

Denamur S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. ***Antimicrobial Agents and Chemotherapy (2008) 52 : 2236 – 2238.***

Finally, to better understand the link between lysosomal membrane permeabilization and activation of mitochondrial pathway of apoptosis by gentamicin, we explored several pathways that could be implicated in gentamicin-induced apoptosis. We considered the possibility of the involvement of p53 signaling pathway, of the implication of an ER stress and of the inhibition of proteasome by gentamicin. *These results have not yet been published and are described in chapter IV.*

CHAPTER II. Gentamicin-induced lysosomal membrane permeabilization and mechanism involved

Previous works of Servais and colleagues have suggested that gentamicin accumulated in lysosomes after endocytosis could induce lysosomal membrane permeabilization (Servais et al., 2005). These conclusions were based on the observation of a change in emission fluorescence ratio of acridine orange, a membrane-permeant weak base that emits red fluorescence when accumulated in acidic vacuoles due to a proton-trapping mechanism, and green fluorescence if released from lysosomes to the cytosol. As the acridine orange emission fluorescence is both pH- and concentration-dependent, whether these results reflected gentamicin-induced loss of acidic lysosomal pH or effective lysosomal membrane permeabilization remained unclear.

In this context, we bestow this study to investigate gentamicin-induced lysosomal permeabilization and to explain the underlying mechanism, particularly focusing on the role of ROS.

Manuscript 1 : Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic.

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Original Contribution

Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic

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ABSTRACT

Gentamicin, an aminoglycoside antibiotic used to treat severe bacterial infections, may cause acute renal failure. At therapeutic concentrations, gentamicin accumulates in lysosomes and induces apoptosis in kidney proximal tubular cells. In gentamicin-treated renal LLC-PK1 cells, acridine orange release from lysosomes, previously interpreted as lysosomal membrane permeabilization, precedes the apoptotic cascade that develops during incubation with gentamicin. However, the link between gentamicin lysosomal accumulation and apoptosis remains unclear. We here examined if reactive oxygen species (ROS) production could account for gentamicin-induced acridine orange release and apoptosis, and the implication of iron in these events. We found that gentamicin induced ROS production prior to, and at lower drug concentrations than required for, acridine orange release and apoptosis. ROS antioxidant or scavenger, catalase, and *N*-acetylcysteine largely prevented these events. Vital confocal imaging revealed that gentamicin-induced ROS production occurs in lysosomes. Deferoxamine, an iron chelator, which is endocytosed and accumulates in lysosomes, largely prevented gentamicin-induced ROS production as well as apoptosis. Direct evidence for gentamicin-induced permeabilization of lysosomal membrane was provided by showing the release into the cytosol of Lucifer yellow, a membrane-impermeant endocytic tracer with a comparable molecular weight as gentamicin. Altogether, our data demonstrate a key role of lysosomal iron and early ROS production in gentamicin-induced lysosomal membrane permeabilization and apoptosis.

Aminoglycosides including gentamicin have been successfully used for decades in the treatment of Gram-negative bacterial infections [1,2] and currently regain popularity because of widespread resistance to other antibiotic classes [3,4]. Unless careful serum monitoring and optimized administration, aminoglycosides can, however, induce acute nephrotoxicity in 5–25% of treated patients, and even more in populations at risk [2]. Nephrotoxicity induced by gentamicin is due to uptake of the ultrafiltrated polycationic drug by proximal tubular cells, via adsorptive/ receptor-mediated endocytosis upon binding to acidic phospholipids and megalin at the brush border [5,6]. As a result, gentamicin accumulates to large extent in lysosomes [7], although cell culture studies suggest a way to the cytosol by retrograde transport via the Golgi complex and the endoplasmic reticulum [8]. Accumulation of gentamicin in proximal tubular cells rapidly leads to lysosomal phospholipidosis, due to inhibition of acid phospholipases, followed by mixed apoptosis/necrosis together with signs of tubular regeneration/peritubular inflammation and fibrosis that ultimately lead to renal dysfunction (see review in [9]).

Apoptosis induced by aminoglycosides can be reproduced *in vitro* using cells of both renal (LLC-PK1 and MDCK) and non-renal (fibroblasts) origin and correlates with the level of drug accumulation [10]. One mechanism proposed to link aminoglycoside lysosomal accumulation with apoptosis is permeabilization of the lysosomal membrane based on change in the fluorescence emission of acridine orange. In LLC-PK1 cells [11], this shift is already detectable after 2 h of drug exposure, increases over time and precedes appearance of other changes directly related to apoptosis triggering and execution, including loss of mitochondrial potential, release of cytochrome *c* and activation of caspase-9 [11]. That permeabilization of lysosomes causing release of gentamicin into the cytosol may trigger apoptosis is supported by direct cytosolic introduction of the antibiotic via electroporation [12]. Cytosolic gentamicin could either act directly on mitochondria by causing the release of intermembrane proteins, as shown for a variety of polycations including aminoglycosides [13], or indirectly through impairment of Bax proteosomal degradation [12] upon drug binding to the β-9 proteasome subunit [14]. Alternatively,

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apoptosis could also be triggered by lysosomal release of cysteine- and aspartyl-cathepsins, known to directly activate cell death signaling pathways [15,16].

Lysosomal membrane permeabilization by gentamicin remains, however, hypothetical and its mechanism is obscure. A simple explanation invokes membrane destabilization upon drug binding to the inner luminal leaflet phospholipids [17], which would be favored by the acidic pH [11]. However, several observations point to a role of reactive oxygen species (ROS) in this process. Indeed, lysosomes generally contain a high iron pool [18,19] which, combined with a reducing potential and acidic pH [20], should favor ROS production. This reaction could be enhanced by gentamicin when forming a ternary complex with iron and membrane phosphoinositides [21].

In the present study, we have first examined the induction by gentamicin of ROS production in the kidney proximal tubular cells-derived cell line, LLC-PK1. We next assessed their role, as well as that of iron, in triggering lysosomal membrane destabilization and cell apoptosis by using antioxidants and deferoxamine. Given the importance of lysosomal permeabilization to the proposed model as well as the concerns raised by change in the fluorescence emission of acridine orange release for studying lysosomal membrane permeabilization, we also documented this critical event by following the release of the membrane bilayer-impermeant lysosomal vital tracer, Lucifer yellow. Of interest, the molecular weight of Lucifer yellow is comparable to that of gentamin. Our results demonstrate a role for ROS in a cascade linking gentamicin lysosomal accumulation and membrane permeabilization with apoptosis.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and trypsin-EDTA were purchased from Life Technologies, Paisley, UK. Gentamicin sulfate (GEOMYCINE®) was from GlaxoSmithKline, Belgium. 4',6'-diamidine-2'-phenylindole (DAPI) was from Roche (Basel, Switzerland). Acridine orange, bafilomycin A1, catalase, deferoxamine (DFO), 1,4-diazabicyclo [2.2.2]octane (DABCO), Lucifer yellow, monensin, *N*-acetylcysteine and probenecid were from Sigma-Aldrich (St-Louis, MO, USA). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), MitoTracker Deep Red and LysoTracker Red were from Invitrogen (Paisley, UK). Unless stated otherwise, all other reagents were of analytic grade and purchased from Merck (Darmstadt, Germany).

Cells and gentamicin incubation

All experiments were performed with LLC-PK1 cells (Lilly Laboratories, Culture-Pig Kidney Type 1) from ATCC (CL-101). This cell line was isolated from, and displays some attributes of kidney proximal tubular cells [22]. Cells were cultivated in DMEM supplemented with 10% foetal calf serum (FCS) in 95% air - 5% CO_2. They were subcultured twice a week and used at ~80% confluence. All gentamicin solutions were adjusted to pH 7.4 prior addition to the culture medium. Electroporation was performed with cells detached by trypsinization as previously described [12].

Oxidative stress assay

ROS were detected by means of the oxidation-sensitive fluorescent probe, 2',7'-dichlorodihydrofluorescein (H_2DCF). The membrane-permeant fluorigenic precursor (2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is deacetylated by cytosolic esterases to H_2DCF, which is further oxidized into the fluorescent compound, 2',7'-dichlorofluorescein (DCF) when and where cellular peroxides are produced [23]. In our fluorimetric experiments, cells were cultured in 96-wells microplates, rinsed, and preincubated at 37 °C for 30 min with 10 μM H_2DCFDA in Krebs-Ringer-HEPES buffer (KRH) to avoid extracellular hydrolysis of the probe. They were then incubated with the indicated compounds in Hanks'Balanced Salt Solution (HBSS), and examined at 1-h intervals with a Fluorocount Microplate Fluorometer (Packard Instrument Company, Downers Grove, IL, USA) with excitation wavelength at 485 nm and emission recorded at 530 nm.

Acridine orange release

Acridine orange is a fluorescent membrane-permeant weak base, which reversibly accumulates into acidified membrane-bound compartments [24]. The fluorescence emission of acridine orange is concentration-dependent, from red at high concentrations (e.g. in lysosomes) to green at low concentrations (e.g. in the cytosol) [24,25], with yellow as intermediate (e.g. upon trapping in nucleoli). However, fluorescence of acridine orange is also dependent of changes of pH, as reported in literature [26]. Shift in red-to-green emission ratio in comparison to controls may thus either monitor lysosomal leakage or change in lysosomal pH.

In our fluorimetric studies, cells cultured in 24-wells culture plates were first loaded with acridine orange (5 μg/mL) in DMEM with FCS at 37 °C for 15 min, rinsed, then incubated in HBSS with or without gentamicin for the indicated times. Whole cell sheets were examined at 1-h intervals with the Fluorocount Microplate Fluorometer with excitation wavelength at 485 nm and emission recorded at 530 nm and 620 nm.

Counting of apoptotic cells

Apoptotic nuclear fragmentation, revealed by DNA staining with 4',6'-diamidine-2'-phenylindole (DAPI) [11], was identified during random counting of 500 cells per condition. Clusters of apoptotic bodies were given as a single count. Data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

Vital imaging of lysosomal alterations

Cells were cultured in Lab-Tek II chambers. For acridine orange labeling, cells were pre-incubated with 5 μg/mL acridine orange for 15 min, rinsed and replaced in culture medium supplemented with 10% fœtal calf serum containing 3 mM gentamicin, 100 nM bafilomycin or 50 μM monensin, for 2 or 6 hours as mentioned. Cells were briefly washed and immediately observed with a LSM 510 META confocal microscope (Zeiss, Jena, Germany) using a Plan-Apochromat 63X/1.4 oil DIC objective, with imaging in the green and red channels simultaneously.

For Lucifer yellow labeling, cells were pre-incubated with 2 mg/mL Lucifer yellow overnight (pulse), then chased for 6 hours in medium alone or supplemented by 3 mM gentamicin, 100 nM bafilomycin or 50 μM monensin. One hour before the end of this incubation, cells were rinsed with serum-free DMEM, incubated with 250 nM MitoTracker Deep Red in serum-free DMEM for 30 minutes, rinsed and reincubated in complete growth medium for 30 minutes, both steps maintaining same gentamicin, bafilomycin or monensin concentrations.

In a second part of experiments, pulse and chase were both made in absence or presence of 2.5 mM probenecid, to sensitize detection of Lucifer yellow release by preventing endosomal/lysosomal recapture and/or efflux from cytosol [27]. At the end of the chase, cells were briefly washed and immediately observed by vital imaging.

Vital imaging of oxidative stress

Cells cultured in Lab-Tek II chambers were preincubated or not for 3 hours with deferoxamine, then incubated with or without 200 μM H_2O_2 or 2 mM gentamicin for the indicated times, maintaining the same concentrations of deferoxamine. One hour before the end of this

incubation, cells were rinsed with serum-free DMEM, incubated with 250 nM MitoTracker Deep Red in serum-free DMEM for 30 minutes, rinsed and reincubated with 3 μM H_2DCFDA and 50 nM LysoTracker Red in KRH for 30 minutes. H_2O_2, gentamicin and deferoxamine were maintained up to imaging with the confocal microscope using a Plan-Apochromat 63X/1.4 oil DIC objective. Because H_2DCFDA is susceptible to photo-oxidation, images were sequentially collected in the green, red and blue channels at low laser power and with a single scan under identical conditions for all samples.

Determination of gentamicin cell content

The cellular gentamicin content was assessed by a disc-plate microbiological technique using *Bacillus subtilis* (ATCC 6633) as test

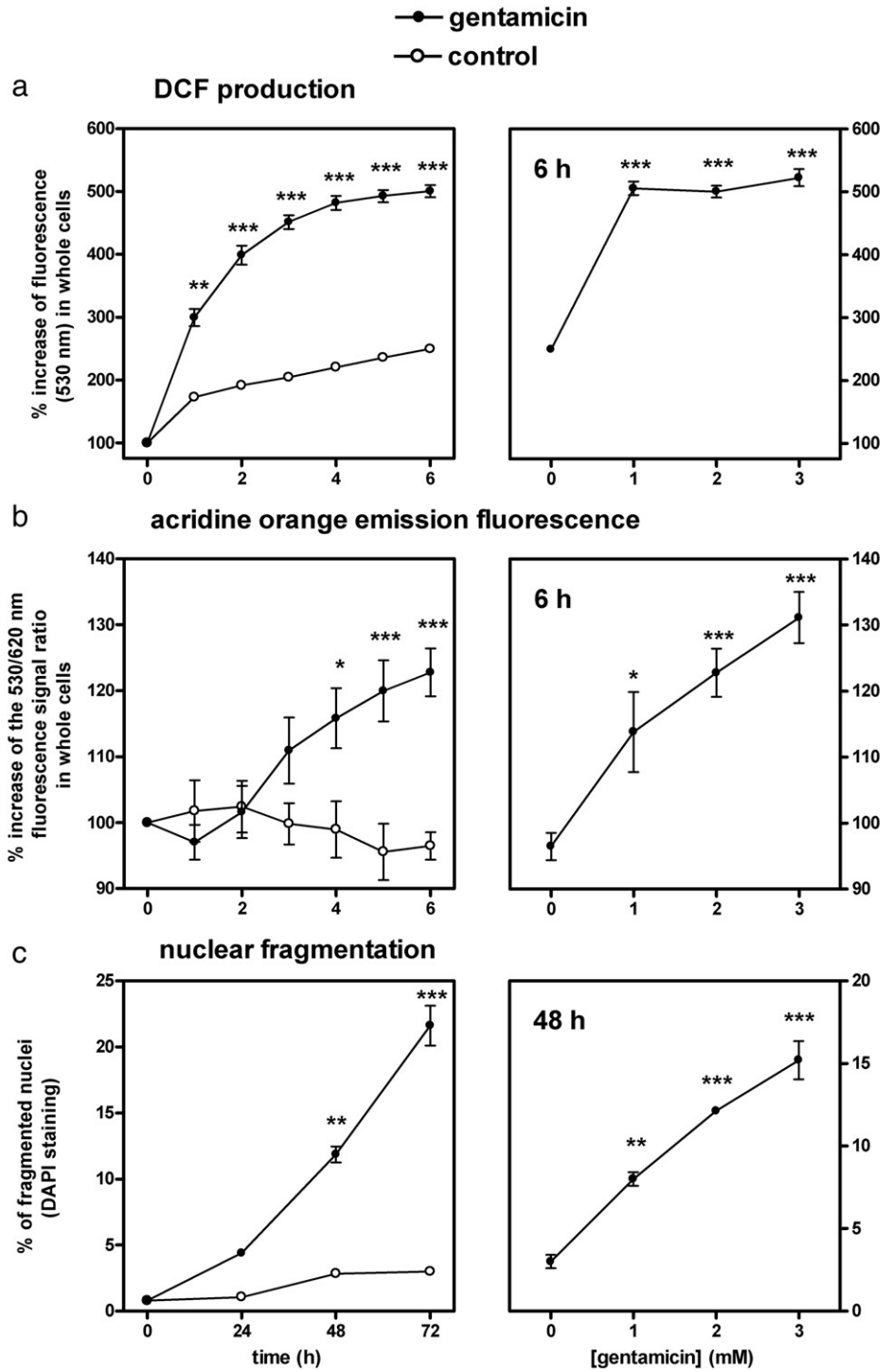


Fig. 1. Effect of gentamicin on ROS production, acridine orange emission fluorescence shift, and apoptosis in LLC-PK1 cells. Left, effect of incubation time with 2 mM gentamicin (closed symbols) as compared with untreated controls (open symbols). Right, effect of gentamicin concentration after 6 h (ROS production and acridine orange emission fluorescence shift) and after 48 h (apoptosis). Top panels. ROS production was monitored by 2',7'-dichlorofluoresceine production (DCF). Central panels. Effects on lysosomes were evaluated by the shift of acridine orange emission ratio, as percentage of value at time 0. Bottom panels. Apoptosis was measured as the percent of cells with fragmented nuclei. Data are means ± SD (n = 6 from 2 separate experiments for ROS; n = 9 from 3 separate experiments for lysosomal permeabilization; n = 3 for apoptosis; where not visible, error bars are included in the symbols).

organism [28]. Cell protein was measured by the Folin-Ciocalteu method and gentamicin cell content expressed as µg antibiotic / mg cell protein. A posteriory mixing of naive cells with defined amounts of fresh gentamicin yielded about 90% of the expected values.

Statistical analysis

All statistical analyses were perfomed with GraphPad Prism version 4.02 and GraphPad InStat version 3.06 (GraphPad Prism Software, San Diego, CA, USA). The comparison of 3 or more groups of data was performed using one-way ANOVA with Tukey's multiple comparison post-tests. The significance of the differences between two sets of data was tested using two-way ANOVA followed by Bonferroni's post-test. Paired data were compared using repeated-measures ANOVA.

Results

Gentamicin sequentially triggers ROS production, shift of acridine orange emission fluorescence and apoptosis

In a first series of experiments, we examined the time- and concentration-dependence of gentamicin-induced (i) ROS production, (ii) acridine orange emission fluorescence shift, and (iii) apoptosis. To these aims, cells were incubated with 2 mM gentamicin for increasing periods of time, or with 0–3 mM gentamicin for a fixed interval, then tested for (i) increase in fluorescence due to oxidative conversion of H_2DCF into 2,7-dichlorofluorescein (DCF); (ii) change in the ratio of the 530 to 620 nm fluorescence signals of acridine orange indicating lysosomal leakage or change in lysosomal pH; and (iii) increase in the proportion of DAPI-labeled fragmented nuclei indicating apoptosis. As shown in Fig. 1 (left panels), 2 mM gentamicin rapidly increased ROS production (half-maximal response within 1 h) to level off at 4 h (top; no further increase in fluorescence up to 8 h). Cells incubated with 200 µM H_2O_2 for 6 h, used as positive control, showed a comparable but slightly higher ROS production as upon parallel incubation with 2 mM gentamicin for the same interval (data not shown). After a lag period of ~2 h, acridine orange fluorescence emission started shifting to greener values; the green/red ratio then increased almost linearly with time (central panel; incubation for up to 24 h showed a continuous increase of the difference between gentamicin-treated and control cells (from 122 ± 2% at 6 h to 262 ± 4% at 24 h [n = 3]). Appearance of apoptotic cells was further delayed, being significant after only 24 h, then increased continuously from 24 to 72 h (bottom). These three events occurred thus sequentially. The right panels of Fig. 1 show that ROS production measured at 6 h (top) was maximal for the lowest gentamicin concentration tested (1 mM). In contrast, 530/620 nm acridine orange fluorescence ratio, also measured at 6 h (central) was roughly proportional to concentration, as was apoptosis measured at 48 h (bottom). These data thus showed that gentamicin-induced ROS production occurred both prior to, and at lower drug concentrations than the 530/620 nm ratio shift of acridine orange emission fluorescence, used as a marker of lysosomal alteration, itself followed by nuclear fragmentation reflecting apoptosis.

Gentamicin induces the release of Lucifer yellow

Although acridine orange is widely used as a marker of lysosomal "permeabilization", recognized by a decrease in red acridine orange fluorescence while maintaining high green fluorescence [29], the reversion of acidotropic sequestration due to change of pH across a still unpermeable membrane remains an alternative explanation. This possibility was tested by vital imaging using two well-established procedures to collapse lysosomal acidic pH : bafilomycin A1 and monensin. Furthermore, to avoid confusion between a change in lysosomal pH and true leakage, we followed lysosomal membrane permeabilization by vital imaging after loading cells with the pH-insensitive membrane bilayer-*impermeant* lysosomal vital tracer, Lucifer yellow (LY). The sensitivity of this approach was greatly increased by inhibiting the organic anion transporter with probenecid, as originally reported by Steinberg and his colleagues [30].

When lysosomes had been loaded by acridine orange, collapsing lysosomal pH by bafilomycin A1 (100 nM for 2 h), an inhibitor of the vacuolar proton-pump [31], or monensin (50 µM for 2 h), an electro-neutral ionophore [32], caused the virtual disappearance of dotty red signal, with full relocation of acridine orange as green signal into the cytosol, nucleosol and especially nucleoli (compare at Fig. 2, panels c, d *vs* a) whereas Lucifer yellow remained fully trapped in lysosomes (Fig. 2, panels g, h) demonstrating their integrity.

Thus, shift of red to green does not necessarily reflect lysosomal rupture and should be used with caution. Upon gentamicin treatment, lysosomes remained labelled by acridine orange, but partial relocation to nucleoli was obvious, revealing release from preloaded lysosomes and preferential trapping in nucleoli as a sensitive read-out (compare Fig. 2b *vs* Fig. 2a).

This result is consistent with either partial membrane leakage, a discrete change in lysosomal pH, or even conceivably trapping into a modified lysosomal matrix. To circumvent these undertainties, we turned our attention to Lucifer yellow, a bona fide membrane-impermeant lysosomal tracer.

Upon gentamicin treatment, we noticed that lysosomes loaded with Lucifer yellow were more clustered (Fig. 2, panel f *vs* e). When carefully examined, clusters frequently showed fuzzy boundaries, first suggesting acute Lucifer yellow release, with rapid dilution in the cytosol (Fig. 2, panel f, arrows). The X-Z series shown in Supplementary Fig. 1 suggested that the fuzzy boundaries were not artefacts due to fluorescence generated by voxels above or below the center of the focal plane.

To sensitize detection of Lucifer yellow release, we used probenecid, a well-established inhibitor of general organic anion transporters, which will prevent Lucifer yellow transfer across membranes [27,30]. As a control, we used Mitotracker to test if mitochondria could be affected in cells where ROS was induced (see Fig. 1a). We found no change in distribution and abundance of labeled mitochondria, in contrast to lysosomes (compare at Fig. 2, panels j *vs* i).

Under these conditions, cytosolic labeling by Lucifer yellow upon gentamicin treatment became prominent in a large fraction of cells (Fig. 2, panel j), whereas it was never observed upon lysosomal pH neutralization (Fig. 2 panels k,l). This set of data provides the first visual evidence that gentamicin is able to permeabilize membranes in living cells as to release small molecular weight tracers of the size of gentamicin. The reversibility of the effect of probenecid is shown in Supplementary Fig. 2.

ROS induced by gentamicin are localized in lysosomes

To test whether gentamicin-induced oxidative stress was specifically localized into lysosomes, ROS production was analyzed by vital confocal imaging based on H_2DCF conversion after lysosomal labeling with LysoTracker [in red] and mitochondria labeling with MitoTracker [in blue]. H_2O_2 was used as a control for mitochondrial ROS production. As shown in Fig. 3, lysosomes and mitochondria were clearly resolved from one another and no detection of DCF could be detected in control cells (no gentamicin added; upper row). When H_2O_2 was added (2^d row), a marked green staining appeared, which largely co-localized with mitochondria but not with lysosomes (see inset in merge). When cells had been incubated with gentamicin (lower three rows), green staining appeared instead in lysosomes, at the exclusion of mitochondria (see inset in merges). In situ imaging of ROS production in lysosomes was already obvious within 1 h of exposure to gentamicin, matching fluorimetric results of Fig. 1, increased after 6 h but decreased after prolonged incubation with gentamicin (18 h). At this late interval, while ROS and MitoTracker were never found to colocalize upon gentamicin treatment,

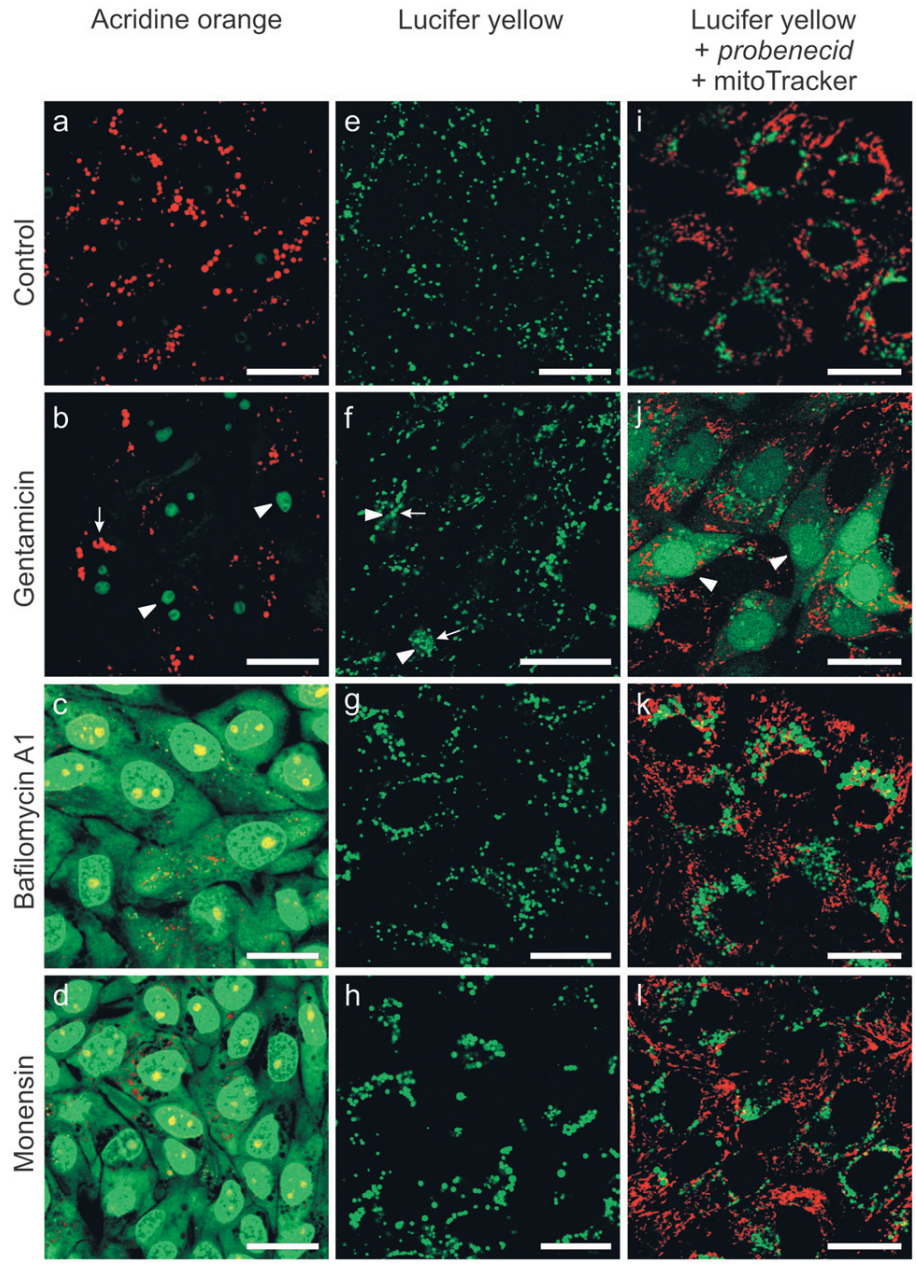
MitoTracker signal had completely vanished (lowest row), pointing to membrane potential perturbation and providing indirect evidence for a secondary role of mitochondria in apoptosis induced by gentamicin, as previously reported [11]. These morphological analyses indicated a sequence of organelle-specific perturbations: lysosomal ROS production, followed by a much delayed mitochondrial loss-of-function.

Antioxidant treatments largely prevent gentamicin-induced ROS production and partially protect against apoptosis

To test for a causal role of ROS in lysosomal membrane permeabilization, we next examined if pretreating cells with catalase or *N*-acetylcysteine as ROS scavengers in lysosomes could prevent gentamicin-induced ROS production, thereby conferring protection against lysosomal permeabilization and apoptosis. This set of experiments was conducted with 2 mM gentamicin, 1,000 U/mL catalase and 1 mM *N*-acetylcysteine and the exposure time was selected for optimal measurement of corresponding signals for ROS and lysosomal permeabilization (6 h) or apoptosis (48 h). As shown by Fig. 4 (upper panel), gentamicin-induced ROS production was largely prevented by catalase, and essentially abrogated with *N*-acetylcysteine. These anti-oxidant treatments also partially protected against apoptosis (lower panel). This protective effect of antioxidant molecules was also demonstrated by vital imaging (Supplementary Fig. 3). Protection by antioxidants did not result from a decreased cellular accumulation of gentamicin : after 48 h with 2 mM gentamicin, the cellular concentration was 22.3 ± 3 μg / mg protein without antioxidant, 20.6 ± 4 μg / mg protein with *N*-acetylcysteine and 24.3 ± 3 μg / mg protein with catalase.

Deferoxamine partially impairs gentamicin-induced ROS production and partially protects against apoptosis

Because iron chelators have been long suggested to protect against gentamicin-induced nephrotoxicity [33], we further tested deferoxamine,



well-known to accumulate in lysosomes [34]. As shown in Fig. 5 left, treatment with 10 μM deferoxamine significantly decreased the production of ROS at all time points examined. Upon vital confocal imaging (Fig. 6), deferoxamine prevented gentamicin-induced ROS staining (below detection level), indicating that the iron chelator was acting on the gentamicin-induced lysosomal production of ROS. Deferoxamine also partially prevented apoptosis induced by gentamicin (Fig. 5; right). Increasing the concentration of deferoxamine to 25 μM caused cell toxicity (data not shown).

As for the antioxidants, deferoxamine did not decrease the cellular accumulation of gentamicin under the conditions used (22.3 ± 3 μg / mg protein after 48 h with 2 mM gentamicin alone *vs.* 25.9 ± 0.5 μg / mg protein in the presence of deferoxamine).

Deferoxamine and N-acetylcysteine do not protect against gentamicin-induced apoptosis when the drug is directly introduced in the cytosol by electroporation

To test whether the (partial) protection conferred by antioxidants was dependent from the lysosomal localization of gentamicin, the antibiotic was directly introduced in the cytosol by electroporation, thus by-passing the lysosomal compartment [12]. We previously reported that gentamicin triggers apoptosis at much lower concentrations when used in cells subjected to electroporation rather than when added to the culture medium. Therefore, cells were preincubated with deferoxamine or *N*-acetylcysteine, then electroporated with gentamicin and returned for 24 hours to gentamicin-free medium containing deferoxamine or *N*-acetylcysteine (Fig. 7). As predicted, whereas electroporation was innocuous (< 3% apoptotic nuclei) in the absence of gentamicin, electroporated cells were much more susceptible to gentamicin : 30% of apoptotic nuclei when the cells were electroporated at 0.1 mM *vs* 15% under endocytic uptake at 3 mM. Yet, deferoxamine or *N*-acetylcysteine were unable to confer any protection in electroporated cells, consistent with a role in lysosomes.

Discussion

Nephrotoxic drugs including aminoglycosides remain a major cause of acute renal failure in critically ill patients [35,36]. A large body of *in vitro* and *in vivo* evidence indicates that oxygen reactive species are important mediators of gentamicin nephrotoxicity [33,37]. Iron-gentamicin complex can increase reactive oxygen species [38,39] and a beneficial effect of ROS scavengers to protect against tubular necrosis induced by gentamicin in animals has been demonstrated [40]. The present study was carried out with LLC-PK1 cells, a model widely used for the study of various aspects of gentamicin-induced nephrotoxicity [41,42] and apoptosis [10,43] and extends over these observations demonstrating the role of ROS in the early signs of gentamicin cellular toxicity including lysosomal permeabilization, mitochondrial loss-of-function and apoptosis [44], and underlying the physiopathological role of lysosomes in this cascade.

The "lysosomal pathway of apoptosis" [15] proposes that cells can undergo apoptosis upon moderate lysosomal damage but will suffer necrosis if the damage is extensive [45,46]. The release of lysosomal constituents such as cathepsins could be sufficient to trigger apoptosis, since these enzymes can (i) cleave Bid, an antiapoptotic protein of the Bcl-2 family [47], (ii) directly activate pro-caspase-3 and −7 [48,49], and generate a cytochrome *c*-releasing factor from the cleavage of pro-caspase 2 [50]. The hypothesis that apoptosis can result from oxidative stress associated with lysosomal membrane permeabilization has been investigated since two decades, and led to emphasis on iron as a cause of generation of deleterious ROS (see [45] for review).

The data of the present report show that gentamicin induces ROS production very early on after cell exposure to the antibiotic, specifically in lysosomes (colocalization with Lysotracker), and can be partly prevented by experimental antioxidants or by the iron chelator used in clinical settings, deferoxamine. That lysosomal iron is a critical actor in gentamicin-induced early ROS production is supported by the following observations: (i) gentamicin starts accumulating in lysosomes [51]; (ii) lysosomes are organelles extremely active in redox reaction and containing significant amounts of transition metals, like iron [18]; and (iii) deferoxamine enters lysosomes by endocytosis [52] and can mobilize iron stores [34].

The use of H_2DCF to monitor ROS production fluorimetrically and morphologically does not provide direct information as to which type of ROS is being produced in lysosomes when cells are exposed to gentamicin. In the presence of iron and at acidic pH, H_2O_2 may form other reactive species such as $HO^{\cdot}$ and HO^{-} through the Fenton reaction [19]. Even more, oxidation of H_2DCF can be triggered without generation of ROS intermediates as described for hemoproteins like cytochrome c [53], nitric oxide [54], or pyocyanine [55]. H_2O_2 can be tentatively identified as one of the involved species because of protection by catalase against gentamicin-induced H_2DCF oxidation. Production of ROS in lysosomes upon gentamicin accumulation is critical, since addition of antioxidants or deferoxamine cannot protect against apoptosis when gentamicin is delivered directly in the cytosol by electroporation, thus by-passing lysosomes. Our observations thus lead to opposite conclusions than what has been derived from the effect of the acidotropic-sequestered detergent, MSDH on lysosomal and mitochondrial membranes permeabilization in which relocation to the cytosol of redox-active iron and cytochrome c has been considered responsible for H_2DCF oxidation [56]. The difference could possibly stem from the experimental systems with MSDH which has been reported to cause a massive disruption of lysosomes [11], whereas gentamicin led to a more subtle and slower lysosomal membrane permeabilization. In none of our conditions could detectable ROS production occur without being followed by significant increase in acridine orange release after > 2 h nor of apoptosis after > 1 day. However, this robust link in LLC-PK1 cells remains to be documented in vivo.

In vitro studies [21] show that ROS can be formed by aminoglycoside antibiotics in the presence of iron and polyunsaturated lipids, as electron donors. Phosphoinositides- or arachidonic-iron-gentamicin

Fig. 2. Subcellular localization of acridine orange and Lucifer yellow upon gentamicin *versus* bafilomycin A1 and monensin treatments. Acridine orange (a-d). Cells were preincubated with 5 μg/ml acridine orange for 15 min, then rinsed and replaced by culture medium supplemented with 10% foetal calf serum (control; a), or further treated with 3 mM gentamicin for 6 h (b), 100 nM bafilomycin A1 for 2 h to inhibit the vacuolar ATPase (c), or 50 μM monensin for 2 h to collapse proton gradients (d). Cells were briefly washed and immediately examined by vital imaging in the green and red channels simultaneously. Comparison of (a) with (b) suggests that gentamicin causes clustering of lysosomes (arrow). As to acridine orange redistribution, notice the modest green labeling of nucleoli upon gentamicin alone (arrowheads at b), contrasting with the strong green labeling in the entire cytosol and especially nucleosol in all cells upon lysosomal pH neutralization, with yellow signal indicating the higher nucleolar acridine orange concentration (c,d). Lucifer yellow (e-h). Cells were labelled with 2 mg/ml Lucifer yellow overnight (pulse), then chased for 6 hours in medium alone (e) or supplemented by 3 mM gentamicin (f), 100 nM bafilomycin A1 (g) or 50 μM monensin (h). Careful examination reveals at (f) a diffuse labeling (arrowheads) at the immediate vicinity of clustered lysosomes (arrows), but not upon lysosomal pH neutralization (g;h), suggesting lysosomal leakage of Lucifer yellow under gentamicin (for higher magnification and serial optical sectioning, see Suppl. Fig. 1). Sensitization of Lucifer yellow detection in the cytosol/nucleosol upon inhibition of organic anion transporters by probenecid (i-l). Cells were labeled with Lucifer yellow as above, except that pulse and chase were performed in the presence of 2.5 mM probenecid to sensitize detection of Lucifer yellow cytosolic release by preventing endosoma/ lysosomal recapture and/or cell expulsion by organic anion transporters. One hour before the end of chase, cells were further incubated for 30 min with MitoTracker red to simultaneously visualize mitochondria. After a final rinse, cells were immediately observed by confocal microscopy vital imaging, with sequential recording in the green (Lucifer yellow) and red (MitoTracker) channels. Notice the strong labeling of cytosol and especially nucleosol by Lucifer yellow in a large fraction of gentamicin-treated cells (j), but not upon lysosomal pH neutralizing agents (k;l). All scale bars, 20 μm.

ternary complexes [57] bring into a close proximity the redox center (Fe_{II}/Fe_{III}) and the electron donor [38]. This process is favored by acidic pH, the presence of reducing equivalents for generation of reactive hydroxyl radicals through the Fenton reaction [19], and low-molecular-weight iron, three conditions that are met in lysosomes [18,58,59]. Iron may, however, not be the only factor favouring the

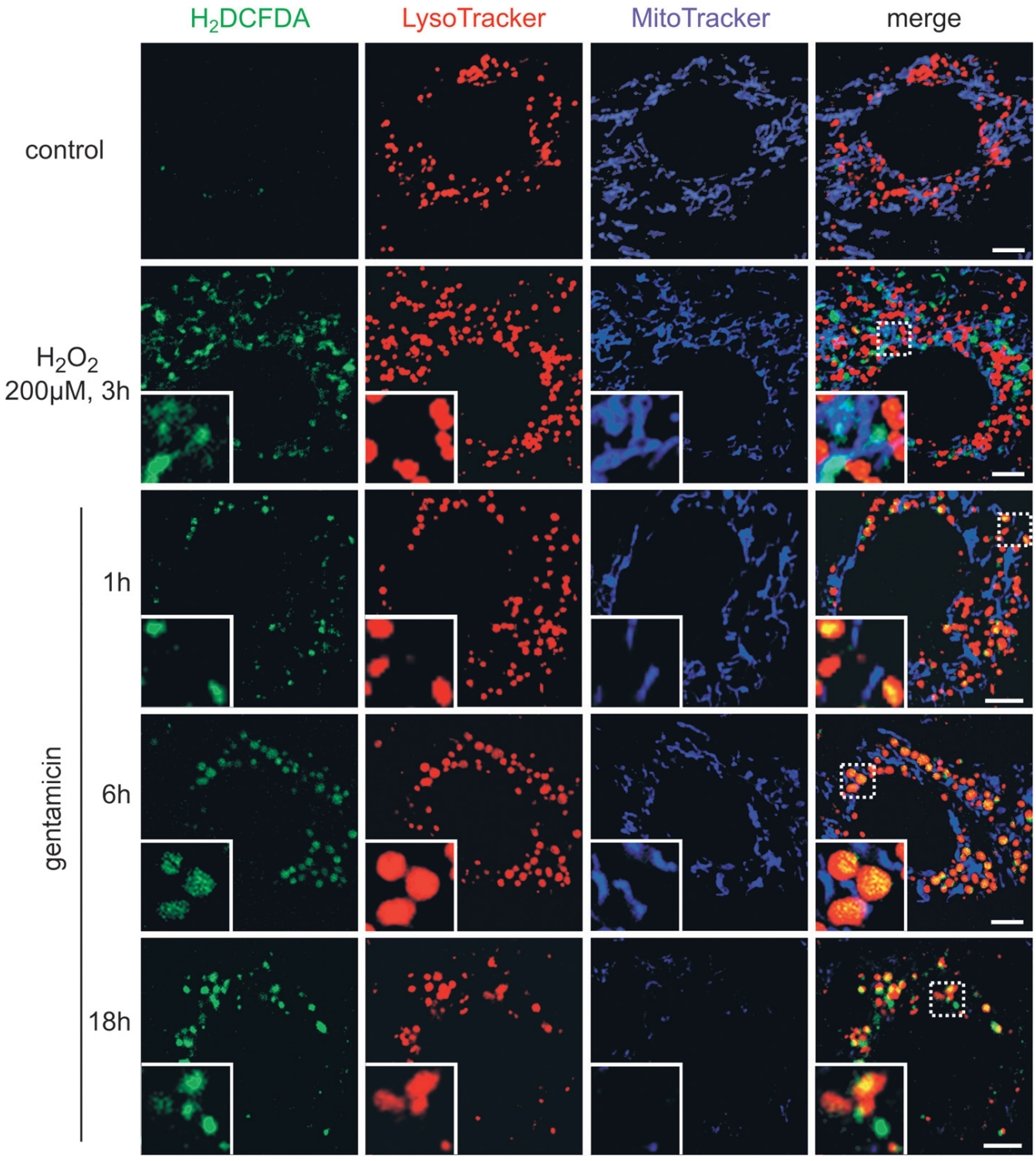


Fig. 3. Intracellular localization of ROS upon gentamicin and H_2O_2 treatments. Cells were kept untreated (control), or treated with either 200 µM H_2O_2 for 3 h or 2 mM gentamicin for the indicated times. One hour before the end of this incubation, cells were washed and sequentially incubated with MitoTracker for 30 min (to evidence mitochondria), then with a combination of LysoTracker (to detect lysosomes) and H_2DCFDA (to detect ROS production) for another 30 min, while maintaining the same concentrations of H_2O_2 or gentamicin. After washing, cells were immediately observed by confocal microscopy with sequential recording in the green (oxidized product of H_2DCF), red (LysoTracker) and blue (MitoTracker) channels. Merged images are shown at right. Background was set to the level of untreated cells. Upon H_2O_2 treatment, notice the complete dissociation of ROS from lysosomes, but its large codistribution with mitochondria. Upon gentamicin treatment, notice instead that ROS (green) fully overlap with LysoTracker (red) at 6 h, as obvious in the merge inset at the 4th row. All scale bars, 5 µm.

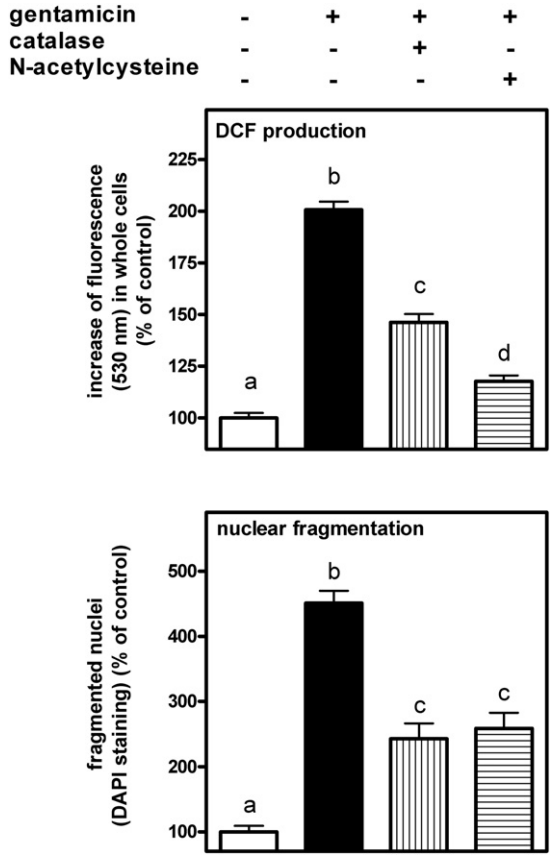


Fig. 4. Prevention by catalase and *N*-acetylcysteine of gentamicin-induced ROS production and apoptosis. Cells were preincubated for 3 h with 1,000 U/mL catalase or 1 mM *N*-acetylcysteine, then incubated with 2 mM gentamicin in the continued presence of catalase or *N*-acetylcysteine for 6 h (ROS) or 48 h (apoptosis). ROS production and apoptosis were measured as in Fig. 1. Data are expressed as percent of values in untreated cells (no gentamicin and no antioxidant; the addition of antioxidants alone had no effect) and are given as means ± SD (n = 6 from 2 separate experiments for ROS; n = 5 from 2 separate experiments for apoptosis). Statistical analysis: One-way analysis of variance and Tukey's Multiple comparison test; bars with different letters are significantly different from each other ($p<0.05$).

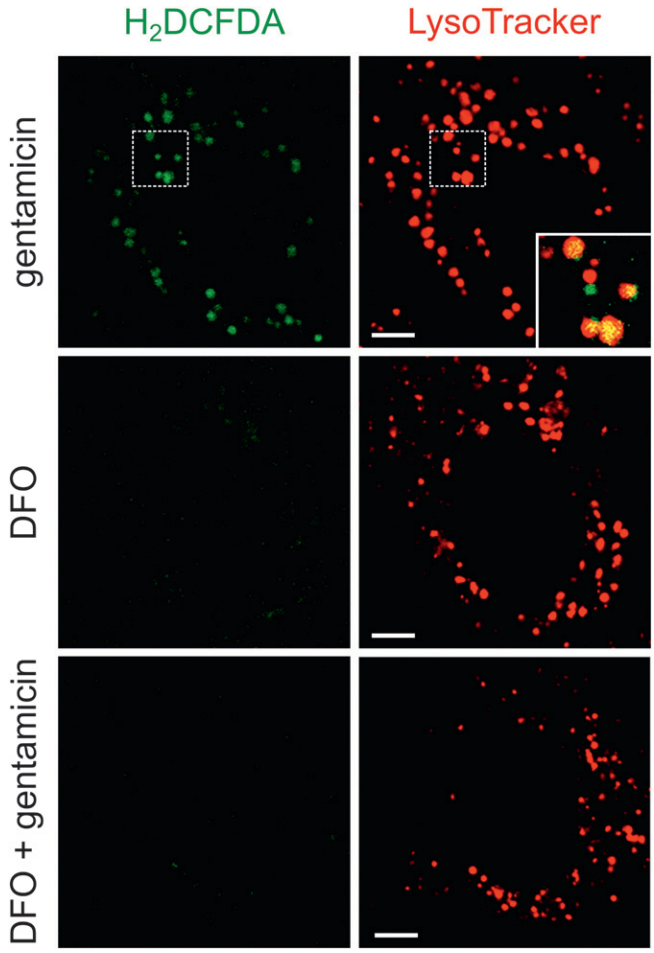


Fig. 6. Prevention by deferoxamine of gentamicin-induced ROS production in lysosomes. Cells were preincubated with 10 μM deferoxamine (DFO) for 3 h, then incubated with 2 mM gentamicin in the continued presence of deferoxamine (bottom row). Controls included cells exposed to gentamicin alone (upper row) or cells exposed to deferoxamine alone (middle row). Vital imaging was performed as in Fig. 3. All scale bars, 5 μm.

production of ROS, as deferoxamine afforded only a partial protective effect. Thus, while only iron-rich lysosomes would be amenable to protection by antioxidants and/or deferoxamine, those containing only negligible amounts of it, and for which labilization of membrane could occur through an iron-independent mechanism, would not be protected [60].

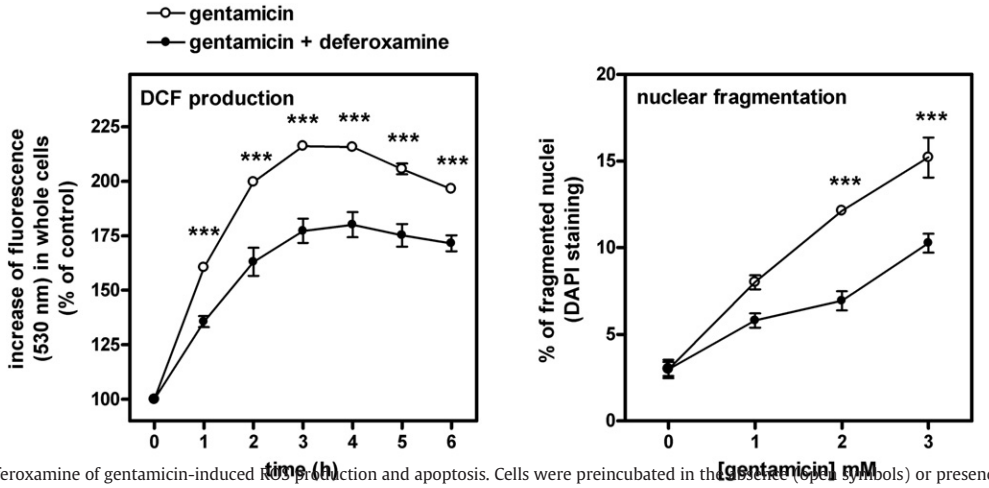


Fig. 5. Prevention by deferoxamine of gentamicin-induced ROS production and apoptosis. Cells were preincubated in the absence (open symbols) or presence (closed symbols) of 10 μM deferoxamine for 3 h, then exposed to gentamicin (2 mM) in the continued absence or the presence of deferoxamine for the indicated times (left) or gentamicin concentrations (right panel). ROS production and apoptosis were measured as in Fig. 1. Data are expressed as percentages of the values in untreated cells (no gentamicin and no deferoxamine; the addition of deferoxamine alone had no effect) and are given as means ± SD (n = 3). Where not visible, error bars are included in the symbols.

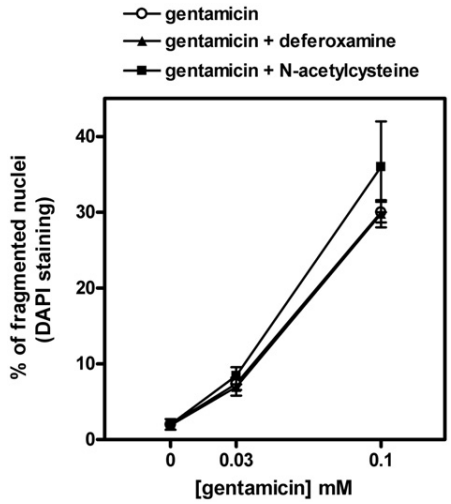


Fig. 7. Absence of protection by deferoxamine and *N*-acetylcysteine on apoptosis induced by gentamicin electroporation. Cells were preincubated or not with 10 μM deferoxamine or 1 mM *N*-acetylcysteine for 3 h, electroporated with gentamicin at the indicated concentrations, then returned for 24 h in gentamicin-free corresponding medium (deferoxamine or *N*-acetylcyteine, closed symbols; no further addition, open symbols). Apoptosis was measured as the percent cells with fragmented nuclei. Data are means ± SD (n = 3).

The generation of ROS caused by gentamicin may induce peroxidation of the lysosomal membranes and their permeabilization, as evidenced from studies using isolated lysosomes [61]. Acridine orange was often used as a probe for lysosomal membrane permeabilization. Arguably, the method does not allow to differentiate between (i) dilution upon release into the cytosol via membrane permeabilization, (ii) cytosolic dilution upon loss of lysosomal sequestration due to an oxidation of V-ATPase or ClC7, or (iii) alteration in emission spectrum of acridine orange, due to lysosomal alkalinization. However, while release and changes in lysosomal pH appear in parallel in cells upon photoactivation of the probe [62], studies using cultured proximal tubular cells failed to demonstrate an influence of gentamicin on lysosomal pH [63]. Moreover, cytosolic labeling of Lucifer yellow upon gentamicin treatment was clearly evidenced whereas it is completely absent upon changes in lysosomal pH induced by bafilomycin A1 or monensin.

ROS production in lysosomes seems to be a key pathogenic event for gentamicin toxicity leading to apoptosis. However, several data, including the partial protective effect afforded by *N*-acetylcysteine or deferoxamine and the incomplete correlation between the amount of ROS producted and the percentage of apoptotic cells detected at increasing concentrations of gentamicin, suggest that this mechanism is not the only one implicated in gentamicin-induced toxicity. It is well known that ultrafiltrated gentamicin is partially endocytosed by kidney epithelial cells lining the S1 and S2 segments of the proximal tubules where the drug enters by adsorptive/receptor mediated endocytosis after binding to acidic phospholipids and megalin respectively and eventually accumulates in lysosomes where it induces readily detectable phospholipidosis. However, and in contrast to fibroblasts and MDCK cells [10], only marginal phospholipidosis was found in LLC-PK1 cells, yet the level of apoptosis was similar in all three cell lines, suggesting that phospholipidosis is only one of the contributors of aminoglycoside toxicity at low therapeutic doses. The present paper depicts ROS generation selectively in lysosomes followed by lysosomal membrane permeabilization as a complementary mechanism for gentamicin-induced toxicity. One could tentatively reconcile these two mechanisms to account for the differential concentration dependence on ROS and apoptosis in LLC-PK1 cells, if ROS effects were maximal at a lower (~1 mM) extracellular gentamicin concentration and were synergized by a non-saturating concentration-dependent phospholipidosis, which develops as a slower process.

The clinical significance of our results remains to be evaluated. Although the extracellular concentrations of gentamicin used may seem overwhelming as compared to clinical serum concentrations, they were selected to match cellular concentrations reached in animals and humans treated with therapeutic doses (see discussion in [11]) and correspond to those eliciting apoptosis of proximal tubular cells in experimental animals [44]. Thus, ROS generation we evidenced may well occur *in vivo* under conditions pertinent of the clinical use of gentamicin. Moreover, the concentrations of *N*-acetylcysteine (1 mM) and of deferoxamine (10 μM) used to obtain a protective effect are in the range or below those observed in the serum of humans receiving intravenous therapeutic doses of these agents (0.2-3 mM for *N*-acetylcysteine [64]; up to 200 μM for deferoxamine [65]. Our results might therefore also have potential clinical implications in human aminoglycoside therapy; even if only partial cell protection can be reasonably expected, it could make a clear difference in overall clinical outcome.

In conclusion, ROS are rapidly produced in lysosomes of cultured LLC-PK1 cells incubated with gentamicin, and secondarily lead to lysosomal permeabilization followed by apoptosis. These effects can be largely prevented by preincubation with antioxidants or deferoxamine. Our data further point to lysosomal iron as a key actor in triggering the pathogenic cascade. Through the potential formation of a ternary complex gentamicin-iron-phosphoinositides, the accumulation of gentamicin *per se* is probably one of the critical event leading to lysosomal phospholipidosis, ROS production and lysosomal membrane permeabilization. We expect this study may help elucidate the subcellular mechanism responsible for activation of the lysosomal pathway of apoptosis and nephrotoxicity induced by aminoglycoside antibiotics, prompt further experimental work on the relation between permeabilization and apoptosis, and possibly justify clinical investigations.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.07.015.

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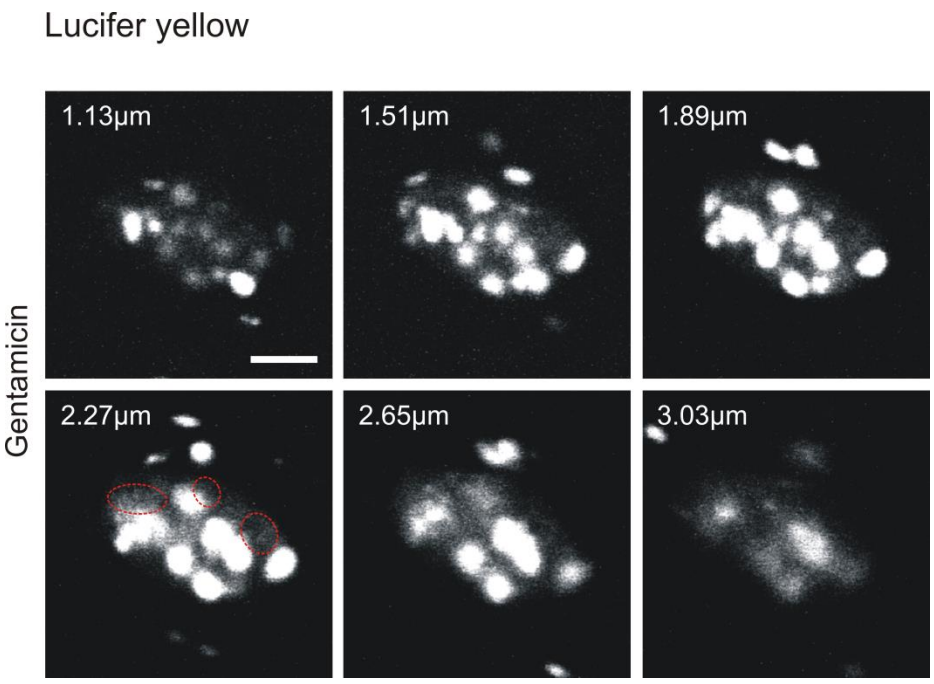
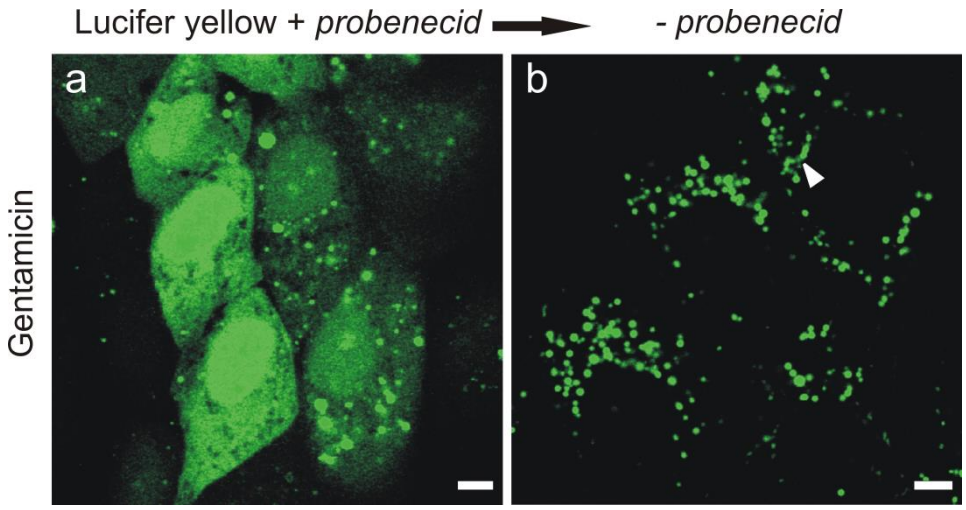
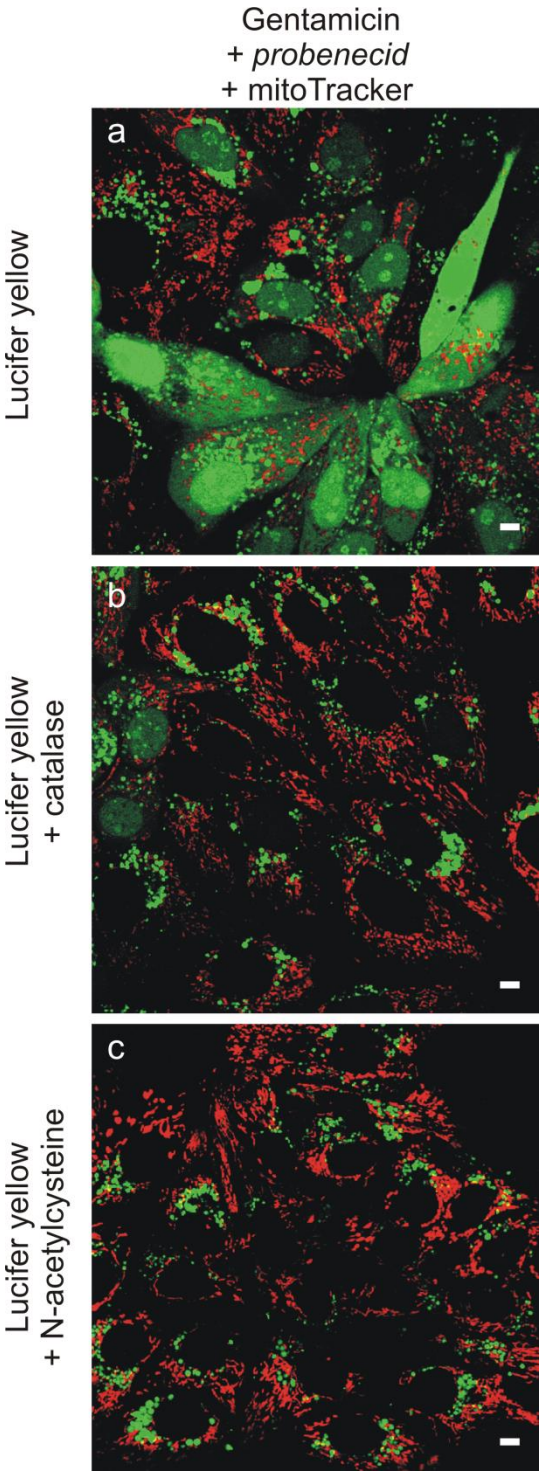
Supplementary material



Supplementary Figure 1: Gentamicin induces the clustering of lysosomes labeled by Lucifer yellow and a weak diffuse labelling at their immediate vicinity. Cells were labelled with 2 mg/ml Lucifer yellow overnight (pulse), chased for 6 h in the presence of 2 mM gentamicin, then immediately examined by vital imaging by serial horizontal optical sectioning (distance of the center of the focal plane is indicated on each panel). Dotted lines indicate fuzzy labelling which cannot be accounted for by fluorescence emission within the optical section from voxels above or below the center of the focal plane. Scale bar, 2 µm.



Supplementary Figure 2 : Reversibility of probenecid effect on Lucifer yellow relocalization in gentamicin-treated cells. Cells were labeled with Lucifer yellow and chased with 3 mM gentamicin together with probenecid to sensitize detection of Lucifer yellow release. At the end of chase, some cells were reincubated for 1 h in the absence of probenecid (but in the continued presence of gentamicin) (right). After brief washing, cells were immediately examined by vital imaging in the green channel. The strong cytosolic/nucleosolic labeling by Lucifer yellow observed in cells upon gentamicin treatment in the presence of probenecid quickly disappears upon probenecid removal, except around a few clusters as in cells never exposed to probenecid (arrowhead at b). All scale bars, 5 µm.



Supplementary Figure 3:

Prevention of gentamicin-induced Lucifer yellow lysosomal leakage by ROS scavenging. Cells were labeled with 2 mg/ml Lucifer yellow overnight (pulse), then chased for 6 h in the presence of 3 mM gentamicin and probenecid (a) or combined with catalase (b) or *N*-acetylcysteine (c). One hour before the end of chase, cells were further incubated for 30 min with MitoTracker red to simultaneously visualize mitochondria. Notice that the labeling of cytosol and nucleosol by Lucifer yellow is largely prevented by catalase and by *N*-acetylcysteine. All scale bars, 5 µm.

Additional data

To complete the experiments with antioxidant molecules (*N*-acetylcysteine and catalase) presented in the published manuscript, we also tested the effect of superoxide dismutase on apoptosis induced by gentamicin.

The protocol used was the same as the one described in the manuscript for *N*-acetylcysteine and catalase experiments. Briefly, cells were pre-incubated for 3 h with 300 U/ml superoxide dismutase, and incubated for 48 h with 2 mM gentamicin in the presence of superoxide dismutase. Fig.11. shows a partial protective effect afforded by SOD, of the same order of magnitude as the protection afforded by catalase and N-acetylcysteine presented in the manuscript.

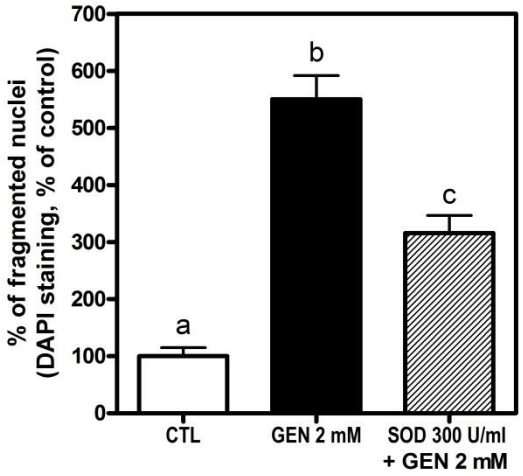


Fig.11. Protective effect of superoxide dismutase on gentamicin-induced apoptosis. Cells were pre-incubated for 3 h with 300 U/ml superoxide dismutase and then incubated with gentamicin 2 mM with the continued presence of SOD for 48 h. Results are presented as percent of values in untreated cells (control, CTL) and are given as means ± SD (n = 5 from 2 separate experiments). Statistical analysis : One-way analysis of variances with Tuckey's multiple comparison test; bars with different letters are significantly different from each others ($p<0.05$).

The effect of superoxide dismutase on gentamicin-induced ROS production and acridine orange release should also be evaluated to complete this result.

The following conclusions can be highlighted from this work :

- gentamicin induces **lysosomal membrane permeabilization**, as demonstrated by the release of the fluorescent dye Lucifer yellow from lysosomes to the cytosol.

- the mechanism of gentamicin-induced lysosomal membrane permeabilization involves the **production of reactive oxygen species (ROS)** in lysosomes.

- this production of ROS probably implicates **iron** as suggested by the protective effect observed in presence of the iron chelator deferoxamine. The underlying mechanism could be the formation of a ternary complex between gentamicin, iron and acidic phospholipids, as demonstrated *in vitro* by Lesniak and colleagues (Lesniak et al., 2005).

- antioxidant molecules and the iron chelator deferoxamine afford only **partial protective effect** against gentamicin-induced apoptosis, suggesting this pathway is not the only one implicated in lysosomal membrane permeabilization.

CHAPTER III. Could we generalize the effect of cytosolic gentamicin to other aminoglycosides ?

We concluded in the previous chapter to the induction by gentamicin of lysosomal membrane permeabilization (Denamur et al., 2011). The release of the fluorescent dye Lucifer yellow from lysosomes could imply a possible release of aminoglycosides into the cytosol after the lysosomal membrane permeabilization. Moreover, the electroporation of gentamicin induces apoptosis at very low concentration of drug (Servais et al., 2006). To investigate the role of cytosolic aminoglycosides in apoptotic processes, we compared the percentages of apoptosis induced by aminoglycosides of different nephrotoxic potential after incubation and electroporation in LLC-PK1 cells.

We first compared gentamicin-induced apoptosis, evaluated in terms of caspase-3 activity induction and fragmentation of nuclei, with those induced by amikacin, a less nephrotoxic aminoglycoside. Aminoglycoside concentrations ratio used for incubation of gentamicin and amikacin take in account the most common dosage ratio for humans, which are 4 mg/kg (8.56 µmol/kg) for gentamicin, and 15 mg/kg (25.6 µmol/kg) for amikacin.

Secondly, to confirm the role of cytosolic antibiotics, we compared apoptosis induced by aminoglycosides of different nephrotoxicity potential when they reach directly the cytosol, after electroporation. For this, we selected neomycin B, as example of nephrotoxic aminoglycoside other than gentamicin, and amikacin and isepamicin, known to induce less nephrotoxicity in patients. LLC-PK1 cells were then electroporated with these four aminoglycosides.

Electroporating cells with aminoglycosides allows introducing the antibiotics directly in the cytosol, avoiding endocytosis pathway and potential influence of differences in the ability of each antibiotics to induce lysosomal membrane permeabilization.

Manuscript 2 : Apoptosis induced by aminoglycosides in LLC-PK1 cells : comparative study of neomycin, gentamicin, amikacin and isepamicin using electroporation.

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Apoptosis Induced by Aminoglycosides in LLC-PK1 Cells: Comparative Study of Neomycin, Gentamicin, Amikacin, and Isepamicin Using Electroporation▽†

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Levels of apoptosis induction (4′,6′-diamidino-2-phenylindole staining, activation of caspase 3) for aminoglycosides were compared by using renal LLC-PK1 cells. Amikacin caused less apoptosis than gentamicin in incubated cells. In electroporated cells, neomycin B and gentamicin caused apoptosis in the 0.03 to 0.1 mM range, isepamicin required larger concentrations (0.2 mM), and amikacin was without effect.

Multiresistance in gram-negative bacteria (1, 11) and a lack of truly novel compounds (24) have led to 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, 5, 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 can also be differentiated from gentamicin for apoptosis by using incubated and electroporated cells. In the latter model, we included neomycin B (a well-known nephrotoxic aminoglycoside) (7) and isepamicin (which shares many of the properties of amikacin, including its lower potential for nephrotoxicity compared to gentamicin) (13, 15).

All methods and products were as previously described (20, 22) except for minor modifications (see the supplemental material). Cell-associated aminoglycosides were measured by a microbiological technique (20; linear response for both gentamicin and amikacin [$R^2 > 0.99$]). 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 are expressed as free base (see the 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/liter]) caused a marked, time-dependent increase in the percentage of apoptotic cells (as in reference 5), whereas amikacin (6 mM [3.516 g/liter]) was without effect at days 1 and 2 and caused only a small increase at day 3. Gentamicin also caused a marked increase in caspase 3 activity at day 1, followed by a maximum at day 2 and a decrease thereafter. Caspase 3 activity in cells incubated with amikacin was slightly lower than or similar to that of controls. Apoptosis, measured after 2 days of incubation, proceeded in a concentration-dependent manner with gentamicin (0 to 3 mM [0 to 1.389 g/liter]), whereas amikacin was without a significant effect at concentrations of up to 9 mM (5.274 g/liter). The accumulations of both drugs measured at 48 h were linearly related to their extracellular concentrations, with slopes of 11.9 ± 0.9 nmol · mg of protein^{-1} · 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 the values were compared at an extracellular concentration molar ratio of 3:1 (corresponding to their most common dosage ratios for humans, which are 4 mg/kg [8.56 μmol/kg] for gentamicin and 15 mg/kg [25.6 μmol/kg] for amikacin). Lactate dehydrogenase release (index of necrosis [20]) remained nonsignificantly different from that from the matching controls under 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 0.032 and 0.128 mM, with a maximum at 0.064 mM (39.2 mg/liter) for neomycin B and at around 0.1 mM (46.7 mg/liter) for gentamicin (the bell-shaped curve of apoptosis versus concentration is due to the development of necrosis once the concentration reaches a critical threshold; see reference 20 for a discussion). Isepamicin showed a considerably less-marked effect and larger concentrations (between 0.192 and 0.384 mM [109 to 218 mg/liter]) were required. 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 the supplemental material). The appar-

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† Supplemental material for this article may be found at http://aac.asm.org/.

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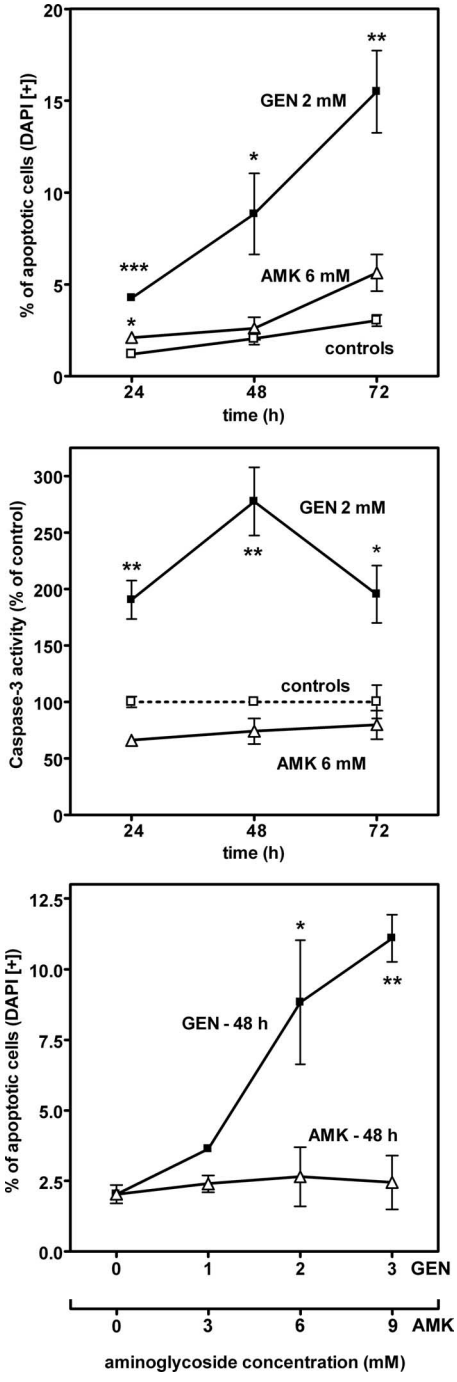

FIG. 1. Percentage of apoptotic cells (upper and lower panels) and increase of caspase 3 activity (middle panel) in LLC-PK1 cells incubated in the absence of aminoglycoside (controls; open squares) or in the presence of gentamicin (GEN; closed squares) or amikacin (AMK; open triangles). Upper panel, cells were incubated for up to 3 days without or with 2 mM gentamicin (0.926 g/liter) or 6 mM amikacin (3.51 g/liter), and the percentage of apoptotic nuclei was determined by microscopic examination after 4′,6′-diamidino-2-phenylindole (DAPI) staining (14.3 μM camptothecin [5 mg/liter] was used as the positive control (22) and yielded values of 43.1% ± 1.8%, 40.8% ± 2.2%, and 48.7% ± 2.8% of control values at 24, 48, and 72 h, respectively). Middle panel, same conditions of incubation as in the upper panel. Caspase 3 activity was assayed by using *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (see the supplemental material). Cells incubated with camptothecin yielded values of 2,767% ± 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 to 3 mM; 0.463 to 1.39 g/liter) or amikacin (3 to 9 mM; 1.76 to 5.26 g/liter). All values are means ± standard deviations ($n = 3$). Statistical analysis (two-tailed analysis of variance) 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 the text).

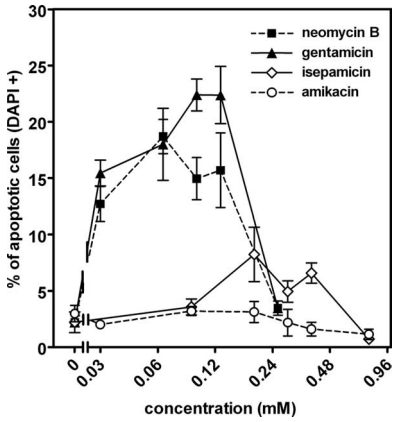

FIG. 2. Apoptosis in electroporated cells. Cells were electroporated in the absence (controls) or in the presence of neomycin B, gentamicin, isepamicin, or amikacin and returned to aminoglycoside-free medium, and apoptotic nuclei were enumerated 24 h later. Values are means ± standard deviations ($n = 3$). Statistical analysis was performed by two-tailed analysis of variance ($P < 0.01$). All values for neomycin B and gentamicin, except those observed for the largest concentration tested (0.256 mM), are significantly different from those of the controls; isepamicin values observed for 0.192, 0.288, and 0.384 mM concentrations are significantly different from those of controls; amikacin values did not differ from control values. The 0.12 mM concentration corresponds to approximately 74 mg/liter for neomycin B, 56 mg/liter for gentamicin (taking into account the respective contents of the commercial gentamicin in C1, C1a, and C2 components), 68 mg/liter for isepamicin, and 70 mg/liter for amikacin. See the supplemental material for structures of tested compounds.

ent cell concentrations for 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 than for gentamicin (53.3 ± 1.7 versus 26.7 ± 1.5 nmol · mg of protein^{-1} · 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 with rats, which showed that amikacin induces less apoptosis than gentamicin when tested at clinically relevant dosages (4). Under our culture conditions, LLC-PK1 cells take up aminoglycosides slowly and to a limited extent (20, 22), making it necessary to use extracellular concentrations that largely exceed those observed for 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 it possible (i) to compare

drugs at more clinically relevant concentrations (the percentage of apoptotic cells being already about sevenfold larger than in controls for a gentamicin concentration as low as 0.03 mM [approximately 14 mg/liter]); (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 one hand, and of amikacin and isepamicin, on the other hand, suggest specific interactions of these drugs with those intracellular constituents that are susceptible to the triggering of apoptosis (18, 20, 22). These should be further explored though systematic structure-activity relationship studies, but it already appears that the number of ionizable groups (and perhaps also their positions) could be critical (see the supplemental 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 mechanisms other than apoptosis (7, 21), making clinically pertinent drug ranking quite complex, the method developed here may help in further refining approaches toward the selection of safer derivatives. Generally speaking, it may also prove useful for the study of other drugs which, under normal conditions, would only slowly or poorly reach their intracellular pharmacological or toxicological target.

Marie-Claire Cambier provided dedicated technical assistance for cell culture, as did Martial Vergauwen and Vincent Rucchin for the apoptosis studies.

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Supplementary material:

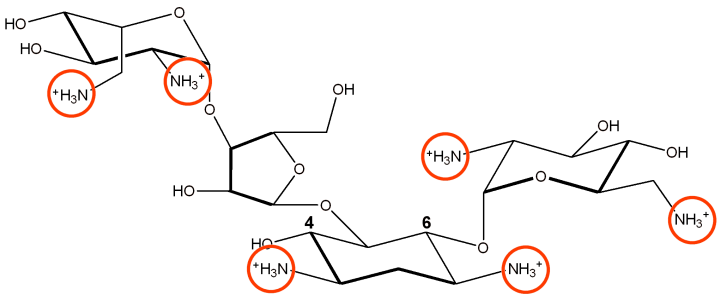
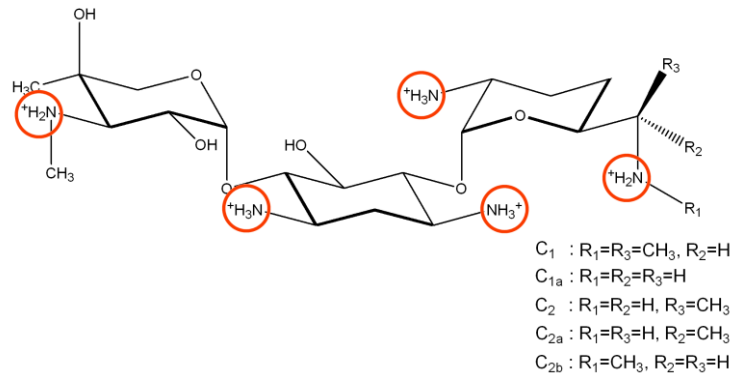
Materials and Methods

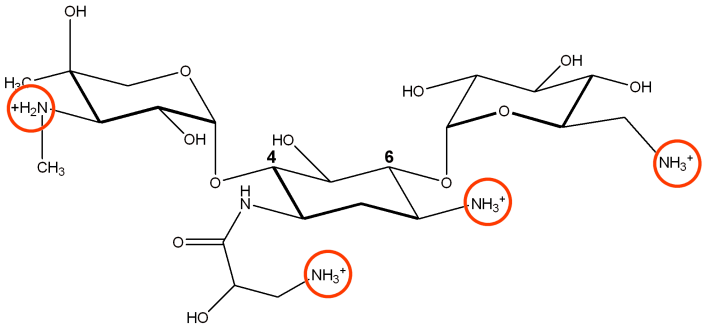
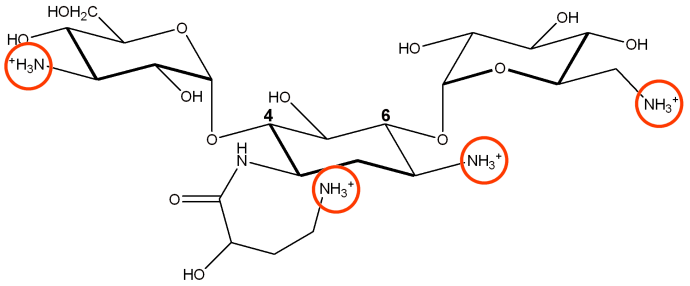
Conditions of assay of caspase-3:

Caspase-3 activity assay (20 µg of cell proteins/assay; measured with the Quick Start Bradford protein Assay kit [Biorad Laboratories, Hercules, CA]) was assayed using *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC [Caspase-3 Assay Kit Fluorimetric product code CASP-3F, Sigma Aldrich, St-Louis, MO]. Lysis buffer: 50 mM HEPES [pH 7.4], 5 mM CHAPS ([3-[(3-chloraminopropyl)dimethylammonio]-1-propane-sulfonate], 5 mM DTT (1,4-dithiothréitol); final reaction mixture: 17 µM Ac-DEVD-AMC, 20 mM HEPES [pH 7.4], 2 mM EDTA, 0.1% CHAPS, 5 mM DTT. Positive control: 12.5 µg/L lyophilized caspase-3 [product code C5974]. Blanks: addition of the caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde [Ac-DEVD-CHO; 2 µM]).

Figure SP-1:

Structure and systematic names of the aminoglycosides studied. Molecules are represented using the chair conformation for each 6-membered cyclic structures (with the forefront bonds in bold), as proposed in [1,2], and centered on the 2-deoxystreptamine (for which the numbering of the atoms used in the present representation (and as in [1]) is indicated). The structures and the configurations were cross-checked using Sci Finder Scholar version 2006 [American Chemical Society, Washington, DC]). The systematic names (obtained from Sci Finder Scholar) start from the left substituent(s) of the 2-deoxystreptamine to continue with the right substituent. The ionizable aminogroups are circled in red.

neomycin B [O[-2,6-diamino-2,6-dideoxy-β-L-idopyranosyl-(1→3)]-O-β-D-ribofuranosyl-(1→5)]]-O-[2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)]-2-deoxy-D-streptamine MW = 614.64	
gentamicin Commercial preparations are a mixture of 3 major (C1, C1a, and C2; approx. molar ratio, 30:15:50) and 2 minor components (C2a, C2b) [3] Average MW used in the present paper: 467 <u>Systematic name of the C1a component</u>: O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→4)]-O-[2,6-diamino-2,3,4,6-tetradeoxy-α-D-erythro-hexopyranosyl-(1→6)]-2-deoxy-D-Streptamine	 C_1 : R_1=R_3=CH_3, R_2=H C_{1a} : R_1=R_2=R_3=H C_2 : R_1=R_2=H, R_3=CH_3 C_{2a} : R_1=R_3=H, R_2=CH_3 C_{2b} : R_1=CH_3, R_2=R_3=H

isepamicin O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→4)]-O-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→6)]-N1-[(2S)-3-amino-2-hydroxy-1-oxopropyl]-2-deoxy-D-streptamine MW = 569.60	
amikacin O-[3-amino-3-deoxy-α-D-glucopyranosyl-(1→4)]-O-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→6)]-N1-[(2S)-4-amino-2-hydroxy-1-oxobutyl]-2-deoxy-D-streptamine MW = 585.60	

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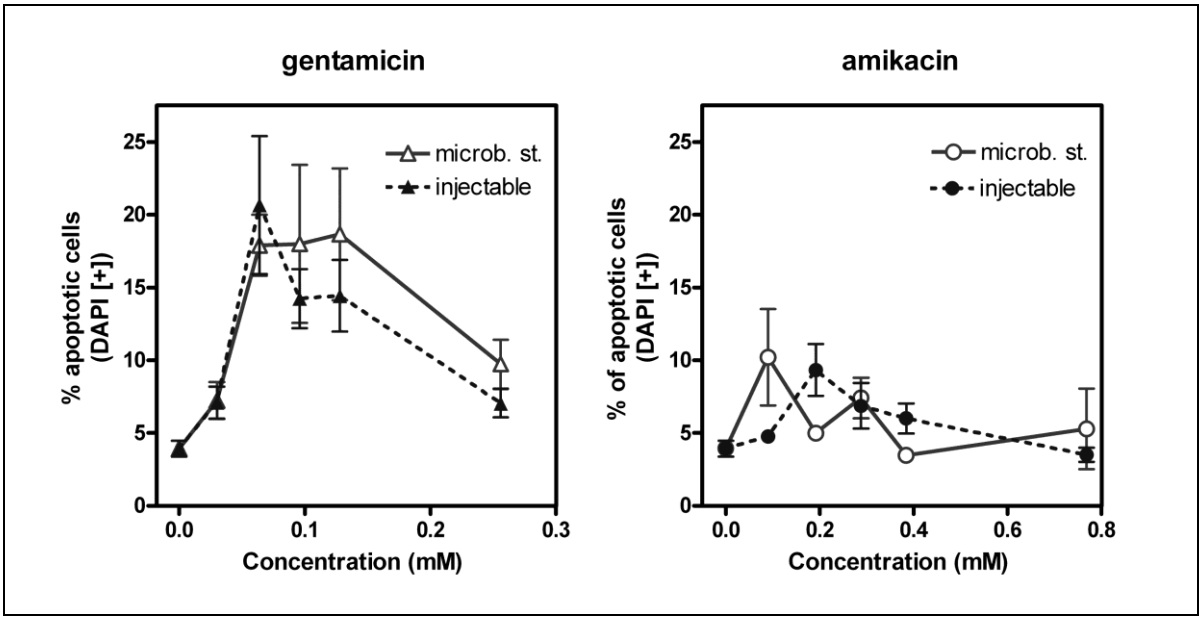
3. according to the European Pharmacopoeia, the amounts of C1, C1a and the sum of C2, C2a and C2b are limited to 20–40%, 10–30% and 40–60% of the total sample content [Gentamicin Sulphate, Monograph 01/2005:0331 Corrected, European Pharmacopoeia, 5th ed., European Department for the Quality of Medicines, Strasbourg, France, 2005, pp. 1653–1654]. The United States Pharmacopeia specifies that gentamicin C1 should be limited to 25–50%, C1a to 10–35% and the sum of C2 and C2a to 25–55% [Gentamicin Sulphate, Official Monograph, United States Pharmacopeia 26, United States Pharmacopeial Convention, Rockville, MD, 2003]. A recently published analysis of a typical standard sample of gentamicin sulphate (obtained as Chemical Reference Standard [CRS] from the European Pharmacopoeia Laboratory [http://crs.edqm.eu]) yielded values of 28.8, 16.3, 45.8, 2.7, and 9.8 % (w/w) for C1, C1a, C2, C2a, and C2b, respectively, whereas that of 3 bulk samples from Schering-Plough-Belgium (responsible for the distribution of clinical and microbological samples in Belgium) yielded values of 31.3-32.8, 26.1-28.8, 36.3-38.2, 1.6-1.8, and 1.4-1.7 % (Curiel H, Vanderaerden W, Velez H, Hoogmartens J, Van Schepdael A. 2007. Analysis of underivatized gentamicin by capillary electrophoresis with UV detection. J Pharm Biomed Anal. **44**:49-56).

Results

Figure SP-2



Comparison between the apoptotic response observed in cells electroporated with gentamicin (left) and amikacin (right) using either a microbiological standard (closed triangles; "microb. st.") or a clinically-used preparation (open squares; "injectable"; preparation complying with the European Pharmacopoeia and available for the corresponding marketing authorization holders in Belgium). Cells were electroporated in the absence (controls) or in the presence of gentamicin (0.03 mM-0.25 mM) or amikacin (0.09-0.9 mM), returned to aminoglycoside-free medium, and apoptotic nuclei enumerated 24 h later. Values are means ± SD (n=3). Statistical analysis (ANCOVA) showed no statistically-significant difference between the respective responses obtained with either type of product source.

The following conclusions can be highlighted from this work :

- **amikacin induces less apoptosis** than gentamicin when tested at clinically relevant dosages.

- the **low apoptogenic potential of less nephrotoxicant aminoglycosides** amikacin and isepamicin, and higher apoptosis induction by gentamicin and neomycin B, which are most nephrotoxic, are confirmed using electroporation.

- **observations made on rats** by El Mouedden regarding the ranking of the ability of aminoglycosides to induce apoptosis (El Mouedden et al., 2000a) can be extended to cultured and electroporated cells.

- **specific interactions** of the drugs with intracellular components able to trigger apoptosis, beyond their capacity to permeabilize lysosomal membrane, and independently of the drug accumulation can be hypothesized from the results obtained.

CHAPTER IV. What could link gentamicin-induced lysosomal membrane permeabilization and mitochondrial pathway of apoptosis ?

Lysosomal membrane permeabilization has been related with apoptosis induction in many models, mainly via the release of lysosomal proteases cathepsins and Bcl-2 proteins family implication. In the model of gentamicin induced apoptosis, several results suggest a direct role for cytosolic gentamicin (Mather and Rottenberg, 2001; Servais et al., 2006; Denamur et al., 2008) furthermore the possible release of cathepsins. To better understand the link between gentamicin-induced lysosomal membrane permeabilization and activation of mitochondrial pathway of apoptosis, we explored several pathways that could be implicated in gentamicin-induced apoptosis in LLC-PK1 cells treated with 1-3 mM.

We investigated the potential implication of p53 signaling pathway by the use of pifithrin α, a p53 inhibitor, on apoptosis induced by gentamicin. We also evaluated p21 and p27 cellular levels, as well as the potential induction of ER stress or ER-induced apoptosis by looking for upregulation of the chaperones GRP78 and GRP 94, phosphorylation of eIF2α and activation of caspase 12 by western blot. Finally, we considered a possible inhibition of the proteasome degradation pathway by gentamicin. In this respect, we measured the inhibiting potential of gentamicin on the three proteasome catalytic activities in LLC-PK1 cells lysates, we then evaluated cellular impact of the inhibition observed by detection of ubiquitinated proteins in cells incubated or not with gentamicin.

This part of the work has not yet been published and further experiments are in progress.

IV.1. Material and methods

IV.1.1. 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 and glucosamine hydrochloride were from Sigma Aldrich (St-Louis, MO, USA). 4',6'-diamidine-2'-phenylindole (DAPI) was from Roche (Basel, Switzerland).

IV.1.2. Cells and treatments

All experiments were performed with LLC-PK1 (Lilly Laboratories Culture-Pig Kidney Type 1) cells as 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% CO2. 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.

IV.1.3. Counting apoptotic cells

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

IV.1.4. Western blot analysis

Accumulation of ubiquitinated proteins, of p21 and p27, phosphorylated eIF2α and overexpression of ER chaperones GRP78 and 94 were analyzed by western blot analysis. After incubation for 24 hours with gentamicin, tunicamycin (as positive control of eIF2α phosphorylation and ER stress (Peyrou and Cribb, 2007)), cisplatin (as positive control of p21- and p27-induced apoptotic pathway (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 saved. Cells were detached by trypsinization, pelleted by centrifugation at 290 g for 7 minutes and washed two times with gentle resuspension and repelleting in ice-cold Phosphate Buffer Saline (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 minutes at 14000 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 4X NuPAGE LDS sample buffer and 10X NuPAGE reducing agent, then heated at 70°C for 10 minutes. Samples were loaded on acrylamide gels (NuPAGE 1% Bis-Tris Gel, Invitrogen). After migration, proteins were electro-transferred onto PVDF membrane (0.45 µm, Pierce), which was blocked by a 1 h incubation in 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 hours at room temperature with the primary antibodies anti-ubiquitin (sc-47721, Santa Cruz Biotechonolgies, Santa Cruz, CA, USA), anti-GRP78 (76-E6) (sc-13539, Santa Cruz Biotechnologies), and anti-GRP94 (SPA-850F, Stressgen, Enzo Life Sciences, Farmingdale, NY, USA), or overnight at 4°C with anti-phosphorylated eiF2α antibody (3597, Cell Signaling Technology, Danvers, MA, USA), to detect ubiquitin, GRP78, GRP94, and phosphorylated form of eiF2α respectively, and exposed to appropriate horseradish peroxidase-coupled secondary antibodies for 1h. Blots were revealed by chemiluminescence (SuperSignal West Pico, Pierce). Membranes were then washed with Restore Western Blot Stripping Buffer (Pierce) for 30 minutes, and anti-actin polyclonal antibodies (sc-1616, Santa Cruz Biotechnologies) were used as loading control. Films were scanned and subjected to a densitometric analysis using image J software (version 1.3.1; available from the Research Service branch of the National Institute of Mental Health at http://rsb.info.nih.gov/ij).

IV.1.5. Proteasome activities 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 scraped 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 seconds and centrifuged for 15 minutes at 15000 g and 4°C. Supernatants were collected and proteins were assayed realized using 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 1h 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)). 50 µl of reaction buffer (Tris-HCl 50 mM pH 7.4, EDTA 0.5 mM) containing suitable substrate concentration (100 µM Suc-LLVY-amc, 200 µM Ac-RLR-amc and 100 µM Ac-nLPnLD-amc) were then added in the wells. Fluorescence was read immediately and after 4 hours incubation at 37°C with a FluoroCount Microplate Fluorometer (Packard Instruments Company, Downers Grove, IL, USA) using the excitation wavelength of 380 nm and reading at 460 nm. We checked that neither gentamicin nor epoxomicin interfered with AMC fluorescence.

IV.1.6. Statistical analysis

All statistical analyses were perfomed with GraphPad Prism version 4.02 and GraphPad InStat version 3.06 (GraphPad Prism Software, San Diego, CA, USA). The comparison of 3 or more groups of data was performed using one-way ANOVA with Dunnett's multiple comparison post-tests to compare values to control, and with Tukey's multiple comparison post-tests to compare all values.

IV.2. Results

IV.2.1. Implication of p53 signaling pathway

p53 is a transcription factor which is implicated in cellular response to stress. It can be activated by lysosomal membrane permeabilization induced by H_2O_2 (Eno et al., 2013). To determine if p53 is involved in apoptosis induced by gentamicin, we treated cells with the p53-inhibitor pifithrin α (Yano et al., 2007).

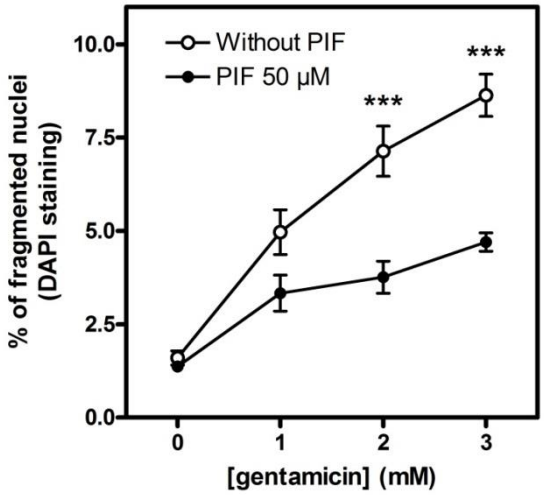


Fig.12. Prevention by the p53 inhibitor pifithrin α of gentamicin-induced apoptosis. Cells were pre-incubated or not for 1h with 50 µM pifithrin α and then incubated with gentamicin at indicated concentrations for 24h, in continued presence of pifithrin α. Data are given as means ± SD (n=6 from 2 separate 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, where not indicated, differences are not significant).

As shown in Fig.12, pre- and co-incubation of cells with pifithrin α decrease from about half apoptosis induced by gentamicin after 24h incubation. The same observation of a partial protective effect can be made from cells incubated for 48h with gentamicin with 17.15% apoptosis in cells incubated with 3 mM gentamicin and 9.6% apoptosis in the same conditions gentamicin but after treatment with pifithrin α. 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). The p53 inhibitor did not induce any change in gentamicin accumulation (data not shown).

IV.2.2. Slight accumulation of cyclin-dependent kinases inhibitors.

p53 can interfere with the cell-cycle progression through the induction of the cyclin-dependent kinase inhibitor p21. The expression levels of the p21 family CDK inhibitors play an important role in the regulation of cyclin-dependent kinases activity, which are responsible for cell cycle progression.

We thus investigated cellular levels of p21 by western blotting after gentamicin treatment (Fig.13a). We observed a very slight increase in p21 protein levels. This increase seems to be concentration-dependent but is statistically non-significant.

Cellular levels of p27, another cyclin-dependent kinases inhibitor, also slightly increased in response to gentamicin (Fig.13b). However, levels of p27 in cells treated with 3 mM were a trifle lower than those observed in cells treated with 2 mM of the drug. Cisplatin was used as positive control of p21 and p27 overexpressing agent (Wang et al., 2004).

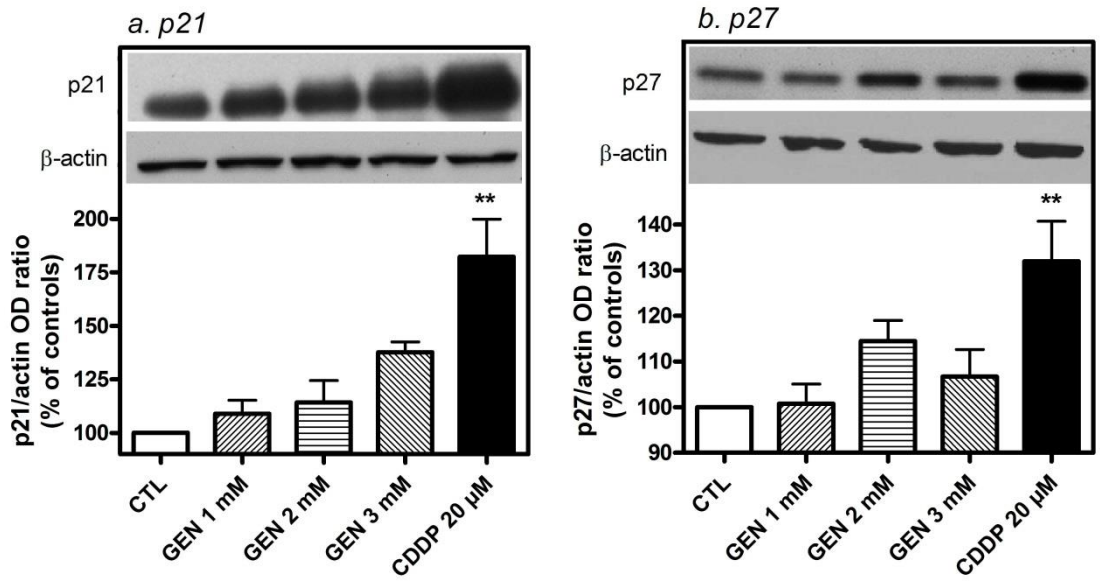


Fig.13. Effect of gentamicin on p21 and p27 protein levels. Cells were incubated for 24h without (controls, CTL) or with gentamicin (GEN 1-3 mM) or cisplatin (CDDP 20 µM). p21 and p27 protein band were detected at 21kDa and 27kDa respectively; densitometric values are normalized to β-actin. The blot presented is representative from 3 independent experiments, data are means ± SD (n=3) (*p<0.05, ***p<0.001, where not indicated, differences are not significant).

IV.2.3. Absence of accumulation of ER chaperones and slight accumulation of phosphorylated eIF2α

Another possible hypothesis to explain induction of apoptosis by cytosolic gentamicin is an implication of ER. Several elements are consistent with this, such as 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 100mg gentamicin (Peyrou et al., 2007). To test this hypothesis, we evaluated cellular levels of ER chaperones as markers of endoplasmic reticulum stress.

To this aim, cells were incubated with or without gentamicin (1-3 mM) for 24h, and expression levels of the ER chaperones GRP78 and GRP 94 were examined by western blot (Fig.14, 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.

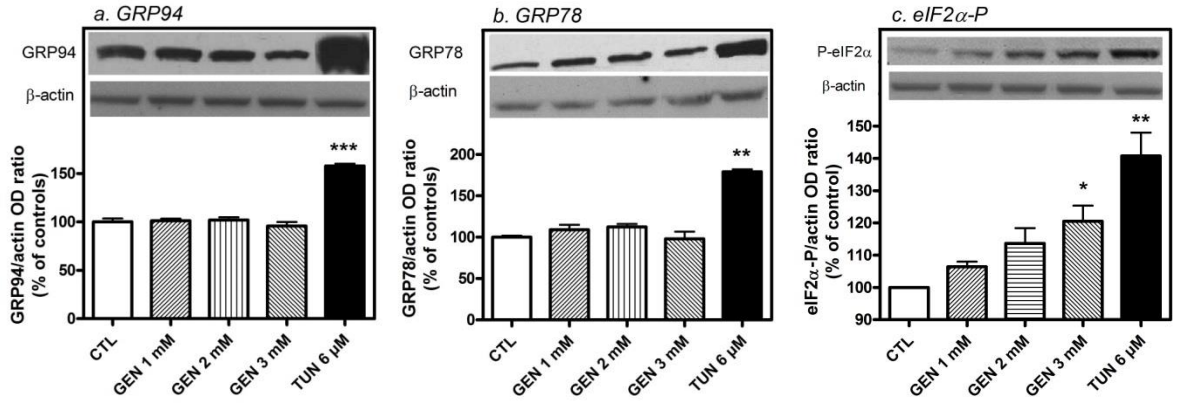


Fig.14. Effect of gentamicin on cellular levels of GRP94 (a), GRP78 (b), and phosphorylated eIF2α (c). Cells were incubated for 24h with or without (control, CTL) gentamicin (GEN) or tunicamycin (TUN) at indicated concentrations. Densitometric values are normalized with β-actin, results are presented as percentage of OD ratio obtained for control cells. Values are means ± SD (n=3, from 3 separate experiments). GRP94, GRP78 and p-eIF2α were detected at 94, 78 and 38 kDa respectively.

As shown in figure 14, no significant change in GRP 94 (Fig.14a) and 78 (Fig.14b) protein levels were detected in cells incubated with gentamicin, while tunicamycin induced a marked increase of both chaperones. These experiments were also realized on cells incubated for 48h with gentamicin, and no significant change was observed (data not shown).

Western blot analysis of cellular levels of phosphorylated eIF2α shows an increase in protein levels which became significant in samples incubated with 3 mM gentamicin (Fig.14c). However, in order to interpret this increase, total eIF2α will also be quantified by western blotting.

IV.2.4. Inhibition of trypsin- and caspase-like activitites of proteasome at high doses in cellular lysates

p21, p27 and the eIF2α kinase GCN2 are all targets for degradation by the proteasome. A plausible explanation to their accumulation and to p53 pathway activation could be an inhibition of the proteasome activity by gentamicin. This hypothesis is supported by the accumulation of ubiquitinated Bax in cells incubated and electroporated with gentamicin (Servais et al., 2006).

To test this hypothesis, we assayed the three different catalytic activities of proteasome, LLC-PK1 cells lysates were collected and crude extracts were incubated for 1 h at 37°C with increasing concentrations of gentamicin (0 up to 30mM) before adding specific fluorogenic substrates of each catalytic activity. Epoxomicin, a potent inhibitor of the proteasome (Giguere and Schnellmann, 2008) was used as positive control.

Among the substrates used for detection of proteasomal catalytic activities, Suc-LLVY-amc has been described to be not only cleaved by the proteasome but also by the non-lysosomal cysteine protease calpain (Giguere and Schnellmann, 2008). To evaluate the contribution of proteasome in Suc-LLVY-amc cleavage in our experiments, we co-incubated cellular lysates with epoxomicin and gentamicin, epoxomicin inhibiting only chymotrypsin-like activity of the proteasome. Fluorescence in samples co-incubated with gentamicin and epoxomicin detected was the same as in substrate alone. We thus concluded that Suc-LLVY-amc was only cleaved by proteasome in our experiments (see Fig. 15).

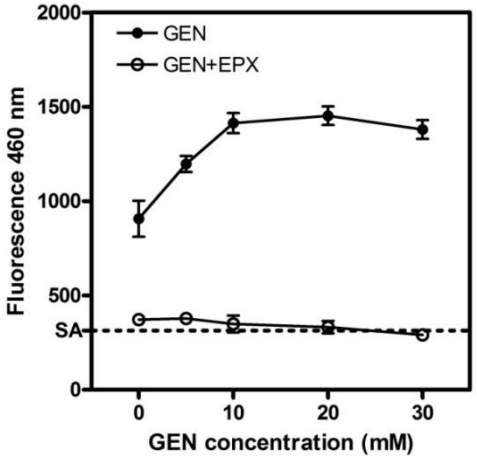


Fig.15. Specificity of Suc-LLVY-amc to detect chymotrypsin-like proteasome activity. LLC-PK1 cellular lysates were incubated at 37°C for 1h with gentamicin (GEN, 0-30 mM) in presence (open symbols) or in absence (closed symbols) of epoxomicin (EPX) 20µM. Suc-LLVY-amc was then added and fluorescence was read after 4 hours incubation at 37°C. Fluorescence level of the substrate alone (SA, substrate not incubated with cell lysate) is represented by a dotted line.

While chymotrypsin-like activity was completely inhibited by epoxomicin as the fluorescence level detected with this inhibitor is comparable to the fluorescence emitted by the substrate alone (SA, Fig.16a), incubation with gentamicin induced a non significant increase of chymotrypsin-like activity from 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 became complete (same fluorescence level as the substrate alone) from 15 mM. Note that trypsin-like activity was not inhibited by epoxomicin (Fig.16b), as described previously (Naujokat et al., 2007).

Effect on caspase-like activity was bimodal, increasing at 5-10 mM, then falling below control (untreated), but no change was statistically significant (Fig16c). As for chymotrypsin-like activity, epoxomicin induced an important inhibition of this proteasomal activity.

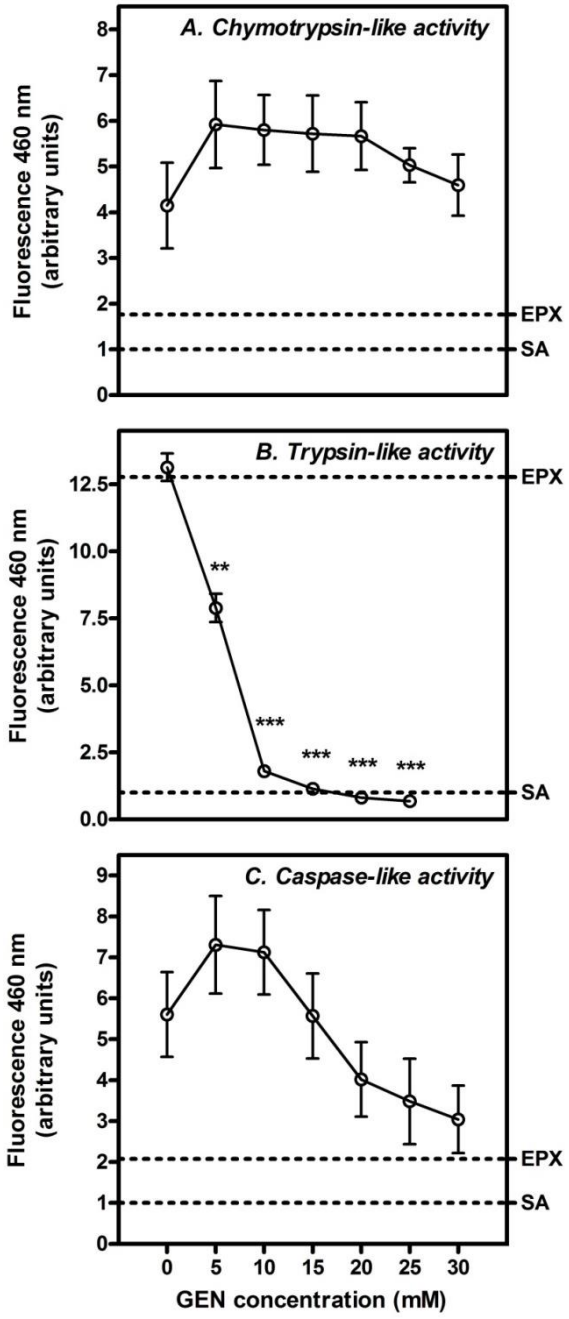


Fig.16. Effect of gentamicin on proteasome catalytic activities, determined by measuring fluorescence produced by fluorogenic specific substrates of the three proteasomal activities. Results are expressed in arbitrary units, fluorescence of the substrate alone being considered as having a value of 1. Cellular lysates were incubated at 37°C for 1h 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 4h incubation at 37°C after substrate addition. 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).

IV.2.5. Slight accumulation of ubiquitinated proteins

Gentamicin thus inhibited trypsin- and caspase-like activities at high concentrations in cellular lysates. To evaluate the impact of this phenomenon in cultured cells treated with gentamicin, we explored by western blotting the levels of ubiquitinated proteins in cells incubated for 24h with the drug. The proteasome inhibitor epoxomicin (EPX, 20 µM) was used as positive control. As shown in Fig.17., epoxomicin induces an important accumulation of ubiquinated proteins in the cells, and the signal measured by densitometry is oversaturated and therefore underestimated in the graph 17B. Gentamicin shows a slight concentration-dependent effect, as 3 mM induces an accumulation of ubiquitinated proteins, even if not statistically significant.

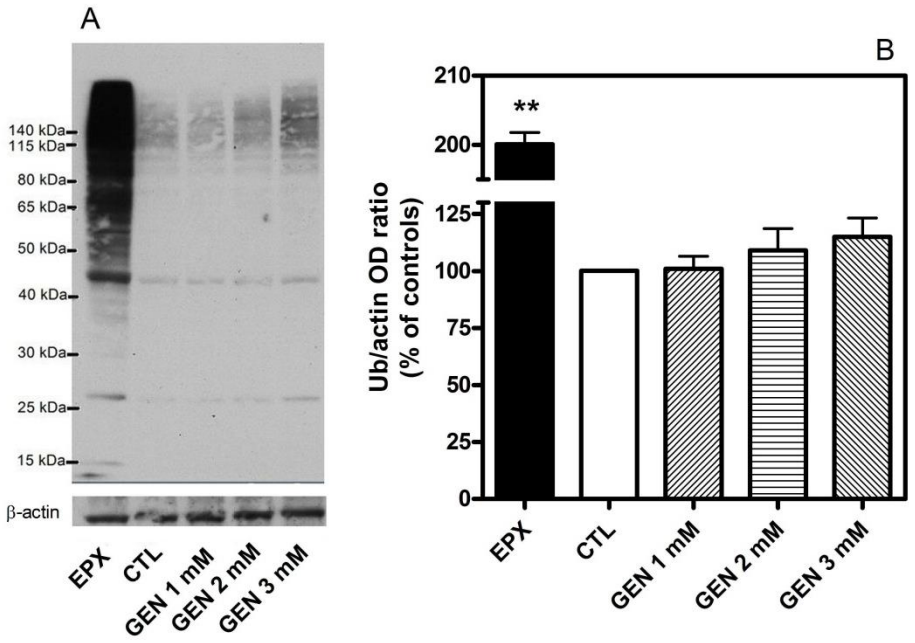


Fig.17. Effect of gentamicin on ubiquitinated proteins, detected by western blot. LLC-PK1 cells were incubated for 24h with or without (control, CTL) gentamicin (GEN 1-3 mM) or epoxomycin (EPX, 20 µM). After revelation, membrane was washed, and equal loading of wells was controlled by detection of β-actin. 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). Statistical analysis was made by one-way Analysis of Variances, followed by Dunnett's multiple comparison post-test to compare each point to control value (without gentamicin) (**p<0.01, where not indicated, differences are not significant).

IV.3. Discussion

Previously, we described gentamicin-induced lysosomal membrane permeabilization and suggested a role of ROS in the underlying mechanism (Denamur et al., 2011). However, the mechanisms through which lysosomal membrane permeabilization is coupled with mitochondrial outer membrane permeabilization and apoptosis are not yet understood. In this work, we explored several pathways that could result from lysosomal membrane permeabilization and could induce apoptosis.

Our results suggest a possible involvement of p53 signaling pathway in apoptosis induced by gentamicin, accompanied by a slight increase in p21 and p27 protein levels. We tested the hypothesis of an inhibition of proteasome by gentamicin to explain the slight accumulation of these proteins. We observed an inhibition of trypsin- and caspase-like activities of proteasome in cellular lysates but only at high concentration of gentamicin. Finally, we did not observe any increase of chaperones proteins GRP78, GRP94 but an increase in the phosphorylated form of eIF2α.

The partial protective effect afforded by the p53 inhibitor pifithrin α suggests an implication of p53 signaling pathway in gentamicin-induced apoptosis. This hypothesis is reinforced by the increase of p53 translation, observed by microarray experiments realized on rats treated with 80 mg/kg gentamicin (Ozaki et al., 2010).

The activation of p53 in cells treated by gentamicin can be explained by proteasomal inhibition (see below), or effects on NFκB or JNK pathways. Activation of the p65 subunit of NFκB is able to activate the p53 promoter (Wu and Lozano, 1994). Implication of NFκB in events cascade that follows gentamicin treatment is suggested by the increase of NFκB activation demonstrated after incubation of renal tubular cells with gentamicin (Chen et al., 2011; Juan et al., 2007). NFκB pathway could be induced by gentamicin-induced lysosomal ROS production (Wang et al., 2002) and facilitated by eIF2α phosphorylation (Jiang et al., 2003). Regarding the JNK-signaling pathway, a potential stabilization and activation of p53 is also possible (Fuchs et al., 1998). JNK implication has been described after gentamicin treatment in mesangial cells (Martinez-Salgado et al., 2005). Moreover, an increase of Jun translation has been evidenced by microarray studies (Ozaki et al., 2010), even if no modification of JNK or pJNK was observed in proximal tubular cells after 24h of incubation with gentamicin 3 mM (Chen et al., 2011).

p53 can activate the transcription of number of protein-coding target genes implicated in apoptosis induction. Activation of p53 signalling pathway lead to Noxa transcription and inhibition of anti-apoptotic members of the BCL-2 family (Shibue et al., 2003). On one hand, Noxa is able to interact with and causes the release of cytochrome c from mitochondria, causing induction of apoptosis and its role in gentamicin-induced apoptosis would be interesting to elucidate. On the other hand, the protective role of Bcl-2 against gentamicin-induced apoptosis has been suggested earlier (El Mouedden et al., 2000b), and its inhibition by p53 signalling pathway could promote apoptosis induction by gentamicin.

Pifithrin α has been described to protect against genotoxic agents, including cisplatin (Jiang et al., 2004) or UV-induced damages (Komarov et al., 1999). However, it only provides a partial protective effect against gentamicin-induced apoptosis. This partial effect could be explained by (i) the stability of the compound and (ii) implication of p53-independent pathways in gentamicin-induced apoptosis. First, pifithrin α has been reported to be unstable at long term in cell culture medium and to convert to its condensation product pifithrin β (Walton et al., 2005). This element could explain that we lost, after the 24 hours of incubation, some protective effect afforded by the inhibitor, and that we still have apoptosis in cells incubated with gentamicin and pifithrin α. Second, regarding the multiplicity of apoptotic pathways, even if p53 activation is inhibited by pifithrin α, gentamicin probably activates p53-independent other apoptotic pathways by (i) inhibition of degradation of pro-apoptotic proteins by the proteasome (Servais et al., 2006), or direct interaction of gentamicin with mitochondria (Mather and Rottenberg, 2001). Lysosomal membrane permeabilization can, besides the release of gentamicin to the cytosol, also allow the release of lysosomal content and translocation to the cytosol of lysosomal proteases such as cathepsins which are known to induce apoptosis (Chwieralski et al., 2006). Moreover, since some authors suggest a protection by pifithrin α by a mechanism independent of p53 (Sohn et al., 2009), the role of p53 in gentamicin-induced apoptosis should be confirmed by more specific techniques such as p53 si-RNAs.

To further test the hypothesis of an implication of p53 in gentamicin-induced apoptosis, we quantified p53 by western blotting. However, numerous unidentified bands appeared in the blots, and we were not able to reach a final conclusion from these experiments. The protective effect observed with the inhibitor is not yet sufficient to characterize p53 implication in gentamicin-induced apoptosis. Other experiments should be conducted in this aim.

A well-described effect of p53 activation is the induction of p21 expression. p21 and p27 are both mentioned for their role in cell cycle inhibition and apoptosis modulation. We showed by western blot analysis a slight increase of both p21 and p27 protein levels. p21 increase can result from accumulation due to (i) p53-induced translation, (ii) gentamicin-induced inhibition of ubiquitin-proteasome degradation pathway or (iii) up-regulation due to JNK pathway activation (Yang et al., 2004). p27 expression is controlled by its degradation rate more than changes in transcription or translation (Pagano et al., 1995). To determine the cause of the increase that we observe, it would be interesting to quantify the mRNA of both proteins.

However, the increase of the levels of p21 and p27 proteins observed is relatively small, and we can wonder about the real impact of these events in the apoptotic cascade induced by gentamicin. Using siRNA would be useful to evaluate the role of p21 and p27 in this phenomenon. We can also wonder if the small effect observed result from a slight increase of p21 and p27 in all the cells of the sample, or from a consequent increase in a minority of the cells. Immunohistochemistry techniques would be helpful to answer this question.

In addition, since the pro- or anti-apoptotic role of p21 is dependent of its nuclear or cytoplasmic localization, it would be interesting to investigate the subcellular localization of the p21 increase observed in our experiments before concluding on influence of this overexpression on gentamicin-induced apoptosis.

To further explore the mechanisms involved in gentamicin-induced apoptosis, we investigated the implication of endoplasmic reticulum. We did not detect changes in GRP94 and GRP78 proteins level in our experimental conditions. Former studies described signs of ER stress induction after treatment of rats with gentamicin (Peyrou et al., 2007), but chaperones induction and caspase 12 activation only appeared after 7 days of treatment with gentamicin at high doses (160 mg/kg), while apoptosis we are describing here is a characteristic of short-term and therapeutic-related dose (Mingeot-Leclercq and Tulkens, 1999). Despite this, in order to exclude an implication of ER stress in the apoptotic process, other markers of ER stress should be investigated such as caspase 12 and CHOP.

Unlike results for chaperones, western blot analysis showed increased levels of phosphorylated eIF2α, which can be caused by ER stress and PERK activation (Sha et al., 2011) or by increased phosphorylation by eIF2α kinases such as GCN2, as earlier described after proteasomal inhibition (Jiang and Wek, 2005).

Taking into account all hypothesis together, a possible link between the activation of p53, the accumulation of p21 and p27 and phosphorylated eIF2α could be the inhibition of

proteasome by gentamicin. Mechanisms underlying the proteasome inhibition by gentamicin could involve a direct interaction of the drug with the proteasome, since Horibe and colleagues have described binding of the drug to proteasome subunit β9 (PSB9) (Horibe et al., 2004). PSB9 consists of the precursor of the proteasome subunit β7 which functions to amplify specific endopeptidases activities of the proteasome and contributes to the hydrolysis of hydrophobic and basic substrates (Van Kaer et al., 1994). Alteration of the proteasomal activity can also be due to the accumulation of misfolded/unfolded proteins (Bence et al., 2001) induced by aminoglycoside interaction with the eukaryotic ribosome (Ryu and Rando, 2001).

Our results show an inhibition of trypsin- and caspase-like activities of proteasome only at high doses of gentamicin in cellular lysates, and a slight accumulation of ubiquitinated proteins is observed in cells.

Trypsin- and caspase-like activities are suggested to have very little role in yeast protein degradation, and chymotrypsin-like is considered as rate-limiting in protein breakdown (Heinemeyer et al., 1997). In contrast, all three types of active sites have been demonstrated to contribute to protein breakdown in mammalian cells. Relative importance of each catalytic site varies widely with the substrate, and inhibition of multiple sites is required to markedly decrease proteolysis. Simultaneous inhibition of trypsin- and caspase-like activities reduce degradation rate of model proteins by pure 26S proteasome by 30 to 56% depending on the substrate observed (Kisselev et al., 2006).

However, we have to be aware that concentrations needed to observe proteasome activities inhibition in cellular lysates are huge. Concentrations of gentamicin reached in lysosomes where it accumulates are evaluated at 30 mM or above (El Mouedden et al., 2000b) after incubation of LLC-PK1 cells with the drug at extracellular concentrations selected to match cellular concentrations reached in animals and humans treated with therapeutic doses (Servais et al., 2005). However, after lysosomal permeabilization, the accumulated gentamicin is diluted about 100 fold into the cytoplasm (Wilmotte et al., 1983) and cytoplasmic concentrations of gentamicin are thus much lower than those we used in cellular lysates, suggesting that direct inhibition of proteasome by gentamicin is probably not a key event in the apoptotic cascade induced.

Regarding thoroughly the inhibitory effects on proteasome induced by gentamicin and especially the inhibition of the trypsin-like activity, we have to paid attention to the role played by the amino functions. The substrate used for this assay (Ac-RLR-amc) contains two arginines, basic amino acids containing a side-chain consisting of a three-carbon aliphatic chain, capped by a guanidinium group, and presenting amine function, as aminoglycosides.

In order to determine if amine functions of gentamicin are important for trypsin-like activity inhibition, and to discriminate if inhibition observed result from competition between gentamicin and the substrate, we compared results obtained with gentamicin with those of glucosamine, which only has one amine function (while five for gentamicin). Glucosamine induced an important inhibitor effect increasing between 5 and 25 mM. No link can thus be established between proteasome inhibitor effect observed and the number of amines presents on the molecule, suggesting thus that inhibition of substrate degradation was not due to competition between amine functions of gentamicin and that of Ac-RLR-amc for proteasome degradation.

Finally, the induced accumulation of ubiquitinated proteins is non significant regardless of the dose of gentamicin, although a slight increase is visible at 3 mM. Since gentamicin induces apoptosis between 1 and 3 mM (Denamur et al., 2008), we can suppose that accumulation of ubiquitinated proteins is not a major step for apoptosis induction by gentamicin.

At this stage of the work, we can summarize our observations as follows :

- p53 inhibitor pifithrin α provided a partial protective effect on gentamicin-induced apoptosis, maybe suggesting **p53 signaling pathway** implication in this process.

- cellular levels of **p21 and p27 cyclin-dependent kinases inhibitors slightly increased** after incubation with gentamicin, and a **slight increase of phosphorylated eIF2α** is induced.

- **GRP78, GRP94 cellular levels does not change** after gentamicin treatment. However, before excluding an implication of ER stress, other markers should be investigated such as caspase 12 and CHOP.

- **inhibition of trypsin- and caspase-like activities of proteasome at high gentamicin doses in cellular lysates**, and slight **accumulation of ubiquitinated proteins** appeared in cells treated with the drug.

CHAPTER V. Main findings of this work

Aminoglycosides antibiotics are important molecules for the treatment of severe infections due to Gram-negative bacteria. Their antiviral properties and their potential antiviral and gene defect-correction properties make a great therapeutic interest, and could lead to development of chronic treatment with aminoglycosides. However, presently, their use is still limited by the induction of nephrotoxicity, whose mechanisms remains uncompletely understood.

The present study was therefore designed to get further knowledge about the intracellular mechanisms underlying gentamicin-induced apoptosis in LLC-PK1 proximal tubular cells. Our aim was to better understand the link between gentamicin lysosomal accumulation and activation of mitochondrial pathway of apoptosis, and identify the main organelles and pathways implicated in aminoglycosides toxicity.

At the end of this project, the main conclusions we achieved about the mechanisms of gentamicin-induced apoptosis in LLC-PK1 cells can be summarized as followed.

V.1.1. Gentamicin induced lysosomal membrane permeabilization

Prior observations made by Servais and colleagues in LLC-PK1 cells highlighted the change of acridine orange fluorescence emission after gentamicin-treatment. Given the concerns raised by the acridine orange technique, we used vital imaging and showed the release of Lucifer yellow, a membrane-impermeant endocytic tracer, from lysosomes into the cytosol. We have interpreted this observation as the induction by gentamicin of lysosomal membrane permeabilization.

V.1.2. ROS and iron played a critical role in lysosomal membrane permeabilization induced by gentamicin

We have demonstrated a critical role of ROS in gentamicin-induced lysosomal membrane permeabilization and apoptosis. We evidenced by fluorimetry assay of the oxidation of the fluorogenic dye H_2DCFDA the production of ROS in LLC-PK1 cells treated with gentamicin. We found that gentamicin induced ROS production prior to, and at lower drug concentrations than that required for acridine orange release and apoptosis. Vital imaging allowed to localize this ROS production in lysosomes.

Use of anti-oxidant or scavenger molecules catalase or *N*-acetylcysteine largely (but not completely) prevented gentamicin-induced ROS production, acridine orange release and apoptosis. Moreover, deferoxamine, an iron chelator which is endocytosed and accumulated in lysosomes, largely prevented ROS production and apoptosis.

Our data demonstrate thus a key role of early ROS production and lysosomal iron in gentamicin-induced lysosomal membrane permeabilization and apoptosis.

V.1.3. Cytosolic aminoglycosides may account for apoptosis

We compared apoptosis induced by gentamicin with the one induced by other aminoglycosides (amikacin, isepamicin and neomycin B). Our results extended to cultured cells the observations made with rats, which showed that amikacin induces less apoptosis than gentamicin. We then observed that electroporation of more nephrotoxic aminoglycosides (gentamicin and neomycin B) induced more apoptosis than low toxic ones (amikacin and isepamicin). The low concentrations needed to observe induction of apoptosis by nephrotoxic drugs after electroporation highlight the high apoptogenic potential of cytosolic aminoglycosides, and the important consequences that could have their release from lysosomes into the cytosol. The common behaviors of neomycin B and gentamicin, on one hand, and of amikacin and isepamicin, on the other hand, suggest specific interactions of these drugs with intracellular constituents that are susceptible to trigger apoptosis and toxicity. Moreover, although the renal nephrotoxicity of aminoglycosides may involve mechanisms other than apoptosis (Servais et al., 2008; Lopez-Novoa et al., 2011), making clinically pertinent drug ranking quite complex, the method developed here may help in further refining approaches toward the selection of safer derivatives.

V.1.4. p53 signaling pathways are candidates for gentamicin-induced apoptosis

Exploration of several pathways that could link lysosomal membrane permeabilization and activation of mitochondrial intrinsic pathway of apoptosis led us to suggest an implication of p53 signaling pathway in gentamicin-induced apoptosis, following the observation of a partial protective effect of the p53 inhibitor pifithrin α on gentamicin-induced apoptosis. This hypothesis remains to be confirmed by specific techniques such as si-RNAs. We also observed slight increases in cellular levels of p21 and p27 after incubation of cells with gentamicin. We made the hypothesis that these effects could be due to an inhibition of the

proteasome by gentamicin, but the high drug concentrations needed to observe proteasomal inhibition in cellular lysates challenge this hypothesis.

Regarding the involvement of an ER stress in apoptosis induced by gentamicin, we did not observe any change in ER chaperones GRP78 and GRP94 but other markers such as CHOP and caspase 12 should be investigated. We also observed an increase in phosphorylated eIF2α which can be caused by ER stress and PERK activation or by increased phosphorylation by eIF2α kinases such as GCN2.

CHAPTER VI. General discussion

Our conclusions are based on results obtained from experiments performed in LLC-PK1 cultured cells. This model presents several advantages and disadvantages.

VI.1. Interest and limits of LLC-PK1 cellular model

LLC-PK1 is a stable porcine tubular proximal well-characterized cell line possessing several characteristics of proximal tubular cells, such as Na^+-dependent transport systems, enzymes located in the apical membrane including alkaline phosphatase and γ-glutamyl transpeptidase (Hull et al., 1976; Rabito, 1986). LLC-PK1 were widely used to study nephrotoxicity of drugs and particularly to study gentamicin nephrotoxicity (Hori et al., 1984; Sandoval and Molitoris, 2004). The ability of aminoglycosides to induce apoptosis in LLC-PK1 has also been characterized previously in our lab (El Mouedden et al., 2000b), and the involvement of lysosomes and mitochondria in gentamicin-induced apoptosis has been described in LLC-PK1 cells by Servais *et al.* (Servais et al., 2005). Our work aimed at better understanding the intracellular cascade previously described and was designed to get further knowledge of the link between accumulation of aminoglycosides in lysosomes and activation of mitochondrial intrinsic pathway of apoptosis. Based on the previous works, LLC-PK1 seems to be an appropriate model to study gentamicin-induced apoptosis, and allowed us to compare and integrate our observations to the former results. More generally speaking, working in cellular model also allowed us to explore more precisely the pathways that could be implicated in gentamicin-induced apoptosis, with, for example the vital imaging in cells that allowed to localize ROS production in lysosomes. Moreover, comparison of apoptosis induced by aminoglycosides from different nephrotoxic potential with nephrotoxicity induced *in vivo* suggests that use of LLC-PK1 cells may help in further refining approaches toward the selection of safer derivatives.

However, a cellular model does not allow reproducing all conditions of *in vivo* experiments. First of all, pharmacokinetics parameters such as administration, distribution, metabolism and excretion (ADME) have to be taken in account to consider the *in vivo* situation. Working with cultured cells also limits the observation to one cell type, and does not take in account all potential effects of gentamicin. In this work, we focused on the mechanisms underlying the apoptotic cell death in proximal tubular LLC-PK1 cells. This model did not integrate the possible influence of glomerular toxicity, vascular effect and inflammation (Lopez-Novoa et al., 2011) on induction of cell death by gentamicin.

A second important difference between LLC-PK1 cells and *in vivo* conditions is the presence of megalin at the apical membrane of the cells. Indeed, expression of megalin in LLC-PK1 cells requires polarized culture cell on inserts (Nielsen et al., 1998), and LLC-PK1 probably

does not express megalin in our culture conditions (Decorti et al., 1999; Girton et al., 2002). The presence of megalin could raise different questions namely as the kinetic of gentamicin uptake. In the present study, we used large concentrations of gentamicin (1-3 mM ; approximatively 0,5 to 1,4 g/L) to obtain in LLC-PK1 cells cellular and intralysosomal drug concentrations of the same order of magnitude as those observed in proximal tubular cells of animals receiving clinically-relevant doses of gentamicin and in which apoptosis has been demonstrated (El Mouedden et al., 2000a; El Mouedden et al., 2000b).Use of cells expressing megalin (LLC-PK1 cells grown on inserts or primary culture of human proximal tubular cells) and comparison with our observations could give us additional informations about the events underlying gentamicin nephrotoxicity and would be more relevant to *in vivo* situations.

A third important difference between cell culture and *in vivo* situation is the lysosomal content. Indeed, lysosomes can present an important heterogeneity depending from their endocytic activity. The lysosomal compartment consists of numerous acidic vesicles (pH ~ 4 to 5) that constantly fuse and divide. Lysosomal content, and particularly iron lysosomal content, depends on decomposition of iron-containing metalloproteins within lysosomes which is accompanied by the release of redox-active iron. Differences in lysosomal iron accumulation are considered as responsible of the pronounced variation in the stability of individual lysosomes against oxidative stress in single cells, and also in the total lysosomal population amongst cells (Nilsson et al., 1997). Iron significant concentrations have been detected in LLC-PK1 cells (Baliga et al., 1996), and the implication of lysosomal iron has been suggested in several models of toxicity induced in LLC-PK1 cells (Baliga et al., 1998; Walker and Shah, 1991). However, since we suggested an important role for lysosomal iron in gentamicin-induced lysosomal membrane permeabilization, it should be important to test our hypothesis on *in vivo* model in which the lysosomal iron accumulation is probably not the same as in culture cells.

Our work thus helps to gain a comprehensive view about the intracellular mechanisms implicated in gentamicin-induced apoptosis. We identified several pathways that could play an important role in gentamicin nephrotoxicity, and it would now be very interesting to investigate their implication and to assess their importance *in vivo* in rats or human models, which are already described in the litterature (Ozaki et al., 2010; Peyrou et al., 2007; de Broe et al., 1984).

VI.2. Pending questions and perspectives

VI.2.1. Could we generalize the concept of ROS production through iron implication to other aminoglycosides?

We proposed here that the mechanism of lysosomal membrane permeabilization induced by gentamicin involves ROS production, maybe through formation of a ternary complex constituted of gentamicin, arachidonic acid of lysosomal membrane and iron, present in important amounts in lysosomes due to the degradation of metalloproteins (Yu et al., 2003b).

The binding mode of iron with arachidonic acid and gentamicin at low pH involves two oxygen donated by the carboxyl group of arachidonic acid and two electron donors from gentamicin molecules, namely the 3-NH_2 group (pKa = 5.75) and the glycosidic oxygen connecting the 2-deoxystreptamine and purpurosamine ring located in equatorial positions. Upon transition to a higher pH, the 2'-aminogroup of the purpurosamine moiety undergoes deprotonation (pKa = 7.35) and assumes an appropriate orientation to participate in the coordination sphere in the axial position. Under physiological conditions around pH 7.4, an equilibrium of these two forms will exist.

Regarding amikacin or isepamicin, their ability to establish a similar complex with iron could be impaired. Indeed, the preference of the *S*-4-amino-2-hydroxybutyric acid chain in amikacin or of the *S*-4-amino-2-hydroxypropionic acid chain in isepamicin would hinder the coordination between the aminoglycoside and iron (Lesniak et al., 2003), by inducing a steric shielding. Another hypothesis should be that one of the conformers of the 4-amino group of the side chain might be involved in a less efficient way in a complex with iron impairing the interactions with the two other binding sites. Intriguingly, the moiety in position C_2' is an hydroxyl for amikacin (derived from kanamycin A) and for isepamicin (derived from gentamicin B) instead an aminogroup in gentamicin C and neomycin B derivatives. The aminogroup in position 2' could thus be critical for the formation of the iron complex.

Based on these data, it would be interesting to investigate ROS production and lysosomal membrane permeabilization in cells incubated with aminoglycosides of different nephrotoxic potential to determine if the difference in apoptogenic potential of the molecules results from different potential to permeabilize lysosomal membrane or only from a different behavior once released in the cytosol.

VI.2.2. Is cathepsin release implicated in gentamicin-induced apoptosis?

A role for **cathepsins** in gentamicin-induced apoptosis is an interesting not yet demonstrated possibility. The release of cathepsins after lysosomal membrane permeabilization has been largely described (see (Repnik and Turk, 2010) for review), and cytosolic cathepsins can cleave various protein substrates, thereby propagating apoptosis.

We evidenced lysosomal membrane permeabilization through the release of Lucifer yellow, whose molecular weight is about 460, thus very close to that of gentamicin, but much lower than that of cathepsins (between 25 and 50 kDa) (Barrett, 1970; Barrett, 1973). Lysosomal release associated to apoptosis has been described as size-selective, as 10 and 40 kDa FITC-dextran molecules are released from lysosomes during staurosporine-induced apoptosis, while 70 to 250 kDa FITC-dextran molecules are retained (Bidere et al., 2003). However, this size-selectivity seems to be model- and cell-dependent, as the release of the 150kDa-large lysosomal protein, N-acetyl-β-glucosaminidase, has been described in other experimental conditions (Blomgran et al., 2007; Nylandsted et al., 2004; Kagedal et al., 2005). We can thus not be sure that the lysosomal membrane permeabilization induced by gentamicin is sufficient for the release of cathepsins and subcellular localization experiments would be needed in this aim. Although probably involved, the cathepsins are not the only way responsible for the mitochondrial apoptotic pathway induced by gentamicin since the electroporation of the drug, bypassing the endocytic route, induces apoptosis at very low concentrations (Servais et al., 2006).

VI.2.3. What is the role of p53?

The role of p53 in gentamicin-induced apoptosis is suggested by our observation of the partial protective effect afforded by the p53-inhibitor pifithrin α.

Activation of p53 signaling pathway could result from an increase of p53 gene expression, as demonstrated in rats treated with 80 mg/kg/day (Ozaki et al., 2010), or from an inhibition of p53 degradation by the proteasome (An et al., 2000; Yu et al., 2003a; Hideshima et al., 2003). Assay of p53 mRNA remains to be performed to test the hypothesis of increase of gene expression at lower doses.

The control of p53 function is achieved through several mechanisms that include p53 regulation of transcription, translation, protein stability, subcellular localization and activity (Ryan et al., 2001).

Since Mdm-2 is a key regulator of p53 transcriptional activity and degradation by proteasome, its role should be investigated, namely by western blot analysis to detect an possible increase of Mdm-2 protein levels. Increase of Mdm-2 gene expression was reported after treatment with high doses of gentamicin (Ozaki et al., 2010) supports the hypothesis of p53 activation, but this point remains to be tested at low doses of gentamicin.

Since p53 function depends on nuclear location and its degradation on cytoplasmic location, the subcellular localization of p53 could be investigated in cells treated with gentamicin in addition to the evaluation of p53 accumulation. Immunohistochemistry experiment with p53 and Bax location after gentamicin exposure with and without pifithrin α would also strengthen the role of p53.

Besides the regulation of p53 stability and localization, the ability of p53 to bind DNA and to exert its role of transcription factor can also be modulated. Many post-translational modifications into the carboxyl terminus of p53 have been shown to enhance p53 binding to DNA, such as phosphorylation (Meek, 1999), sumoylation (Gostissa et al., 1999) or acetylation (Gu and Roeder, 1997).

Regarding all possibilities for p53 function control and modulation, we evaluated the implication of p53 in gentamicin-induced apoptosis using co-incubation of cells with pifithrin α, a p53 inhibitor currently used in this aim (Yano et al., 2007; Kelly et al., 2003). However, since some authors suggest a protection by pifithrin α by a mechanism independent of p53 (Sohn et al., 2009), the role of p53 in gentamicin-induced apoptosis should be confirmed by specific techniques such as gene knockout models or impaired p53 translation by siRNAs or shRNAs.

Main p53 transcriptional targets are Puma and Noxa Bcl-2 family proteins, FAS, DR5 and DR4 death receptors and p21cyclin-dependent kinase inhibitor. In this work, we focused on p21 proteins levels, but transcription of the other proteins should also be investigated to evaluate the activation of p53 by gentamicin.

VI.2.4. How could NFκB be activated by gentamicin?

NFκB has been reported to be involved in gentamicin-induced apoptosis, following the observation of a protective effect of inhibitors of NFκB such as pyrrolidine dithiocarbamate (PDTC) and SN-50 on gentamicin-induced apoptosis (Chen et al., 2011). Increase of the nuclear NFκB p65 subunit, and activation of NFκB have been demonstrated in cultured NRK-52E renal cells treated for 8h and 24h with 3 mM gentamicin (Chen et al., 2011; Juan et al., 2007). Moreover, the induction of cell necrosis *in vivo*, even low, induces macrophage recruitment and release of cytokines such as IL-1β or TNFα, that activate NFκB (Park et al., 2010).

NFκB is known to be activated by numerous stimuli. In unstimulated cells, NFκB exists as heterodimers or homodimers of p50 and p65 subunits that are sequestered in the cytoplasm due to their association with a member of IκB family of inhibitors proteins. Mechanisms of NFκB activation involve two steps: (i) nuclear translocation after release from IκB protein following IκB phosphorylation and ubiquitination, and (ii) regulation of the transcriptional activity. Inhibition of the proteasome has been shown to promote IκB-α phosphorylation, ubiquitination and degradation via a mechanism involving autophagy process (Jia et al., 2012), that could explain NFκB nuclear translocation after gentamicin treatment. The use of autophagy inhibitor before monitoring of NFκB translocation could help to evaluate this hypothesis.

Phosphorylation and activation of p65 by PI3K/Akt has been shown to be required for IL-1 and TNF-α- induced NFκB activity (Sizemore et al., 1999), but this pathway is probably not responsible of gentamicin-induced NFκB activation since gentamicin reduces the level of phosphorylated-Akt (Chen et al., 2011). However, ROS production could be considered as a hypothesis to explain NFκB by gentamicin since the level of ROS may regulate NFκB (Li and Karin, 1999). This hypothesis remains however to be tested as NFκB activation by ROS is highly cell-type dependent and H_2O_2 cannot be considered as a general mediator of NFκB activation (Li and Stark, 2002). Activation of NFκB should be investigated in cells treated with gentamicin in presence of antioxidant molecules.

NFκB activation is decribed with pro- and anti-apoptotic functions. While induction of apoptosis by NFκB occurs via induction of p53 (Wu and Lozano, 1994), the anti-apoptotic role is more described and involves the transcriptional induction of genes encoding for the anti-apoptotic proteins Bcl-x_L, cFLIP, cIAP1/2 and Bcl-2 and antioxidant protein SOD2. The protective effect afforded by NFκB inhibitors (Chen et al., 2011) suggests that the role of

NFκB in cells treated with gentamicin is probably anti-apoptotic, this hypothesis could be tested in cells that do not express NFκB, for example due to transfection with shRNA against this protein, and by looking for transcription levels of NFκB target genes.

VI.2.5. Does p21 and p27 accumulation have consequences on cell-cycle progress?

We showed a slight increase in p21 and p27 protein levels in cells incubated with gentamicin. Regarding the biological roles described for p21 and p27, these increases could have consequences on cell cycle progress and apoptosis.

The increase in p21 protein levels can result from a decrease of protein degradation due to inhibition of the proteasome (Lu and Hunter, 2010). However, the high concentrations of gentamicin needed to observe proteasomal inhibition in cell lysates challenge this hypothesis. Another possibility is that p53 activation induces p21 at the transcriptional level via its binding to p53-responsive element in p21 promoter (Parker et al., 1995), and at the post-transcriptional level via stabilization of p21 mRNA (Shu et al., 2006). This hypothesis should be confirmed by quantitative measurement of p21 mRNA in cells treated with gentamicin using competitive reverse transcriptase PCR (RT-PCR). Quantitative measurement of p21 protein in cells treated with gentamicin and an inhibitor of p53 as pifithrin α could also give an idea of the contribution of p53 in p21 protein levels increase. The phosphorylation of p21 can also be responsible for its stabilization (Sheaff et al., 2000), and western blot to detect p-p21 could be interesting in this context.

In the cell cycle, p21 can be responsible for maintaining cells in G_0, arrest in G_1 phase through association to cyclin D-CDK4/6 complex and E2F, inhibiting Rb phosphorylation and translation of proteins required for S phase as cyclin E. In S phase, p21 can induce the inhibition of DNA replication through binding to PCNA (Jung et al., 2010). To investigate an effect of gentamicin on cell-cycle, several experiments can be interesting such as flow cytometry after iodide propidium staining in a cell line presenting a shorter cycle time, allowing to better highlight a possible slowdown cycle, possibly after prior synchronization of cells with nocodazole, an agent perturbing microtubule formation and blocking the cells before mitosis. Staining of cells with 5-bromo-2-deoxyuridine BrdU could also be useful to evaluate the cell proliferation in presence of gentamicin.

Another possible consequence of the p21 increase is the modulation of apoptosis (Huang et al., 2003). As a role in apoptosis has been demonstrated only for cytosolic p21, the subcellular localization of p21 after incubation with gentamicin should be investigated. p21

has been mainly described as an anti-apoptotic agent, and its increase could limit the apoptosis induction, and could explain the moderate levels of apoptosis detected in cells treated with gentamicin, playing a protective role. The functional role of p21 in the modulation of cell cycle and apoptosis by gentamicin could be evaluated by p21 knockout in cells.

Incubation of cells with gentamicin results in a slight increase of p27 protein levels. This increase probably results from the proteasome inhibition, since the main regulation pathway for p27 is its degradation by the ubiquitin-proteasome pathway after its phosphorylation by cyclins. Phosphorylated p27 is ubiquitinated by distinct E3-ligases when located in the nucleus or exported in the cytoplasm and its degradation occurs mainly in G_2 phase (Chen and Lin, 2004), which could explain, if the increase of cells in G_2 phase is confirmed in cell cycle studies, that the p27 accumulation observed is less important after incubation with 3 mM gentamicin. Although described as constant during the cell cycle, mRNA levels can increase after proteasome inhibition (Baiz et al., 2009), and a quantitative measurement of the p27 mRNA would be interesting to complete our results.

Accumulation of p27 is often reported after a proteasomal inhibition and associated with inhibition of the cell cycle and/or the modulation of apoptosis (Kudo et al., 2000; Bae et al., 2008; Baiz et al., 2009). The p27-induced cell cycle inhibition occurs in G_1 phase mainly through an interaction with cyclin E/CDK complexes. p27 has also been shown to have a critical role in promoting the apoptosis in breast, renal, lung and cancer cells lines (Katayose et al., 1997; Wang et al., 1997), although an anti-apoptototic role was also described for p27 (Hiromura et al., 1999). As for p21, we could confirm the role of p27 protein in gentamicin-induced cell cycle and apoptosis modulation by inhibiting the p27 protein expression by avoiding their translation in cells with antisense oligonucleotides for example. Another important consequence of p27 accumulation could be induction of autophagy. Indeed, phosphorylation of p27 at Thr 198 stabilizes p27, and allows the cells to survive to metabolic stress through autophagy, p27 knockdown resulting in apoptosis (Liang et al., 2007)

VI.2.6. Does gentamicin induce autophagy?

Several elements among our results as the possible proteasome inhibition and the p27 accumulation can suggest the induction of autophagy by gentamicin. Among the various mechanisms that regulate cell death including apoptosis and necrosis, autophagy has emerged as another major programmed mechanism to control life and death. This process involves sequestration of proteins and cell organelles in double-membraned structures

termed autophagosomes, in the cytoplasm to target them to the lysosomes for degradation by formation of autophagic vacuoles or autophagolysosomes. While under normal conditions basal autophagy is a process for the turnover of proteins and elimination of damaged or aged organelles to maintain the cell homeostasis, the autophagy induction under pathological conditions is generally considered to provide a pro-survival role; however, extensive autophagy results in cell death by bulk elimination of cells, and a cell would have a very slim chance of escaping cell death through autophagy if intracellular catabolism was impaired by lysosomal rupture early in the apoptotic signaling pathway (Repnik and Turk, 2010). Cross-talks exists between apoptosis and autophagy pathways and both phenomenon can be associated, as demonstrated in renal tubular epithelial cells treated with cisplatin (Yang et al., 2008).

Autophagy and ubiquitin-proteasome system are functionaly coupled and linked by a multi-domain protein adapter, p62, which is capable to bind ubiquitinated proteins and lead them to autophagosomes for degradation (Moscat and Diaz-Meco, 2009). A proteasomal inhibition by gentamicin could promote autophagy, as demonstrated for the proteasome inhibitor bortezomib in colon cancer cells (Ding et al., 2009). This hypothesis is reinforced by our observation of p27 accumulation, which has been related to autophagy induction (Liang et al., 2007), and could explain gentamicin-induced NFκB activation suggested by several groups (Chen et al., 2011; Juan et al., 2007) through degradation of the NFκB inhibitor IκBα via an autophagic process (Jia et al., 2012).

This pathway is thus an interesting hypothesis to investigate in cells treated with gentamicin. For this purpose, essential markers of autophagy should be evaluated as established steps of the autophagosome formation through microtubule-associated protein I light chain 3 (LC3-I) induction and LC3-I to LC3-II conversion or upregulation of autophagic proteins Beclin 1 and Atg5.

VI.2.7. Hypothetical pathways for gentamicin-induced apoptosis

Our results led us to the conclusion of implication of several pathways in parallel, acting on the pro- and anti-apoptotic balance, and resulting on the well described apoptosis induction by gentamicin. To close this work, Figure 18 integrates our observations within existing knowledge about gentamicin-induced apoptosis in culture cells. Contributions of our work are framed.

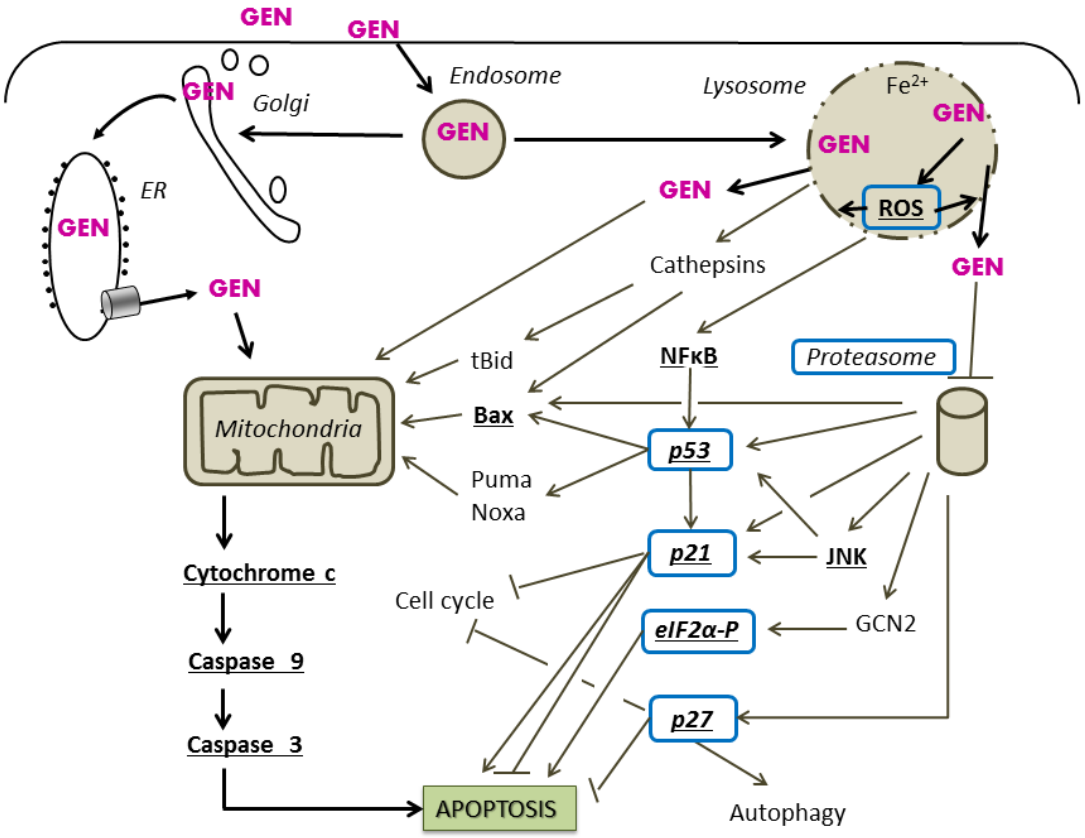


Fig.18. Main mechanisms of apoptosis induced by gentamicin. Contributions of this work are framed. Results obtained in renal cultured cells incubated with gentamicin are in bold and underlined. Proteasome inhibition data were obtained in cellular lysates. Thinner arrows represent hypothetical links. Gentamicin enters into cells by endocytosis after binding to megalin and/or acidic phospholipids of the membrane, and accumulates in lysosomes. Another intracellular pathway describes the transport of a minor fraction of endocytosed aminoglycosides in a retrograde manner through the Golgi complex and the Endoplasmic Reticulum (ER). From there, aminoglycosides would be transported to the cytosol for delivery thorough the cell. In lysosomes, accumulated gentamicin induces **ROS production** by a mechanism implicating **iron** participation, probably via the formation of an oxidant ternary complex between gentamicin, iron and arachidonic acid contained in lysosomal membrane phospholipids. Lysosomal ROS production induces lipid peroxidation and **lysosomal membrane permeabilization**, allowing the release of lysosomal content to the cytosol. After lysosomal membrane permeabilization, several pathways could explain the activation of mitochondrial intrinsic pathway of apoptosis. (i) Lysosomal membrane permeabilization could allow the release of cathepsins into the cytosol, where they could induce pro-apoptotic Bcl-2 family proteins Bax and Bid. (ii) The release of gentamicin itself could permeabilize the outer mitochondrial membrane through direct interaction of

gentamicin with the membrane, or inhibit, at least in part, the degradation activity of proteasome, which would be responsible of accumulation of several proteins such as p53, p21, phosphorylated eIF2α and p27. Both hypothesis are thinkable with the observations of induction of the intrinsic mitochondrial apoptotic pathway, with loss of mitochondrial outer membrane potential, release of cytochrome c that form in the cytosol the apoptosome with Apaf-1 and the pro-caspase 9 allowing caspase 9 activation, which will in turn, activate caspase 3 and bring cell to apoptosis.

In conclusion, our work provides new evidence in the puzzle of cross-talks between the cellular organelles during the apoptosis induced by the aminoglycoside antibiotic gentamicin in LLC-PK1 cells. It highlights the roles of ROS production, lysosomal membrane permeabilization and suggests p53 signaling pathway activation in apoptosis induction, accompanied by a slight increase in p21 and p27 and in the phosphorylated form of eIF2α. To explain the effects of gentamicin on p53, p21 and p27, we tested the hypothesis of an inhibition of the proteasome. Such effect would be responsible for a protein accumulation resulting from a decrease of their degradation. However, we demonstrated an inhibition of trypsin- and caspase-like activities of proteasome in cellular lysates only at concentrations of gentamicin, much higher than the cytosolic concentration that could be found in cells incubated with the drug, and treatment of cells with gentamicin induced only a very slight increase of ubiquitinated proteins. In this context, investigation of the implication of other signaling pathways as NFκB and autophagy implication could be very interesting to further improve our understanding of the subcellular mechanisms underlying the nephrotoxic phenomenons induced by aminoglycoside antibiotics, in a context in which the nephrotoxicity remains a limiting factor for the use of these valuable drugs for treatment of life-threatening infections, as for their promising roles as antiviral and for the treatment of genetic diseases.

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SUMMARY

In a context of resurgence in use of aminoglycosides for their effectiveness against bacterial strains resistant to other classes of antibiotics, as well as for their activities in the treatment of genetic diseases or viral affections, the nephrotoxicity induced by these antibiotics remains so far a barrier against their use. Aminoglycosides are eliminated by glomerular filtration and picked up, mainly by receptor-mediated endocytosis, by proximal tubular epithelial cells that can poison. The tubular toxicity involves the death of epithelial cells, and apoptosis has been observed in rats and cultured cells treated with therapeutically relevant doses.

The present study was therefore designed to gain further knowledge about the intracellular mechanisms underlying gentamicin-induced apoptosis in LLC-PK1 proximal tubular cells. In the first part of this work, we evaluated the role and the mechanisms of lysosomal membrane permeabilization induced by gentamicin. Direct evidence for gentamicin-induced permeabilization of lysosomal membrane was provided by vital imaging, and we showed an involvement of ROS by an iron-dependent mechanism in this process.

In a second part of this work, we focused our interest on the role that can play cytosolic aminoglycosides. We observed that electroporation of more nephrotoxic aminoglycosides (gentamicin and neomycin B) induced more apoptosis than low toxic ones (amikacin and isepamicin). The low concentrations needed to observe induction of apoptosis by nephrotoxic drugs after electroporation highlights the high apoptogenic potential of cytosolic aminoglycosides, and the important consequences that could have their release from lysosomes into the cytosol.

Finally, we explored several pathways that could link lysosomal membrane permeabilization and activation of mitochondrial intrinsic pathway of apoptosis. The partial protective effect of the p53 inhibitor pifithrin α on gentamicin-induced apoptosis led us to suggest an implication of p53 signaling pathway in this process, while inhibition of the proteasome by gentamicin did not appear to be key mechanism of gentamicin-induced apoptosis in LLC-PK1 cells.

Our work helps to gain a comprehensive view about intracellular mechanisms implicated in gentamicin-induced apoptosis. These observations highlight the important role of lysosomes in aminoglycoside-induced apoptosis, and it would now be very interesting to investigate their implication *in vivo* in rats or human models.

Dans un contexte de résurgence de l'usage des aminoglycosides, tant pour leur efficacité contre les souches bactériennes résistantes aux autres classes d'antibiotiques, que pour leurs effets prometteurs dans le traitement d'affections génétiques et virales, la néphrotoxicité induite par ceux-ci reste jusqu'à présent un frein majeur à leur usage. Les aminoglycosides sont éliminés par filtration glomérulaire et en partie captés, principalement par un mécanisme d'endocytose médiée par un récepteur, par les cellules tubulaires proximales qu'ils peuvent intoxiquer. La toxicité tubulaire implique la mort des cellules épithéliales, et l'induction d'apoptose a été mise en évidence chez des rats traités à doses thérapeutiques, ainsi que dans des cellules en culture.

Notre travail avait pour but d'améliorer la connaissance des mécanismes intracellulaires responsables de l'apoptose induite par la gentamicine dans des cellules tubulaires proximales de type LLC-PK1. Dans la première partie du travail, nous avons mis en évidence la perméabilisation lysosomiale par microscopie confocale, et nous avons montré une implication de la production de ROS par un mécanisme dépendant du fer dans ce processus.

Dans un second temps, nous nous sommes intéressés au(x) mécanisme(s) potentiel(s) par lesquels les aminoglycosides, une fois dans le cytosol, peuvent induire l'apoptose. Nous avons observé qu'après électroporation, les aminoglycosides les plus néphrotoxiques (gentamicine et néomycine B) induisaient plus d'apoptose que les moins néphrotoxiques (amikacine et isépamicine). Les faibles concentrations nécessaires pour observer de l'apoptose après électroporation soulignent le potentiel apoptogénique élevé des aminoglycosides cytosoliques, ainsi que les conséquences importantes que peut avoir leur relargage des lysosomes vers le cytosol.

Enfin, nous avons exploré plusieurs voies qui pourraient expliquer le lien entre la perméabilisation de la membrane lysosomiale et l'activation de la voie apoptotique mitochondriale démontrée par Servais et al. L'effet protecteur partiel contre l'apoptose induite par la gentamicine apporté par la pifithrin α suggère l'implication de la voie de p53 dans ce processus. Par contre, selon nos résultats, une inhibition directe du protéasome par la gentamicine ne semble pas être un élément clé.

Notre travail apporte de nouveaux éléments dans la compréhension des mécanismes intracellulaires impliqués dans l'apoptose induite par la gentamicine. Nos observations soulignent le rôle important des lysosomes dans ce phénomène, et il serait maintenant intéressant d'investiguer leur implication *in vivo* dans des modèles animaux et humains.