In Vitro Study of Potential Nephrotoxicity Biomarkers through Gene Expression Using Amphotericin B

Grossi MF, Campos MAA, Soares S, Silva SCT, Nunes MS, Almeida LA and Tagliati CA*

1Toxicology in vitro Laboratory, Department of Clinical and Toxicalogical Analysis, School of Pharmacy - Federal University of Minas Gerais, Belo Horizonte, Brazil
2Immunology Laboratory, Department of Microbiology and Immunology, Institute of Biomedical Sciences - Federal University of Alfenas, Alfenas, Brazil

*Corresponding author: Carlos Alberto Tagliati.
Email: carlostagliati@gmail.com
Received: 14 February 2017; Accepted: 16 May 2017; Published: 22 May 2017

Abstract

Amphotericin B (AmB) is one of the most effective drugs for the treatment of systemic fungal infection. However, its use causes adverse events, especially by nephrotoxicity. Drug-induced nephrotoxicity is one of the most frequently observed side effects in long-term pharmacotherapy. Such situations have been discovered late due to the methods currently used to determine the toxicity of pharmacotherapy. The present study was designed to propose an in vitro alternative method for the early identification of AmB cell toxicity. Therefore, two different renal cell lines, LLC-PK1 (proximal tubule) and MDCK.2 (distal tubule), were exposed to nine different concentrations (2, 4, 6, 8, 10, 12, 15, 20, and 30 μg/mL) of AmB for 24 hours. Gene tests were carried out according to results from MTT assays. A panel of sensitive and specific nephrotoxic genes was selected based on earlier in vitro and in vivo studies. The search for sequences of mRNAs encoding proteins that had previously been associated with kidney damage was conducted in the databases of the National Center for Biotechnology Information (NCBI - USA). The total RNA was extracted from the cells, and RT-PCR was performed to evaluate differential gene expression profiles of the selected genes. Genes with the highest fold change include HAVCR1 (KIM1), CASP3, ANXA5, and VDAC1. According to this study’s results, we suggest that these genes could play an important role in the mechanism of in vitro nephrotoxicity caused by AmB and can represent a starting point for subsequent studies in the development of safer drugs.

Keywords: Amphotericin B; LLC-PK1; MDCK.2; In vitro nephrotoxicity; Gene expression

Abbreviations: AmB: Amphotericin B; cDNA: Complementary deoxyribonucleic acid; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; EMA: European medicines agency; FDA: Food and Drug Administration; LLC-PK1: Porcine kidney cell line; MDCK.2: Madin-Darby canine kidney; MEM: Minimum Essential Medium; mRNA: Messenger ribonucleic acid; MT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); NCBI: National center for biotechnology information; PBS: Phosphate buffer saline; RNA: Ribonucleic acid; RT-PCR: Quantitative Real-Time Polymesase Chain Reaction

Introduction

Amphotericin B (AmB) is a polyene antifungal agent that has been used as the gold standard for more than 40 years to treat many serious systemic fungal infections. However, this drug’s mechanism of action can lead to toxic effects, particularly nephrotoxicity, which is the main effect observed in clinical practice [1,2].

Nephrotoxicity is one of the most frequent side effects observed after the long-term use of this medicine, especially when the methods for determining toxicity, using animal models, have failed in their core aims. There is a need to constantly improve safety-testing strategies. Thus, the validation of sensitive and alternative methods for the early identification of toxic effects is as important as restrictions on the use of animals [3].

Several renal epithelial cell lines have been employed as alternative methods for the study of nephrotoxicity caused by therapeutic products [4-6]. The Madin-Darby canine kidney (MDCK.2), a cell line derived from the distal kidney tubule of a normal cocker spaniel in 1958, is one of the best-characterized and most widely used epithelial cell lines in nephrotoxicity studies [7]. Porcine kidney proximal tubular cells (LLC-PK1) have most frequently been used in studies of toxicity caused by medicine compounds in the proximal tubule [8]. Both cell lines are considered acceptable models to study the nephrotoxicity of drugs and have been validated as alternative methods [9-11].

Recently, some urinary biomarker candidates for the assessment of acute kidney toxicity were approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [12]. Identification and evaluation of new and early biomarkers are among the main applications of genomic technologies. Thus, genomic biomarkers have become useful markers for histopathological damage and may contribute to the assessment of toxicity both in vitro and in vivo [13].

Material and Methods

Drug

Amphotericin B was donated from Cristália (Produtos Quimicos Ltda-Itapira, SP, Brazil). A stock solution of 300 μg/mL of Amphotericin B in sterile buffer solution (PBS) and different volumes were added to Minimum Essential Medium - MEM (GIBCO®) to generate nine different concentrations (2, 4, 6, 8, 10, 12, 15, 20 and 30 μg/mL). The choice of concentrations was based on previous studies [1,6,14].

Cells culture

LLC-PK1 (porcine kidney proximal tubular cells) and MDCK.2 (Madin-Darby canine kidney) adherent cell lines were obtained from the Cell Bank at Universidade Federal do Rio de Janeiro. These were cultured in MEM (GIBCO®) supplemented with 4% (v/v) bovine fetal serum (GIBCO®) and 1% (v/v) of antibiotic antimycotic solution stabilized 100x (Sigma–Aldrich, CA, USA). Cells were grown in 75-cm² (Sarstedt, Germany) and incubated at 37°C in a humidifier with 5% CO₂ (Forma Scientific CO2 Jacketed Incubator). When the growth of the culture cells reached a confluence level of approximately 80%, these cells were then exposed to different concentrations of Amphotericin B for 24 hours.

MTT cytotoxicity assay

Cell viability can be assessed using the well-known MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) assay, as first described by Mosmann (1983) [15]. The test has been modified several times since then and was subjected to laboratory validation at the beginning of this study [16].

First, cell lines were placed on 96-well plates in a concentration of 5.0 x 10⁴. Next, 180 μL of MEM culture medium and Amphotericin B (20 μL/well) were added (2, 4, 6, 8, 10, 12, 15, 20 and 30 μg/mL) to
each well and left for 24 hours of exposure. Cells not exposed to drugs were used as negative controls. Medium containing the control and the drug was removed and substituted with 200 μL/well of a fresh solution containing 0.5 mg/mL of MTT in MEM (Gibco®) and incubated for 4 hours at 37°C in humidified 5% CO₂. The MTT solution was then removed, 100 μL of dimethylsulfoxide (DMSO) was added to each well. Detection and quantification of the formazan crystals was performed by a multiwell plate reader (Molecular Devices SPECTRA Max PLUS 384) at 570 nm. The results were expressed as percentage of the viability in treated cells compared with negative control.

**RNA isolation and conversion to cDNA**

For gene expression analysis, the total RNA was extracted from the cells using Trizol reagent (Invitrogen™ - Thermo Fisher Scientific) according to manufacturer recommendations. The quantity of the purified RNA was evaluated using the Nanovue™ Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). Reverse transcription of 1μg from total RNA was performed using illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK).

**Quantitative Real-time PCR**

A literature search was performed to identify genes with the potential to act as biomarkers of nephrotoxicity. A panel of 10 genes was selected based on alterations of gene expression found in vivo and in vitro studies of nephrotoxicity (Table 1). 7,10,17,18 GAPDH was selected based on alterations of gene expression found in vivo and in vitro and used as an endogenous reference gene to normalize the expression of the target genes. LLC-PK1 cells were exposed to the drug in concentrations 4, 8 and 12 μg/mL and MDCK.2 were exposed to 2, 4 and 8 μg/mL for 24 hours at 37°C in humidified 5% CO₂. These concentrations were selected based on the percentage of viability found in MTT assay.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was performed with StepOne™ Systems Real-Time PCR (Applied Biosystems™ - Thermo Fisher Scientific), using the template cDNA, SYBR® Green Master Mix (Applied Biosystems™) and the primers as recommended in the manufacturer’s protocol. The primers were obtained using Primer BLAST (NCBI - http://www.ncbi.nlm.nih.gov/). Gene expression was determined by RT-PCR, and changes in gene expression were compared to the negative control. Among the ten analyzed target genes, amplified primers were restricted to the range of 100 to 200 nucleotides. The sequences of forward and reverse primers are listed in Table 1. Primer specificities were confirmed by the melting curves that appeared after applying RT-PCR. The resulting cycle threshold (Ct) value was processed based on the comparative Ct method.

**Statistical analysis**

The comparative threshold cycle method was used to analyze the data according to the equation 2-ΔΔCt (Relative Quantification getting started guide for the 7300/7500 System and UserBulletin#2: ABI Prism 7700 Sequence Detection System, Applied Biosystem). All data are shown as relative expression units after normalization to GAPDH. The differences in the relative expression of the genes and MTT assay were performed using GraphPad Prism version 5.00 (GraphPad Software, Inc), applying the one-way ANOVA with Tukey post-test. The analysis is considered significant when p<0.05.

**Results**

**Cell viability using MTT Assay**

In the concentration-response curve tests, according to the method used in this study, LLC-PK1 and MDCK.2 cell lines appear to be sensitive to the toxicity caused by AmB in a concentration-dependent manner (Figure 1). After 24 hours of exposure, a significant difference (p<0.05) could be observed among all concentrations tested when compared to control, and between all concentrations, except 15, 20, and 30 μg/mL for LLC-PK1 and 12, 15, and 20 μg/mL for MDCK [2].

No significant difference (p>0.05) could be observed among the responses of these cell lines when exposed to AmB at concentrations below 2 μg/mL. From these results, it can be concluded that AmB is cytotoxic to both cell lines. Thus, three concentrations were selected for the gene expression study: 4, 10, and 30 μg/mL for LLC-PK1 and 2, 4, and 8 μg/mL for MDCK (low, medium, and high MTT metabolism).

**Gene expression**

The relative level of gene expression in LLC-PK1 and MDCK.2 cells was determined by RT-PCR, and changes in gene expression were compared to the negative control. Among the ten analyzed target genes, changes in gene expression were compared to the negative control.

---

**Table 1: Sequences of forward and reverse primers for quantitative real-time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene description</th>
<th>Access</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANXA5</td>
<td>Annexin A5</td>
<td>XM_003129218</td>
<td>GTCGCTATGCGCAAGGTCTTCT</td>
<td>AGGATGCTCTCTCTCTGCCTCAGT</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>XM_003127290</td>
<td>CGCACTGATCGGACCACACT</td>
<td>AAGATGTCGCAATCAGTAAACCC</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>XM_003121700</td>
<td>CCTTGGATGCCAGGAGAACGG</td>
<td>AACACCAAGCTGAGACCTCAGTCA</td>
</tr>
<tr>
<td>BCL2L1 (BCL-XL)</td>
<td>BCL2-like 1</td>
<td>NM_214285</td>
<td>AGGCACATTCTAGTACCTGAC</td>
<td>CCATCCCAGGAAGAGTTGTTGTT</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase 3, apoptosis-related cysteine peptidase</td>
<td>NM_214131</td>
<td>GACGGAACATGGGAATCTGAG</td>
<td>TGGATGAACCAGATCCGTC</td>
</tr>
<tr>
<td>CASP9</td>
<td>Caspase 9, apoptosis-related cysteine peptidase</td>
<td>XM_003127618</td>
<td>CTGCACGAACTATGTCGAGC</td>
<td>CGTCGTGTCATAACAGCCCTCCT</td>
</tr>
<tr>
<td>EXOC6</td>
<td>Exocyst complex component 6</td>
<td>XM_003361565</td>
<td>TCGAAAAGCAACCCCTTCCA</td>
<td>AGGCTCCGTTGAGTGACTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>NM_001206359</td>
<td>GAAGTCGGAGTGAACGG</td>
<td>GCCAAGTAAAAGACACGCCC</td>
</tr>
<tr>
<td>HAVCR1 (KIM1)</td>
<td>Hepatitis A virus cellular receptor 1</td>
<td>NM_001164736</td>
<td>TTGCATCACAACACTGTGTC</td>
<td>CGACGACCTGTAGTGAGCCT</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
<td>NM_213816</td>
<td>ACTTTATGCTGCCAGCCAC</td>
<td>GTCACAGAAACCGGTGTGCG</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage-dependent anion channel 1</td>
<td>NM_213960</td>
<td>GCTGGTCTTCGCGTAAGT</td>
<td>CACCGCGTTGACATTCTTG</td>
</tr>
</tbody>
</table>

---

changes in expression were observed in four cases (Figure 2a and 2b). Gene expression analysis demonstrated that there were relative increases of HAVCR1 (gene originally cloned from African green monkeys as the receptor for hepatitis A, and subsequently identified as a homolog of human KIM1), CASP3, VDAC1, and ANXA5 targets, when compared to expression levels of the transcripts of these same genes in non-exposed cells. These genes showed a significant increase (p<0.05) at 30 µg/mL in LLC-PK1 and 8 µg/mL in MDCK.2 when compared to the control.

**Discussion**

AmB most likely produces renal injury through a variety of mechanisms. Most studies suggest that toxicity occurs primarily in nephron distal tubules. However some studies have shown that these effects can also occur in proximal tubules. In this study, the tubular cell lines of MDCK.2 (distal tubule) and LLC-PK1 (proximal tubule), represented two physiologically relevant units of the nephron, were used to investigate the cytotoxic effects of AmB on different segments of the kidney. Initially, this study applied the MTT reduction assay, a quick metabolic test for this evaluation. Our results are in agreement with previous literature, since toxic effects were observed in both strains, which were higher in MDCK.2 strains when compared to LLC-PK1 cells, as shown in Figure 1.
Recent studies have shown that MTT assay can be used as an initial screening test to establish drug concentrations [1,3,23]. Despite being a subject test influenced by a number of external factors, the MTT assay should be supplemented with another non-metabolic methods [24]. There is a wide discrepancy in the literature about the need to use complementary tests, since the MTT assay, in most of the cited articles, was employed just to define use concentrations. Moreover, among in vitro studies, the concentration and exposure time to the amB remain unclear [6,14]. In order to study gene expression, three concentrations were chosen according to the obtained MTT results were chosen. Different concentrations were also chosen for gene expression, as the aim was to find out whether the tubules of the kidney (proximal and distal) show different or the same gene expressions in similar cell viabilities.

After the concentrations had been selected, the gene expression test was performed in both lineages. The ten genes analyzed in this study were ANXA5, BAX , BCL -2 , BCL -XL , CASP3 , CASP9 , EXOC6 , HAVCR1, ICAM -1, and VDAC1, based on previous studies using gene expression after exposure to AmB in vivo and in vitro [7,8,17,18,25]. The genes with the highest fold change in this study were HAVCR1, CASP3, ANXA5, and VDAC1. This result suggests that these genes might be used as biomarkers of in vitro nephrotoxicity. Genomic predictors of toxicity were defined in this study as genes that showed a significant change in expression levels after 24h exposure to the nephrotoxicant.

Hepatitis A Virus Cellular Receptor 1 (HAVCR1), also named KIM1 in humans, is a PS receptor that recognizes apoptotic cells and directs them to lysosomes. This gene is conserved in several species, including zebrafish, rodents, dogs, primates, and humans, and is the first discovered molecule that transforms kidney proximal epithelial cells into semi-professional phagocytes [25-28]. HAVCR1 has proven to be an early indicator of acute kidney failure, as compared to other traditional biomarkers recommended by the FDA and the EMA. Human and animals studies have also showed promising results for the potential use of urinary KIM1 as a diagnostic biomarker for acute kidney injury (AKI) [29-31]. Thus, it was observed that the in vitro results found in the present study agree with these studies. The increase in the relative expression of HAVCR1 (KIM1) presented by LLC-PK1 (an approximately 4-fold change) and MDCK.2 (an approximately 11-fold change) after exposure to AmB provides evidence that the suggested model may be an alternative method for screening chemical entities containing nephrotoxic potential, such as AmB.

Increased KIM1, a protein encoded by the KIM1 gene (analogous to HAVCR1), after renal injury leads to increased intracellular calcium and induces an expression of Annexin. Annexins are a family of proteins known to bind phospholipids in a Ca^{2+}-dependent manner in many body tissues, including distal tubule cells and glomerular epithelial cells. These annexin proteins participate in various membrane-related events, such as exocytosis, endocytosis, and apoptosis [13,32]. Thus, the expression of Annexin A5 (ANXA5) was measured in order to better understand the toxic renal processes caused by AmB, given that the biological role of protein Annexin A5 was discovered to be connected with apoptosis and is considered to be an early biomarker of that process [33]. The present study identified a 5-fold change in LLC-PK1 cells and a 21-fold change in MDCK.2 cells within an ANXA5 gene expression after higher concentrations of AmB exposure.

Indeed, the reduction in apoptosis by different approaches displays protection against various forms of renal injury. A specific target in the apoptotic pathway is the family of proteases known as caspases. Caspases are a family of intracellular cysteine proteases that play a key role in the execution-phase of apoptosis. Caspases can act as initiators or executioners. Initiator caspases include caspases 8 and 9, and the activation of these caspases results in the activation of executioner caspases, such as caspases 3 and 7. [34] Thus, caspases seem to be involved in apoptosis associated with toxic acute renal failure. The increase in the expression of CASP3 demonstrated in the present study by LLC-PK1 and MDCK.2 cells after higher exposure to AmB are in accordance with the above studies. There is limited information on the role of caspases in ischemic renal tubular cell injury. Previous studies have demonstrated chemical hypoxia and increased caspase activity that precedes DNA damage and renal cell death [35,36]. Moreover, the inhibition of caspases prevented hypoxia-induced DNA damage and cell death in LLC-PK1 cells playing a key role in apoptosis [37]. This study also observed an increase in the expression of CASP9, an activating caspase protein, in LLC-PK1 cell lines (data not shown) suggesting that the AmB nephrotoxicity is most likely to start by the mitochondrial pathway.

Therefore, the key role of the mitochondrial pathway of apoptosis and the importance of membrane ion channels in cell toxicity caused by AmB were tested in other genes involved in the apoptosis/mitochondrial pathway, such as BAX, BCL-2, BCL-XL, and VDAC1. The voltage-dependent anion channels (VDACs) are the most abundant proteins of the mitochondrial membrane in multicellular organisms, and both cell lines used in this study over expressed this gene [38]. The VDAC1 gene encodes an anion channel protein in the mitochondrial membrane, exchanging metabolites and ions across the mitochondrial pathway, and may regulate its functions [39]. It also plays an important role in apoptosis through Ca^{2+} regulation and the binding of anti-apoptotic proteins of the BCL-2 family, proteins that regulate and contribute to programmed cell death or apoptosis. A recent study provided experimental evidence supporting this conclusion: the cleavage of VDAC1 reflects a survival response of hypoxic cells [40]. Once the cellular expression level of VDAC1 is a crucial factor for the process of mitochondria-mediated apoptosis, the findings of this study are in accordance with previous literature and may well suggest that the mechanism of action of AmB toxicity involves the induction of VDAC1 over-expression, as shown in Figure 2.

Although most of the results presented in this study indicate an important role of the apoptotic pathway in the toxicity induced by Amphoterin B, many studies indicate that this drug acts through multiple pathways. The present study, strongly suggests a central role of mitochondrial pathway dysfunction in the mechanism underlying AmB-induced nephrotoxicity, as demonstrated in the results from MTT assays and the over-expressed genes of HAVCR1, CASP3, ANXA5, and VDAC1. However, further studies are necessary to better elucidate AmB’s toxicity mechanisms.

Conclusion

The present study’s results show that the nephrotoxicity of AmB can vary among nephron segments and that the cell lines appear to be sensitive to the toxicity of the drug in a concentration dependent-manner, which could be identified by performing MTT assays and by changing the expression level of the genes tested in this study. These results of gene expression suggest that the selected genes HAVCR1 (KIM1), CASP3, ANXA5, and VDAC1 may provide an innovative approach to detecting early renal injury biomarkers in vitro. Thus, the need for an improved classification of kidney diseases, ideally based on molecular signatures linked to the mode of action and the success of therapeutic approaches, as well as the need for early biomarkers that adequately allow for the timing of therapeutic intervention, is necessary. This study showed the importance of drug screening at toxicological and molecular levels in more than one cell strain. Although further studies are required to confirm the robustness of the predictability concerning these biomarkers, the results of this work can be useful and represent a starting point for subsequent studies in the development of safer drugs.

Acknowledgements

The authors thank CNPq (Process 473125/2012-9 and 310624/2012-6), FAPEMIG (CDS - APQ-01712-14) and PRPq/UFMG (Pró-Reitoria
