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Amphotericin B nephrotoxicity *in vitro*: Differential profile of PKC signaling in VERO and MDCK cell lines

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ABSTRACT

Nephrotoxicity is one of the main effects caused by Amphotericin B, the susceptibility of which can vary among nephron segments. The purpose of this study was to evaluate the effect of Amphotericin B on the signaling pathway of protein kinase C (PKC) of VERO and MDCK. Both were incubated with Amphotericin B (2, 4, 6, 8, 10, 15, 20, and 30 µg/mL) for 1, 18, and 24 h, and the cytotoxicty was measured by Neutral Red. In cell signaling, the cell strains were first preincubated with a PKC inhibitor and then exposed to Amphotericin B. Viability was decreased in both cells at 15, 20, and 30 µg/mL of Amphotericin B. However, at 18 and 24 h, the strains presented a different sensitivity. No difference in sensitivity with the involvement of PKC after 1 h could be observed. However, after 18 h, when the effect of Amphotericin B was increased by applying MDCK, the PKC was inhibited. Therefore, involvement of PKC with Amphotericin B can vary among strains from different regions of the nephron. The present study shows the importance of the screening and protecting of drugs at the molecular level in more than one cell strain.

KEYWORDS: Amphotericin B, MDCK, nephrotoxicity, Protein Kinase C (PKC), VERO

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1. INTRODUCTION

Kidneys represent the most common target for drugs. Due to the functional and biochemical heterogeneity of nephrons, susceptibility to toxicity can vary among nephron segments [1]. Renal toxicity caused by drugs is a common adverse reaction which brings about severe consequences on a patient's health. In 100 drugs used in intensive care units, 25% present a potential for nephrotoxicity [2], amphotericin B being the most common [1]. This drug is the primary choice for the treatment of systemic fungal infections, although the exact mechanisms of nephrotoxicity caused by Amphotericin B are still unclear [3, 4].

Renal cell lines have been employed as alternative methods for the study of therapeutic products that cause nephrotoxicity [5, 6]. The increased use of *in vitro* techniques from specific renal cell types has enhanced the comprehension of molecular mechanisms involved in nephrotoxicity [7].

One assay that appears to offer a new perspective in the study of nephrotoxicity is cell signaling. Protein Kinase C (PKC) plays a crucial role in cell proliferation and differentiation [8], and some studies indicate a PKC participation in apoptotic processes [9].

VERO cell lines and MDCK cells are considered acceptable models to study the nephrotoxicity of drugs [5, 4, 10].

However, no studies can be identified in the literature which evaluate whether or not a difference

in the PKC signaling pathway's involvement in the nephrotoxicity of amphotericin B in fact occurs. These studies are extremely important, given that the cells along the nephron exhibit a differential metabolism, which can result in their participation, or not, in different cellular signaling pathways. Therefore, the core aim of this work is to evaluate if there is in fact a difference between renal cell lines and the involvement of PKC in nephrotoxicity caused by Amphotericin B.

2. MATERIALS AND METHODS

2.1. Drugs

Amphotericin B was donated by Cristália (Produtos Químicos Farmacêuticos Ltda- Itapira, SP, Brazil (purity of 90%). A stock solution of 300 µg/mL of Amphotericin B in sterile buffer solution (PBS) was prepared and different volumes were added to the RPMI-1640 (Sigma St. Louis, MO, USA) to generate eight different concentrations: 2, 4, 6, 8, 10, 15, 20 and 30 µg/mL [11]. The Inhibitor of PKC pathway, Calphostin C (Calbiochem Merck KGaA, Darmstadt, Germany) was dissolved in anhydrous dimethylsulfoxide (DMSO) to form a concentrated solution that was 1000 times the required final concentration. The inhibitor was aliquoted and stored at -20 °C. The concentrated solution was diluted immediately prior to use and the cells were pretreated with 0.15 µM of Calphostin C for 30 minutes.

2.2. Cell culture

The VERO cell lines (mixture of proximal and distal tubulars renal cells from *Cercopitecus aethiops* monkeys – passages 151 to 157) and MDCK (distal tubular cells from dogs – passages 69 to 77) were obtained from the Cell Bank at Universidade Federal do Rio de Janeiro (UFRJ). These were cultivated in a RPMI-1640 culture medium (Sigma St. Louis, MO, USA) and supplemented with 10% (v/v) bovine fetal serum (Invitrogen Co Ltda, Carlsbad, CA, USA), 100 IU penicillin/mL, and 100 µg streptomycin/mL (Sigma St. Louis, MO, USA). Cells were cultivated in 75 cm^2 bottles in an atmosphere of 5% CO_2–95% air at 37 °C.

2.3. Neutral Red cytotoxicity assay

The neutral red color is characterized by its accumulation in the lysosomes of viable cells. For the experiments, the cells were seeded on 96-well plates at a 5.0 x 10^3 cells/well density and were cultured at 37 °C for 24 hours, and then the medium was washed twice with PBS solution and incubated in RPMI-1640 culture medium in the presence of eight different concentrations of the tested drug for 1, 18, and 24 hours at 37 °C in 5% CO_2–95% air. After exposure, the medium containing the drug was removed, 200 µL of the neutral red solution (40 µL/mL) was added, and the plate was incubated at 37 °C in an atmosphere of 5% CO_2–95% air for one hour. Later, the supernatant was removed and 200 µL of formaldehyde solution was added (0.5%, v/v) in $CaCl_2$ (1%). After five minutes, the supernatant was removed, and 100 µL of acid alcohol solution was added [12]. The absorbance was read at 540 nm (Thermo Plate model TP-READER).

2.4. Study of cell signaling via PKC

In an attempt to assess the involvement of the PKC signaling pathway within nephrotoxicity of amphotericin B, the cells (5.0 x 10^3 cells/well) were pretreated for 30 minutes with the respective inhibitor from the PKC pathway (Calphostin C - 0.15 µM). After this period, the cells were incubated in the absence or presence of the most toxic concentration of amphotericin B (30 µg/mL), which was determined by means of the cytotoxicity assay described in item 2.3. The assays were carried out with 1 and 18 hours of incubation. This evaluation was performed using the Neutral Red assay.

2.5. Statistical analysis

The "Mann & Whitney" and "Kruskal-Wallis" non-parametric tests were used in this study. The level of significance was evaluated by determining the p-value, and results were considered significant for values of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Neutral Red cytotoxicity assay

After one hour of exposure at three concentrations (15, 20, and 30 µg/mL) of Amphotericin B, no statistically significant difference could be observed between VERO and MDCK regarding the drug's cytotoxic ability (Figure 1); however, it was important to show how the cytotoxicity of Amphotericin B is

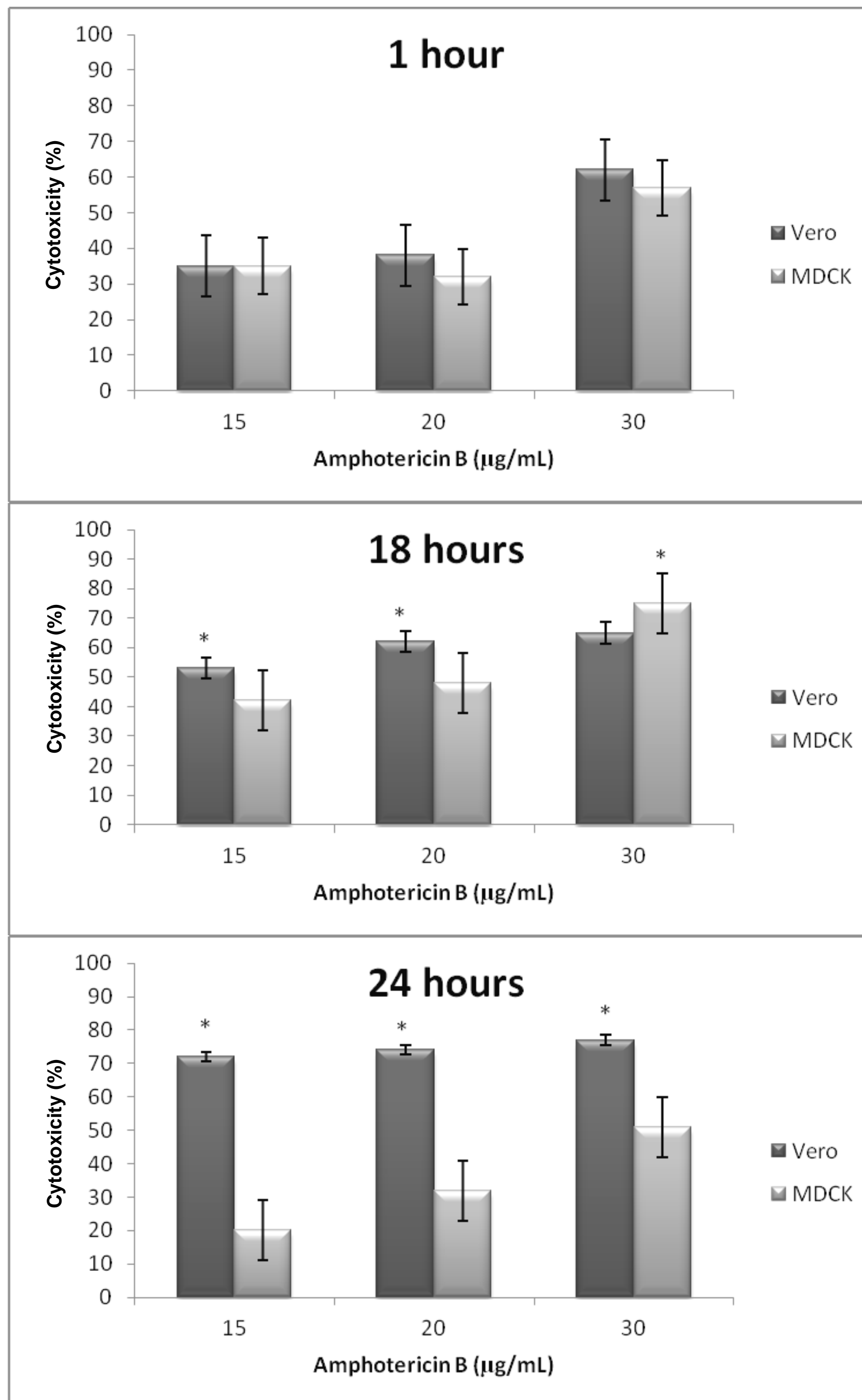

Figure 1. Difference of amphotericin B cytotoxicity between Vero and MDCK cell lines. The cultures were evaluated by Neutral Red assay after 1, 18 and 24 h of exposition to amphotericin B. The media of absorbance of each Amphotericin B concentration was compared to the control group (cells not exposed to the drug) and for this 0% cytotoxicity was considered. *Indicates significant difference ($p < 0.05$) between cell lines, by the nonparametric "Mann & Whitney" test. Results represent mean + SD of triplicates from three independent experiments.

dependent on time, as it increases at a later moment. Nevertheless, at 18 h of incubation, concentrations of 15 and 20 µg/mL of Amphotericin B proved to be more cytotoxic in VERO, while the concentration at 30 µg/mL was more cytotoxic in MDCK. At 24 h of incubation, these three concentrations of Amphotericin B proved to be more cytotoxic to the VERO cell lines (Figure 1). According to [5, 10], the sensitivity detected with the Neutral Red cytotoxicity test in VERO cell lines suggests a specific injury to the lysosomal compartment. Thus, the mechanism of the toxicity of Amphotericin B may be triggered by damage caused to the lysosome. Previous studies have also reported the important role of this organelle in apoptotic processes [1].

Therefore, VERO cell lines exhibited an increased sensitivity to the toxic effects of Amphotericin B, not including trials in which it was applied at a concentration of 30 µg/mL for 18 h, however MDCK cell lines showed an increased sensitivity to the drug, which could be observed through cell signaling.

VERO cell lines are a mixture of proximal and distal tubular renal cells, whereas the MDCK cell lines come from distal tubular renal cells. Cells from distal tubular renal cells, as compared to proximal cells, tend to be more resistant to oxygen deficiencies [7]. Moreover, it is suggested that nephrotoxicity caused by Amphotericin B involves renal arteriolar constriction, which decreases blood flow to the kidney [13, 14] and causes hypoxia or ischemia. This finding suggests a difference in the sensitivity of the cell lines. However, cell lines from proximal tubules are more susceptible to drugs, which may well explain the increased sensitivity to VERO after having been incubated with Amphotericin B at a lower concentration of 15 µg/mL for 18 and 24 h. The MDCK cells exhibited toxicity after having been incubated at a concentration of 30 µg/mL.

Because of the heterogeneous distribution of the transport systems and their different ability to bioactivate or detoxify xenobiotics, the different cell populations (and, therefore, the various regions within the kidney) do not present the same degree of damage after their exposure to toxic substances. Due to the functional and biochemical heterogeneity of the nephrons, their susceptibility

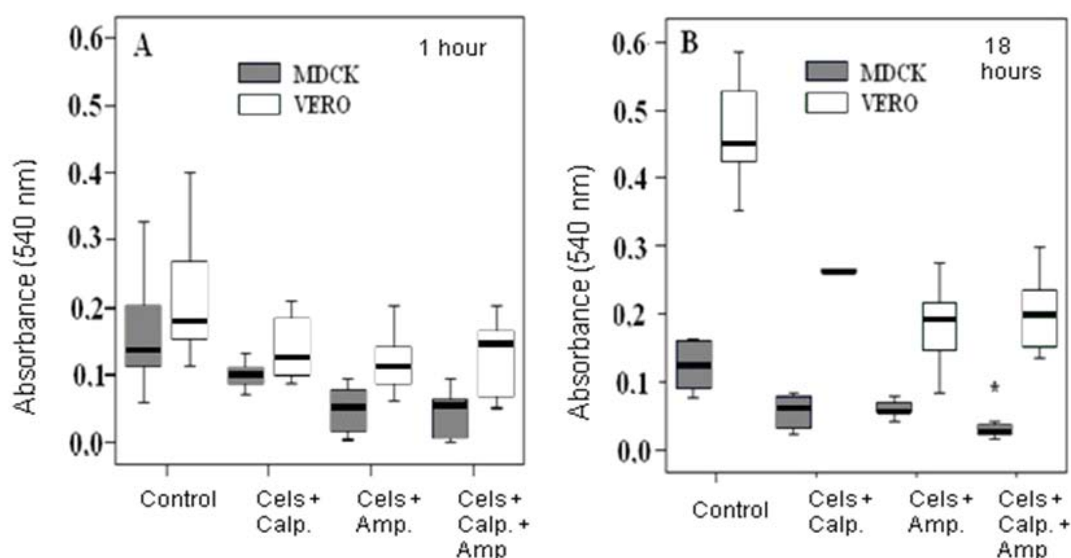

Figure 2. Comparative study of VERO and MDCK cell lines: evaluation of the PKC cell signaling pathway. Cels. + Calp. = Cells + Calphostin C; Cels. + Amp. = Cells + Amphotericin B; Cels. + Calp. + Amp. = Cells + Calphostin C+ Amphotericin B. The cultures were tested using the Neutral Red assay. Absorbance was read at 540 nm and the mean absorbance for each group was expressed. Results represent mean + SD of triplicates from three independent experiments. Asterisk, significantly different from group cells + amp. ($p < 0.05$).

to nephrotoxicity can vary among the nephron segments [1]. Based on this information, it is important to emphasize the importance of standardizing a wide range of cell lines to study nephrotoxicity, given that different cell types are involved in this process.

Therefore, the next step in the process was to determine whether or not these differences, regarding neutral red cytotoxicity, could in some way be related to the change in the PKC signaling pathway.

3.2. Study of cell signaling through PKC

The blocking of the PKC pathways proved to be significant ($p < 0.05$) in the VERO and MDCK cell lines, after 1 and 18 h of incubation. When the comparison was performed in the VERO cells treated with amphotericin B and Calphostin C, as compared to those treated only with amphotericin B, no statistically significant differences could be observed at either of the two evaluated times. In the MDCK cells, when the cells treated with amphotericin B and Calphostin C were compared with the cells that had only been incubated with amphotericin B, a statistically significant blocking ($p < 0.05$) of 52% only occurred at 18 h (Figure 2).

The results from studies on VERO and MDCK cell lines exhibited a similar profile after incubation for 1 hour, which is in accordance with the neutral red assay, which showed that this time period is not enough to detect the effect of amphotericin B on the PKC signaling pathways. Nevertheless, after 18 h of incubation, the inhibition of the PKC signaling pathways can produce a toxic effect of amphotericin B on MDCK cells. Previous studies have also reported that the induction of PKC seems to be protective in renal epithelial cells [8].

Therefore, the present study's results have shown that the nephrotoxicity of amphotericin B can vary among segment nephrons, which could be identified by performing neutral red assays and by changing the pattern of PKC cell signaling. In conclusion, the present study shows the importance of the screening and protecting of drugs at a molecular level in more than one cell strain.

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