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Cytotoxicity assessment of beta-lapachone in endothelial cells

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ABSTRACT. The selective activity of an antineoplastic drug is related to its ability to promote cytotoxic action on tumor cells and preserve the integrity of non-neoplastic cells. Beta-lapachone is extracted from the sawdust of Ipe wood, a thick bark tree from the Ipe wood found in the Brazilian Cerrado biome. This study aimed to evaluate the cytotoxic action of beta-lapachone in an endothelial cell line. The EA.hy926 cells were seeded in two groups, G1 and G2, cultured and exposed to beta-lapachone at concentrations of 0.0, 0.01, 0.03, 0.1, 0.3, 1 and 3 µM for 24 hours. G1 remained under normal cultivation conditions and G2 was subjected to oxidative stress through an ischemia and reperfusion assay, in a deoxygenated sealed chamber. The cytotoxicity assay was performed using the tetrazolium reduction method. In G1, the cytotoxicity ranged from 0.0 to 10.0%; and in G2 between 0.0 and 6.3%. No statistically significant difference was observed between the obtained values. Moreover, we found no cytotoxic action of beta-lapachone on endothelial cells, and the results point out that the drug might have preserved the cell's integrity against oxidative stress under the conditions of this experiment. This promising result suggests the possibility of beta-lapachone as a chemotherapy drug with selective activity.

Keywords: antioxidant; cell viability; beta-lapachone; cytotoxicity; *Tabebuia*.

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Introduction

The selective activity of an antineoplastic drug is related to its capacity to promote cytotoxic action in tumor cells and to preserve the integrity of non-neoplastic cells. Neoplastic cells are sensitive to topoisomerase inhibitors, as they multiply quickly (Kleiner & Silva, 2003). In this context, labile cells, which maintain a constant renewal of tissues, such as the epithelium of the gastrointestinal tract, capillaries, and the immune system, are also damaged during treatment with non-specific antitumor agents. This results in undesirable adverse effects on oncology patients, such as nausea, vomiting, diarrhea, alopecia and decreased immunity (Almeida et al., 2005).

The Cerrado biome has a flora which is rich in biological diversity. Beta-lapachone (BLP), (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione), was isolated in 1882 through the extraction from sawdust of Ipe wood, *Tabebuia* sp., of the family Bignoniaceae (Campanholi et al., 2018). Its chemical structure was unveiled in 1896, but it only started to be easily prepared in 1971. It has a molecular weight of 243.3 KDa (Dalton-A unit of molecular weight) and a melting point between 158 and 159°C. It is also lipophilic and water-insoluble (Pardee, Li, & Li, 2002).

BLP has antibacterial, antifungal, antiretroviral and antineoplastic activities. The mechanism of action includes alkylation of sulfhydryl groups of enzymes, apoptosis and formation of reactive oxygen species (Pardee et al., 2002). Something noteworthy about BLP is that it is a non-competitive inhibitor of the enzyme indoleamine 2,3-dioxygenase (IDO1), which has as one of the functions the suppression of cells of the immune system. It is also possible to obtain synergism between its cytotoxic action and the immunological effects (Martín-Navarro et al., 2010, Flick, LaLonde, Malachowski, & Muller, 2013). Moreover, BLP can also induce oxidative stress in the pathway of the P450 reductase enzyme, by promoting DNA scission (Ferraz et al., 2001). Thus, through the formation of hydrogen peroxide, superoxide and hydroxyl radical, it damages cellular

components, interferes with cell division, alters specific points of natural morphogenetic evolution, makes the cell infeasible and induces apoptosis (Machado, 2000).

In addition, BLP can inhibit the topoisomerase complex, which allows the functions of transcription, repair, replication, and structuring of the chromosome to occur normally. By inhibiting the topoisomerase-DNA complex, checkpoints are formed in the cell cycle, which induce the death of malignant cells (Kleiner & Silva, 2013).Due to the activity of inhibition of DNA topoisomerase I, which is an enzyme that plays an important role in DNA replication and packaging processes, studies on BLP's antineoplastic action in prostate, ovarian, breast, myeloma, lung, canine osteosarcoma and liver cancers have been carried out (Pardee et al. 2002; Kung, Lu, & Chau, 2014; Cruz et al., 2018). In cancer cells, BLP triggers the mechanisms of apoptosis, autophagy, and cell death by necrosis (Park et al., 2014). However, the exact mechanism for triggering cell death remains unknown. On the other hand, it may selectively induce the death of neoplastic cells, without interfering with normal cells (Ríos-Luci & Bonifazi, 2012).

In this study, we aimed to evaluate the cytotoxic action of beta-lapachone in an endothelial cell line, in order to analyze the possibility of using it as a chemotherapy drug with selective activity.

Material and methods

This study was approved by the Research Ethics Committee, CEP-UFG, under number 1,840,500. The cell line EA.hy926, BCRJ 0345, Lot 001100, originating from ATCC (American Type Culture Collection - Manassas, VA, USA), was acquired from the Rio de Janeiro Cell Bank (UFRJ - Rio de Janeiro, Brazil). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS), penicillin and streptomycin (10,000 IU mL^{-1} - 10 mg mL^{-1}), amphotericin B and L-glutamine (all reagents from Cultilab, Campinas, Brazil), and kept in a humidified incubator at 37ºC with 5% CO_2 atmosphere.

BLP was acquired from Santa Cruz Biotechnology (Dallas, Texas, USA). The test aliquots were dissolved in DMSO (Dimethyl sulfoxide, Cultilab, Campinas, Brazil) at a concentration of 1 mM and stored at -20°C.

Two groups, G1 and G2, were prepared in 96-well culture plates, where the cells were seeded at a concentration of $1x10^4$ cells/well. The plates were kept for 24 hours in a humidified incubator at 37°C with 5% CO_2 atmosphere. The cells were treated with BLP at following concentrations of 0.0, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 µM for 24 hours. The control group was BLP-free and received 0.3 µl of DMSO (Figure 1).

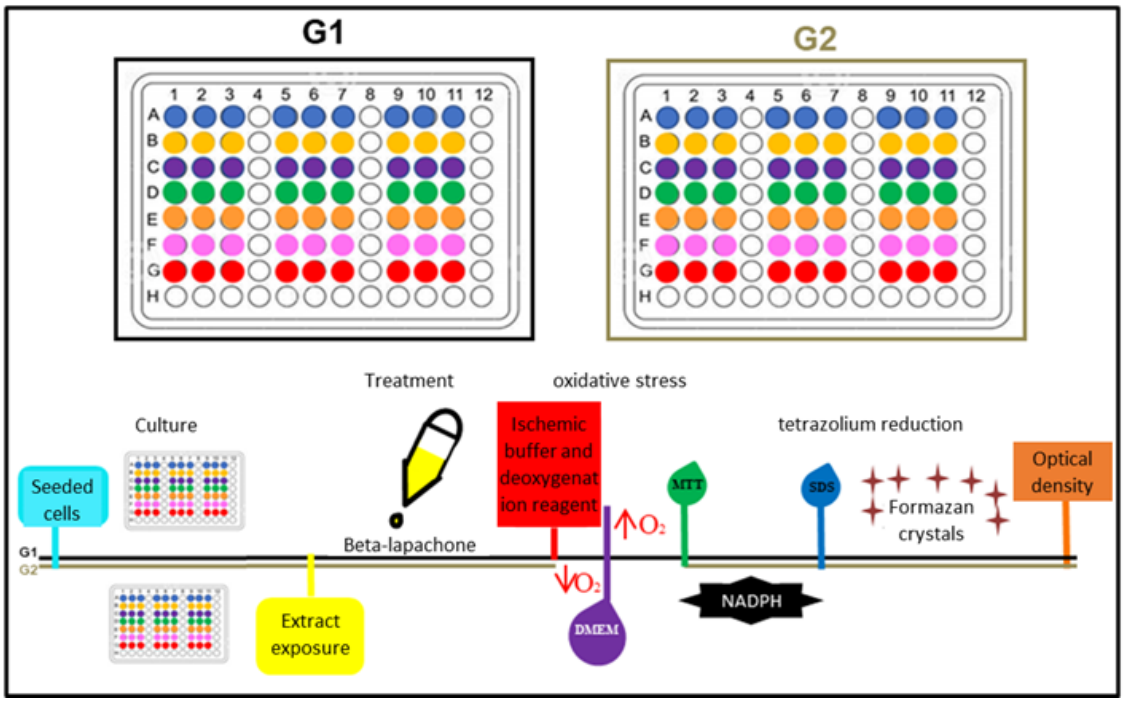

Figure 1. Steps of the experimental treatment containing EA.hy926 cells from established culture, in 96-well plates, for oxidative stress and MTT colorimetric assays. The concentrations of beta-lapachone used were: 0.0 µM (blue); 0.01 µM (yellow); 0.03 µM (purple); 0.1 µM (green); 0.3 µM (orange); 1.0 µM (pink); 3.0 µM (red), during the period of 24-hour exposure time. During oxidative stress, G1 remained in the incubator and G2 was subjected to the process of ischemia and reperfusion in a sealed GasPack chamber. The test was carried out in triplicate, with repetition of three independent experiments.

At the end of the exposition period, the treatment was substituted by a culture medium. G1 remained in the incubator and G2 was subjected to oxidative stress as shown in Figure 1. For this, the DMEM was replaced by an ischemic buffer and the plates were transferred to a GasPack sealed chamber, containing the deoxygenation reagent (Microbiology Anaerocult A, Merck Germany). Thus, the O_2 consumption and the CO_2 production occurred, taking into consideration the ischemia process for one hour. After that, the reperfusion was performed with DMEM medium for two hours.

The cytotoxicity and cell viability assays were performed using the tetrazolium reduction method. After the ischemia/reperfusion treatment period, the media in G1 and G2 plates were discarded and 10 µl of tetrazolium (MTT (3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium) was added to each well. The plates were then incubated for three hours. 50 µl of 10% sodium dodecyl sulfate (SDS - Vivantis Biochemical) diluted in 0.001N HCl was added to finish the MTT reaction. The quantitative determination of the dehydrogenase activity of the NADPH reaction products was performed, which reduced the MTT, and formed purple-blue colored formazan crystals. The plates were kept for 24 hours at room temperature, protected from light. Optical density was quantified in a spectrophotometer (KHB ST-360, 570 nm).

Cytotoxicity was determined using the following Equation 1:

$$\% CT = 100 - [(abs\ treatment / abs\ control) \times 100] \quad (1)$$

Cell viability was determined using the following Equation 2:

$$\% CV = (abs\ treatment / abs\ control) \times 100 \quad (2)$$

where: CT is cytotoxicity; CVis cell viability; and abs is absorbance.

The experiment was performed in three independent experiments in triplicate. We compared the mean values of cytotoxicity and cell viability (%) of the different BLP concentrations (µM) using an Analysis of Variance (ANOVA) and Tukey's test (5% significance). The statistical analyses were performed using the easyanova package (Arnhold, 2013) in the R software (R Core Team, 2017).

Results and discussion

The evaluation of the possible effects of BLP was performed through the MTT assay. In G1, we analyzed the cytotoxic effect in normal cell culture conditions. We observed a slight increase in dose-dependent cytotoxicity (Table 1). However, we found no statistical difference between the treatments with different concentrations and the control group. The BLP did not show cytotoxic action because of the low values we found, which indicates a low cell mortality rate, without significant statistical change.

Table 1. Cytotoxicity in endothelial cells of G1, treated with beta-lapachone (BLP) at different concentrations.

BLP concentration (µM)	Cell cytotoxicity (%)	p*	Coefficient of variation (%)
0 (control)	0		
0.01	3.7^{A}		
0.03	5.3^{A}		
0.1	5.6^{A}	0.0842	3.54
0.3	3.9^{A}		
1	7.4^{A}		
3	10.0^{A}		

*Probability value of Analysis of Variance and Tukey test.

In G2, we analyzed the cytotoxic effect after inducing the oxidative effect, which is a process related to the pathophysiology of several diseases, including cancer. We found an increase in cell viability and a consequent decrease in dose-dependent cytotoxicity (Table 2). However, we found no statistical difference between the treatments with different concentrations and the control group. The cell death is noteworthy in all groups, which validates the action of oxidative stress. However, after the induction period of ischemia and reperfusion, the number of viable cells in the control group was the parameter used to compare and evaluate the cytotoxicity at the different concentrations. In G2, as in G1, we found no cytotoxic effect of BLP on endothelial cells, even after the oxidative stress.

The greatest BLP concentration used in this study was 3.0 µM, following the findings of Byun, Son, & Pae (2014), in which BLP did not have cytotoxic action in dosages of up to 4.0 µM in ECV3048 endothelial cells. Another significant fact is that, unlike the present study, most of the assays assessed the BLP cytotoxic effect on neoplastic cells. Cancer cells are sensitive to BLP action because of the high levels of the enzyme NQO1. However, normal cells contain a lower amount of this enzyme, which probably makes them more resistant to the cytotoxic action of BLP (Kung et al., 2014). The absence of cytotoxic action on endothelial cells found in this study may confirm the discriminatory cytotoxic action of BLP in tumor cells. In other words, this substance likely has antineoplastic activity, with selective non-cytotoxic action on non-neoplastic labile cells.

Table 2. Cytotoxicity and cell viability of endothelial cells of G2, submitted to ischemia and reperfusion, treated with beta-lapachone (BLP) at different concentrations.

BLP concentration (µM)	Cell cytotoxicity (%)	Cell viability (%)	p*	Coefficient of Variation (%)
0 (control)	0.0^{A}	100.0^{A}		
0.01	6.3^{A}	93.7^{A}		
0.03	2.0^{A}	98.0^{A}		
0.1	0.0^{A}	100.0^{A}	0.0842	3.54
0.3	0.0^{A}	102.9^{A}		
1.0	0.0^{A}	101.0^{A}		
3.0	0.0^{A}	103.0^{A}		

*Probability value of Analysis of Variance and Tukey test.

BLP induces death in cancer cells through apoptosis, necrosis and autophagy, with the potential to treat neoplasms (Pinto & Castro, 2009; Cruz et al., 2018). The induction of cell death by necroptosis in SK-Hep1 cells of human hepatocellular carcinoma has recently been demonstrated (Park et al., 2014). BLP effectively inhibited the growth of oral squamous cell carcinoma of the HN22 and HSC4 lines by promoting the DNA condensation and formation of apoptotic bodies in the cell nucleus (Jeon et al., 2015). This substance induced autophagy and suppressed the migration and invasion of HNE1 nasopharyngeal carcinoma cells (Han, Shi, & Li, 2019). In addition to the studies regarding human species, the cytotoxic action of this substance has recently been demonstrated in canine osteosarcoma cells (Cruz et al., 2018). The mechanism of action was the induction of intrinsic apoptosis, by rupturing the mitochondrial membrane potential and blocking the cell cycle in the G0/G1 phase. The cytotoxic dose was 0.3 µM, significantly lower than the maximum dose used in the present study (3.0 µM), which was non-cytotoxic in the endothelial cells analyzed.

The experimental model used in G2 to simulate ischemia was a sealed chamber, similar to Zhu et al., 2015, which created quasi-anaerobic conditions with an oxygen concentration of less than 1%. Reactive oxygen species can be used as a therapeutic agent and as a stimulus to achieve greater efficacy of antitumor drugs (Wang et al., 2019). Oxidative stress, through the process of ischemia and reperfusion, can be induced experimentally, improving the knowledge of mechanisms of action and possible cytotoxicity of antioxidant substances. Another model of oxidative stress was promoted in EA.hy926 endothelial cells, which were treated with hydrogen peroxide (H_2O_2) to simulate the injury and mechanisms of ischemia and reperfusion (Seto et al., 2017).

Other studies show opposite results, in which BLP was cytotoxic due to the high production of reactive oxygen species and induced apoptosis in MDA-MB-231 human breast cancer cells (Zada et al., 2019). In another study, BLP was activated by reactive oxygen species to generate hydrogen, converted into hydroxyl radicals, which increased its anti-tumor cytotoxic activity (Wang et al., 2019).

We did not find any antioxidant effect of BLP in the conditions of this experiment. Other authors have reported antioxidant effects of BLP on ECV 304 endothelial cells and astrocytes, by inhibiting the production of reactive oxygen species and inducing signaling pathways for the production of phase II antioxidant enzymes (Byun et al., 2014; Park et al., 2014). The antioxidant doses of BLP used in studies with BV2 microglial and primary microglial cells were 2 and 4 µM (Lee, Ko, Jeong, Park, & Kim, 2015). In another study, the cytoprotective effect occurred at concentrations above 10 µM (Byun et al., 2014). However, in the present study, in which the maximum dose used was 3.0 µM, we found nocytotoxic action. In addition, higher dosages were not tested to analyze a possible antioxidant effect.

The formation of hydrogen peroxide, superoxide and hydroxyl radical damages cellular components, interferes with cell division, alters specific points of natural morphogenetic evolution, makes the cell infeasible, and induces apoptosis (Machado, 2000). However, we did not observe this formation in our study, and as a consequence, it is possible that, even without statistical proof of antioxidant activity, the integrity of endothelial cells was preserved from oxidative stress, as the BLP concentration increased.

We conclude that further studies are essential to elucidate the mechanism of action, therapeutic effects and adverse consequences for the development and use of BLP as an effective medication for the control of several diseases, including cancer (Castro, Emery, & Silva Junior, 2013; Yu et al., 2014). In this context, the result of the present study is highly promising, because by comparing our results of G1 and G2 treatments with other studies, it is possible to suggest that BLP has a selective capacity for cytotoxic action, at low concentrations, differentiating the endothelial cells of neoplastic cells.

Conclusion

The antineoplastic compound beta-lapachone did not have cytotoxic action on endothelial cells at the dosages studied, which were up to 3 μM. This result suggests that this substance, derived from the sawdust of the Ipe wood, a native tree from the Brazilian Cerrado, might have chemotherapy potential with selective cytotoxic activity.

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