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A toxicological evaluation of hepatocytes exposed to the synthetic resorcinolic lipid 3-Heptyl-3,4,6-trimethoxy-3*H*-isobenzofuran-1-one

Avaliação toxicológica de hepatócitos expostos ao lípidio resorcinólico sintético 3-Heptil, 3,4,6-trimetoxi-3H-isobenzofuran-1-ona

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Resumo

Estudos recentes mostraram que o lipídio resorcinólico 3-Heptil, 3,4,6-trimetoxi-3H-isobenzofuran-1-ona (AMS 35AA) pode ser um importante adjuvante quimioterápico que não é genotóxico e nem mutagênico. Vale ressaltar que, quando a AMS35AA está associada à ciclofosfamida, aumenta o dano mutagênico, a fagocitose esplênica, a neutropenia e induz a apoptose no fígado e nos rins. Tendo em visto isso, o presente estudo realizou uma investigação morfométrica precisa das consequências da associação entre AMS35AA e ciclofosfamida na morfologia dos hepatócitos de camundongos *Swiss*. Nosso resultados fornecem fortes evidências de que a AMS35AA não é tóxico e não induz a hiperplasia celular ou hipertrofia nos hepatócitos, o que pode levar ao desenvolvimento de neoplasia maligna. Então, nosso dados sustentam a visão de que o derivado de lipídio resorcinol não é tóxico para o hepatócito, o que, portanto, pode ter aplicações terapêuticas.

Abstract

Recent studies showed that resorcinolic lipid 3-Heptyl-3,4,6-trimethoxy-3*H*-isobenzofuran-1-one (AMS 35AA) may be an important chemotherapy adjuvant, that is not genotoxic nor mutagenic. It is noteworthy that, when AMS35AA is associated with cyclophosphamide, it increases the mutagenic damage, splenic phagocytosis, neutropenia, and induces apoptosis in liver and kidneys. Taken this into account, the present study performed an accurate morphometric investigation of the consequences of the association between AMS35AA and cyclophosphamide on the hepatocytes morphology of Swiss mice. Our results provide strong evidence that the AMS35AA is not toxic and does not induce cellular hyperplasia or hypertrophy in the hepatocytes, which could lead the development of malignant neoplasia. Then, our data support the view that the resorcinolic lipid derivative is not toxic to the hepatocyte, which therefore could have therapeutic applications.

1. Introduction

Currently there is great interest in isolation and/or synthesis of bioactive compounds with specific characteristics for the treatment and/or prevention of cancer. In general these compounds are considered xenobiotics because they are foreign to the human body. It is noteworthy that the different body tissues are daily exposed to xenobiotics, among which we highlight the environmental contaminants and also medicines. Most drugs are xenobiotics that are used to modulate bodily functions for therapeutic ends, in order to (re)establish homeostasis (Taniguchi et al., 2010).

Drugs and/or drug candidates when penetrate the body are biotransformed by enzymes, in the mammalian liver, which might alter the drug, transforming it in beneficial, harmful or inefficient to the body (Taniguchi et al., 2010). In this context, *in vivo* tests present an important assay to evaluate compounds that are candidate to be used in chemotherapy and/or chemotherapy adjuvant. Among the different substances that should be test is the non-isoprenic resorcinolic lipids. These compounds were first described as products of bacteria, protozoa, algae, fungi, plants and animals (Kozubek and Tyman, 1999. Moreover, synthetic molecules have also been described with similar biological activities to those extracted from living organisms (Navarro et al., 2014).

Recent studies performed by our research group showed that the resorcinolic lipid 3-Heptyl-3,4,6-trimethoxy-3*H*-isobenzofuran-1-one (AMS 35AA, Figure 1), may be an important chemotherapy adjuvant, that is not genotoxic nor mutagenic. The AMS35AA did not alter the histology of liver and kidneys although increased the levels of aminotransferase (AST), creatinine, spleen phagocytosis and apoptosis. It is noteworthy that, when AMS35AA is associated with cyclophosphamide, an important chemotherapy agent, it increases the mutagenic damage, splenic phagocytosis, neutropenia, and induces apoptosis in liver and kidneys (Navarro et al., 2014). This synthetic compound possesses structural similarities to the biologically active natural products named of cytosporones (Santos et al., 2009; Zamberlam et al., 2012).

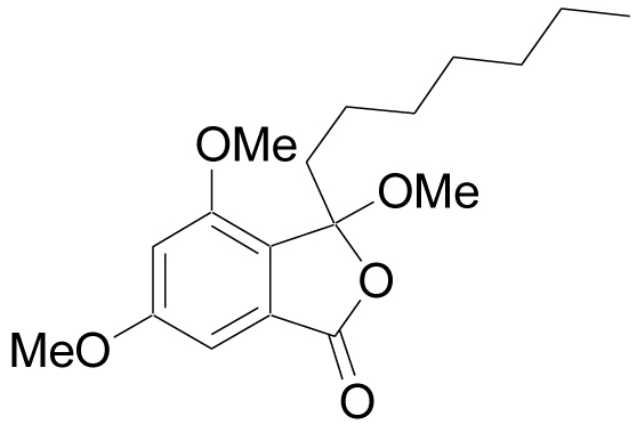

Figure 1 - Chemical structure of AMS35AA.

Taken this into account, there is just one study in the literature that performed a qualitative histopathological evaluation of the liver exposed to the AMS35AA. Assuming that liver is a very important target to drugs and that certain xenobiotics may affect only of the susceptible target tissue but not others, it would be almost impossible to screen for all possible effects (Neubert, 1997). However, it is necessary to perform an accurate histomorphometrical evaluation of the potential AMS35AA influence in the hepatocytes. Although non-significant qualitative histopathological changes were reported, an increase of AST were observed which indicates liver injury, suggesting further studies involving this organ. Therefore, the present study aimed to investigate the consequences of the association between cyclophosphamide, a chemotherapy agent, and AMS35AA on the hepatocytes morphology of Swiss mice exposed animals.

2. Material and Methods

2.1. Chemical compounds

The syntethic resorcinolic lipid 3-Heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one (AMS35AA) was produced as described by Navarro et al. 2014 and was diluted in ethanol (1%) and then in Milli-Q water, and administered by intraperitoneal (*ip*) injection. The chosen doses of AMS35AA has been shown previously to increase the frequency of apoptosis approximately by 16, 17 and 15 fold in the liver at the 10, 20 and 40 mg/kg doses, respectively, without change the absolute and relative weight of the organ (Navarro et al., 2014).

Cyclophosphamide (Sigma), an indirect-acting alkylating agent was used to induce DNA damage. It was prepared in phosphate-buffered saline (PBS) and administered at a dose of 100 mg/kg *bw, ip*.

2.2. Animals and experimental design

Thirty-nine male, 8-week-old, Swiss mice were used in the present studies. These animals were housed in a standard animal facility under controlled temperature (22 °C) and photoperiod (12 h light, 12 h dark) with access to water and rodent food *ad libitum*. All procedures and protocols followed approved guidelines for the ethical treatment of animals, according to the Ethics Committee in Animal Experimentation from the Federal University of Mato Grosso do Sul (Protocol # 399/2012).

The animals were randomly divided into eight experimental groups: the animals of the control group (G1, n=5) received, simultaneously, a physiological solution (the vehicle for the cyclophosphamide, 0.1 mL/10 g *bw, ip*) and hydroethanolic solution (the vehicle for the AMS35AA, Milli-Q water to which 1% ethanol had been added, *ip*); cyclophosphamide group (G2, n=5) received one injection of cyclophosphamide (100 mg/kg *bw, ip*); the AMS35AA groups (G3 n=5, G4 n=5 and G5 n=5) received, simultaneously, one injection of AMS35AA (10, 20, 40 mg/kg, respectively, *bw, ip*) and one injection of physiological solution; in the associated groups (G6 n=4, G7 n=5 and G8 n=5) the animals simultaneously received one injection of AMS35AA (10, 20, 40 mg/kg *bw, ip* and one injection of cyclophosphamide (100 mg/kg *bw, ip*).

The intraperitoneal route was used for all animals to maximize chemical exposure (Oliveira et al., 2012).

2.3. Tissue preparation

Seventy-two hours after the chemical compounds injection, the control and treated animals were anesthetized with 50 mg/kg of ketamine and 10 mg/kg of xylazine and then euthanized by cervical dislocation for liver collection. The liver was fixed in 10% neutral buffered formalin for 24 hours. Tissue samples with dimensions of approximately 3.0 mm in diameter, 5.0 mm in width and 8.0 mm in length were obtained and the fragments were immediately refixated by immersion, in a new formalin solution for at least 2 h. After fixation, the tissue fragments were routinely processed and embedded in paraffin. Subsequently, 6 μm-thick sections were obtained and stained with hematoxylin-eosin.

2.4.Hepatocytes parameters

Ten randomly selected digital images from the liver sections were obtained per animal using a Leica DFC495 digital camera connected to a DM55OOB microscope. Using an analysis program (Program LAS, version 3.8 and Image J) the cells were measured. The individual volume of the hepatocytes was obtained from their nuclear volume and the proportion between nucleus and cytoplasm. To calculate the proportion between nucleus and cytoplasm a 540-point square lattice was placed over the sectioned material at 1000× magnification. At least four thousand points over hepatocytes were counted for each animal. Because the hepatocyte nucleus in mice is spherical, its nucleus volume was obtained from the knowledge of the mean nuclear diameter. For this purpose, the diameters of forty nuclei were measured for each animal. Hepatocyte nuclear volume was expressed in μm^3 and obtained by the formula $4/3\pi r^3$, where r=nuclear diameter/2 (Cunha-Laura et al., 2013).

2.5.Statistical analysis

Values are expressed as mean±SEM and data were analyzed using the one-way ANOVA followed by the Tukey post-test using GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA, USA). The significance level was set at $p<0.05$.

3.Results

The administration of cyclophosphamide and the resorcinolic lipid alone or in combination did not alter the morphology of the liver on mice exposed to the different treatments. All groups investigated in this study showed uni or multinucleate hepatocytes with polyhedral shape, similar to the negative control group (Figure 2).

Regarding the hepatocytes parameters, no significant differences were observed between negative control and treated animals. The exposed animals presented similar nuclear and cytoplasmic volume. As expected, similar tendency was observed for the hepatocyte individual volume (Figure 3).

4. Discussion

It is well established in the literature that liver functions are diverse and most of them are performed by hepatocytes, polyhedral cells which occupy about 70-80% of the hepatic parenchyma. Among the functions executed by possible to mention hemocaterese; the metabolism of lipids, carbohydrates and proteins; storage of vitamins and some minerals and synthesis of growth factors and proteins from blood plasma (Arias et al., 2009). In general, pathologists use size and shape as well as other nuclear features to distinguish benign from malignant or suspected malignant nuclei (Jagoe et al., 1982). Moreover, nuclear pleomorphism may be taken as a measure of differentiation of a tumor (Jagoe et al., 1982; Ptehn and Ptehn, 1975).

Taken to the knowledge that the liver present an ideal tissue for morphometric studies, the primary aim of the present investigation was to evaluate whether liver exposure to the synthetic resorcinolic lipid AMS35AA alone or in combination with cyclophosphamide was able to alter the hepatocyte morphology or even induce hepatocyte damage as hypertrophy, hyperplasia and dysplasia that are frequently associated with development of malignant neoplasia (Farber, 1982). As part of this evaluation, a more general aim was to establish if AMS35AA could be toxic to the liver cells, because this would give new insight into the relative importance of AMS35AA as a chemotherapy adjuvant (Navarro et al., 2014). The present findings show that the resorcinolic lipid does not cause adverse effects in the hepatocyte morphology. There was no enlargement of nuclei of the hepatocyte exposed to AMS35AA, and if it had occurred could be due to the increased metabolism of protein synthesis, that would culminate in hepatocyte hypertrophy (Engelman et al., 2001). Although there is a metabolic heterogeneity in pericentral and perilobular regions of the liver due to the differences of the blood supply and the distribution of glycogen (Kudryavtseva et al., 1996), this study analyzed the morphology of hepatocytes irrespective of their distribution in the organ, which follows the principles of stereology that sampling must be random (Torres et al., 1999).

This study confirms our earlier findings that showed cytoplasm rarefaction only in the livers of animals treated with cyclophosphamide in combination with AMS35AA (Navarro et al., 2014) and extend this data identifying that the hepatocyte nuclear, cytoplasmic and individual volume do not change. Our study reinforces the evidence that AMS35AA is not toxic to the hepatocyte. Although Shokrzadeh et al. (2015) reported necrotic hepatocyte with absence of nuclei in cyclophosphamide treated mice, the results obtained in this research did not show that and this might be explained because Shokrzadeh et al. (2015) used a two times higher dose of cyclophosphamide than that used in our study.

It is important to note that, because of their strong amphipathic properties Kozubek et al., 1995, the resorcinolic lipid has substantial affinity to lipid bilayers and they can cause structural and functional membrane alteration (Stasiuk and Kozubek, 2010). The AMS35AA consists of an alkyl chain of seven carbons attached to the chiral lactone ring and a conjugated system of one dimethoxybenzene ring. As AMS35AA has both a hydrophilic and a lipophilic chemical groups at same molecule, it presents amphiphilic properties, which may favor interactions with cellular structures, composed of lipid membranes (Goñi, 2014) In this context, *in vitro* experiments have shown that resorcinolic lipids can alter membrane-bound acetylcholinesterase enzymatic activity (Stasiuk et al., 2004)

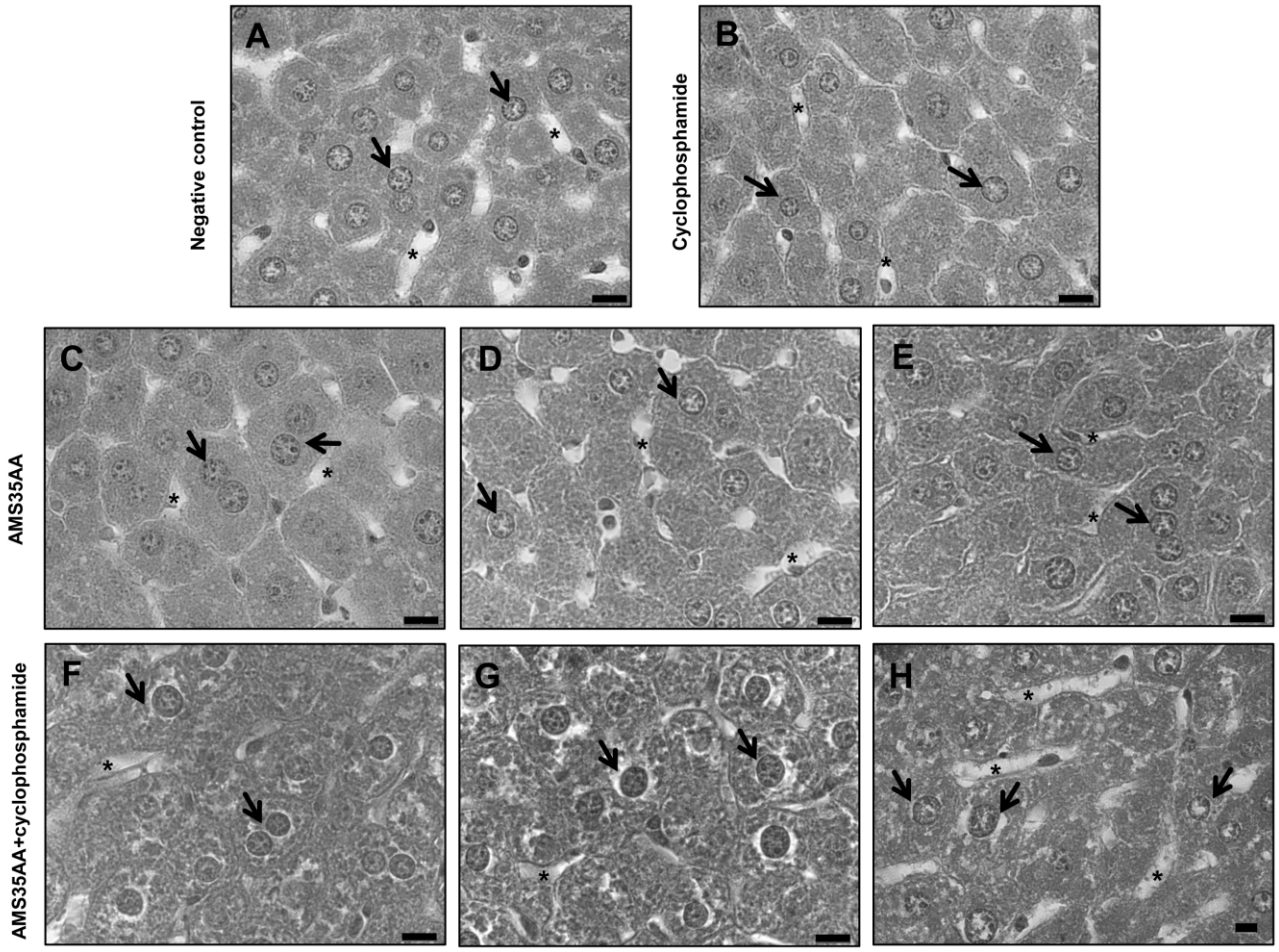

Figure 2 - Light photomicrographs showing liver histology of control (A), cyclophosphamide (B), AMS35AA in 10 (C), 20 (D) and 40 mg/kg (E) and the combination of AMS35AA in 10, 20 and 40 mg/kg associate to cyclophosphamide (F, G, H, respectively) on the adult male mice liver. Observe the normal spherical nucleus (arrow) of the hepatocytes. A rarefaction of the cytoplasm is observed in F, G and H histological sections. The sinusoids and thinner sinusoidal wall (asterisks) can also be observed. Bar, 10 μm.

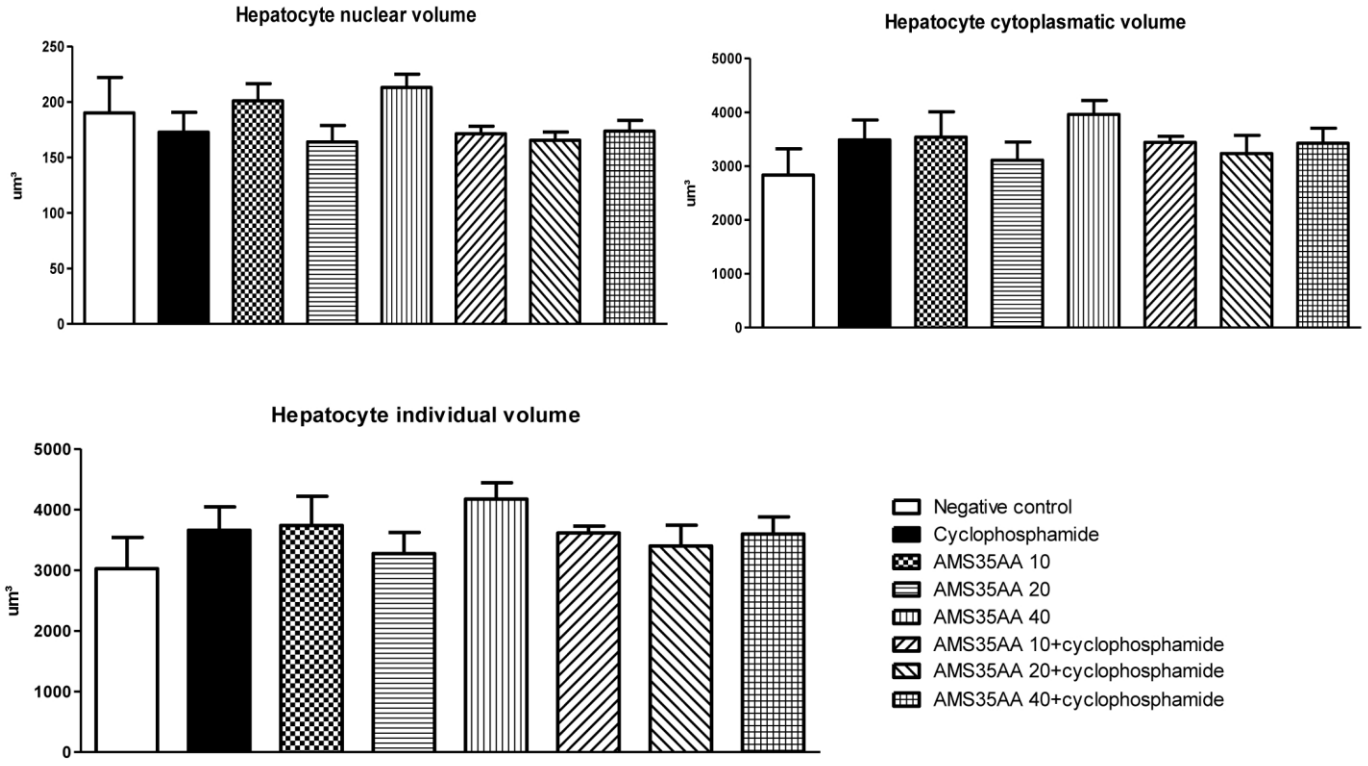

Figure 3 - Morphometric analyses of the hepatocytes nuclear, cytoplasmatic and individual volume on the adult male mice liver. The hepatocytes parameters were similar between control and treated groups. $p>0.05$.

as they can make biomembranes more resistant to peroxidation (Erin et al., 1987). Besides that, when associated to membrane phospholipids and stabilized by hydrogen bonds, they disturb the lipid bilayer and cause changes in the functioning of membrane-bound enzymes (Kaprelyants et al., 1984, Kaprelyants et al., 1987) Moreover, phenolic lipids added to media containing liposomes exhibited the ability to induce increased bilayer permeability for ions and small nonelectrolytes (Kozubek and Demel 1980).

It is worth mentioning that cyclophosphamide was developed with the aim to be activated specifically by tumour phoshoramidase (Hall and Tilby 1992), although it is known today that its activation occurs by a cellular mechanism which involves hepatic microsomal enzymes (Brock,1967). Additionally, the enzymatic oxidation of the chemotherapy render 4-hydroxycyophosphamide and aldophosphamide. Then, the aldophosphamide crosses the cell membrane and forms phosphoramide mustard, which is believed to be the active alkylating component of this pathway (Hall and Tilby, 1992). In this sense, incorporation of resorcinolic lipid AMS35AA in lipidic-proteic membrane cells can alter their structural and functional properties, such as permeability and catalytic properties of associated enzymes. In addition, AMS35AA can act modifying cyclophosphamide and its metabolites permeability across the membrane, since we already demonstrated that it can potentiate mutagenic damage and increase apoptosis caused by cyclophosphamide without causing adverse effects (Navarro et al., 2014).

As already mentioned, because several important cellular metabolic process are related to biological membrane functionality, it is important to establish the effect of AMS35AA on catalytic activity of membrane associated enzymes related to cyclophosphamide metabolism. Also, it is necessary perform the characterization of the membrane properties with respect to biological as well as liposomal membranes in the presence of AMS35AA. In this sense, further studies using biomembrane models approaches, as lipid vesicles and Langmuir monolayer technique must be performed to make sure that AMS35AA can modify membrane properties. Based on these findings, there is a need for more detailed studies, which will be the next steps of our research group with the aim to elucidate the molecular mechanism of action of AMS35AA associated with cyclophosphamide.

In conclusion, although detailed understanding of the molecular action of the AMS35AA into the hepatocyte requires further characterization, the morphometric study of hepatocytes exposed to the resorcinolic lipid provide strong evidence that the AMS35AA is not toxic and does induce cellular hyperplasia or hypertrophy, which could lead the development of malignant neoplasia. Our data support the view that the resorcinolic lipid may be an important chemotherapy adjuvant without causing adverse effects, which therefore have therapeutic applications.

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Declaração: Os autores declaram estar cientes e terem atendido integralmente às normas preconizadas para as pesquisas experimentais de acordo com a Declaração Universal do Direito dos Animais. Os autores declaram ainda ausência de conflito de interesse.

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