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Hepatocellular toxicity of benzbromarone: Effects on mitochondrial function and structure

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ABSTRACT

Benzbromarone is an uricosuric structurally related to amiodarone and a known mitochondrial toxicant. The aim of the current study was to improve our understanding in the molecular mechanisms of benzbromarone-associated hepatic mitochondrial toxicity. In HepG2 cells and primary human hepatocytes, ATP levels started to decrease in the presence of 25–50 μM benzbromarone for 24–48 h, whereas cytotoxicity was observed only at 100 μM. In HepG2 cells, benzbromarone decreased the mitochondrial membrane potential starting at 50 μM following incubation for 24 h. Additionally, in HepG2 cells, 50 μM benzbromarone for 24 h induced mitochondrial uncoupling,and decreased mitochondrial ATP turnover and maximal respiration. This was accompanied by an increased lactate concentration in the cell culture supernatant, reflecting increased glycolysis as a compensatory mechanism to maintain cellular ATP. Investigation of the electron transport chain revealed a decreased activity of all relevant enzyme complexes. Furthermore, treatment with benzbromarone was associated with increased cellular ROS production, which could be located specifically to mitochondria. In HepG2 cells and in isolated mouse liver mitochondria, benzbromarone also reduced palmitic acid metabolism due to an inhibition of the long-chain acyl CoA synthetase. In HepG2 cells, benzbromarone disrupted the mitochondrial network, leading to mitochondrial fragmentation and a decreased mitochondrial volume per cell. Cell death occurred by both apoptosis and necrosis. The study demonstrates that benzbromarone not only affects the function of mitochondria in HepG2 cells and human hepatocytes, but is also associated with profound changes in mitochondrial structure which may be associated with apoptosis.

1. Introduction

The liver represents an important target for drug-mediated toxicity. Accordingly, many drugs are associated with liver injury, which can be hepatocellular, cholestatic or mixed (Navarro and Senior 2006; Suzuki et al., 2010). Importantly, drug toxicity is one of the major causes for fulminant liver failure which may necessitate liver transplantation or even lead to death (Kaplowitz 2005; Ostapowicz et al., 2002). In addition, drug-induced liver injury is one of the most important reasons for withdrawal of drugs from the market (Kaplowitz 2001, 2005). The reason why the liver is a special target for drug toxicity is at least twofold. First, the liver is exposed to high drug concentrations after oral ingestion due to its location between the gut and the systemic circulation. Second, the liver is the major location of drug metabolism. Hepatic metabolism of drugs and other chemical compounds can be associated with the production of reactive metabolites which may be toxic to hepatocytes and/or other cell types located in the liver (Zahno et al., 2013, 2011). For most drugs, the risk for inducing liver injury is small, and the development of liver injury is, however, usually non-predictable and not clearly dose-dependent (de Abajo et al., 2004; Suzuki et al., 2010).

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Benzbromaroneis a benzofurane derivative (Fig. 1) used as a uricosuric for the prophylaxis of acute gout attacks. For many years, benzbromarone was considered to be both effective and well tolerated. However, after several reports of severe hepatotoxicity (Arai et al., 2002; van der Klauw et al., 1994; Wagayama et al., 2000), the drug had to be withdrawn from the market in several countries, e.g. the USA, France and Switzerland. Histological findings in affected patients included microvesicular steatosis of liver (Arai et al., 2002), a finding compatible with inhibition of mitochondrial β-oxidation (Fromenty and Pessayre, 1995; Spaniol et al., 2001, 2003). In a previous in vitro study using isolated rat liver mitochondria and rat hepatocytes, we have compared the hepatotoxicity associated with benzbromarone with that of amiodarone (Kaufmann et al., 2005). Relevant findings in this study were that benzbromarone uncouples hepatic mitochondria and inhibits the respiratory chain and β-oxidation.

Mitochondrial function can be disturbed by chemical compounds via multiple ways. Important mechanisms include inhibition and/or uncoupling of oxidative phosphorylation and inhibition of specific metabolic pathways such as the urea cycle, fatty acid oxidation and/or ketone body production and the citric acid cycle (Krahenbuhl, 2001). While it was clear from our previous study that benzbromarone impairs certain mitochondrial functions such as the respiratory chain and β-oxidation (Kaufmann et al., 2005), it is currently unclear whether the findings in rodent mitochondria and hepatocytes are also present in human liver cell lines, by which mechanisms benzbromarone disturbs mitochondria and how mitochondria react after exposure to benzbromarone. We, therefore, studied the effect of benzbromarone on mitochondrial functions and mitochondrial structure in HepG2 cells after 24 h or 48 h drug exposure.

2. Material and methods

2.1. Cell line and culture

Benzbromarone was purchased from Sigma–Aldrich (Buchs, Switzerland). Primary human hepatocytes were obtained and cultured as described previously in the absence of rifampicin (Zahno et al., 2013). The human hepatoma cell line HepG2 was purchased from American type culture collection (ATCC, Manassas, USA). Cells were kept at 37 °C in a humidified 5% CO_2 cell culture incubator and passaged according to the instructions provided by ATCC using trypsin. HepG2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, containing 1.0 g/l glucose, 4 mM L-glutamine, and 1 mM pyruvate, 10 mM HEPES buffer) from Invitrogen (Basel, Switzerland), which was supplemented with 10% (v/v) heat-inactivated fetal calf serum. Protein concentrations of cells in culture plates were determined with the sulforhodamine Bassay as described by Skehan et al. (1990).

2.2. Isolation of mouse liver mitochondria

Male C57BL/6 mice were kept in the animal facility of the University Hospital of Basel (Basel, Switzerland) with food and water ad libitum. Animal procedures were performed in accordance with the institutional guidelines for the care and use of laboratory animals. Liver mitochondria were isolated by differential centrifugation according to the method described by Hoppel et al. (1979) and the mitochondrial protein content was determined using the bicinchoninic acid protein assay reagent from Thermo Scientific (Wohlen, Switzerland).

2.3. Cytotoxicity and intracellular ATP

Cytotoxicity was assessed using the Toxilight® assay from Lonza (Basel, Switzerland) and carried out according to the manufacturer's manual. In brief, cells grown in 96-well plates were exposed to a range of benzbromarone concentrations for either 24 or 48 h. All incubations contained the same amount of dimethyl sulfoxide (DMSO) (0.1%, v:v), which has been shown not to be toxic to HepG2 cells (Waldhauser et al., 2006). The plate was centrifuged and 20 μl of supernatant per well was transferred to a new 96 well plate. After addition of 100 μl of Toxilight® solution and incubation in the dark at 37 °C for 5 min, luminescence was recorded using a Tecan M200 Pro Infinity plate reader (TecanTraiding AG, Männedorf, Switzerland).

The intracellular ATP content was determined using the CellTiterGlo® Luminescent Cell Viability assay from Lonza (Basel, Switzerland) and carried out according to the manufacturer's manual. In brief, 100 μl assay buffer was added to each 96-well containing 100 μl culture medium. After cell lysisat 37 °C for 30 min, the released ATP was detected by luminescence measurement.

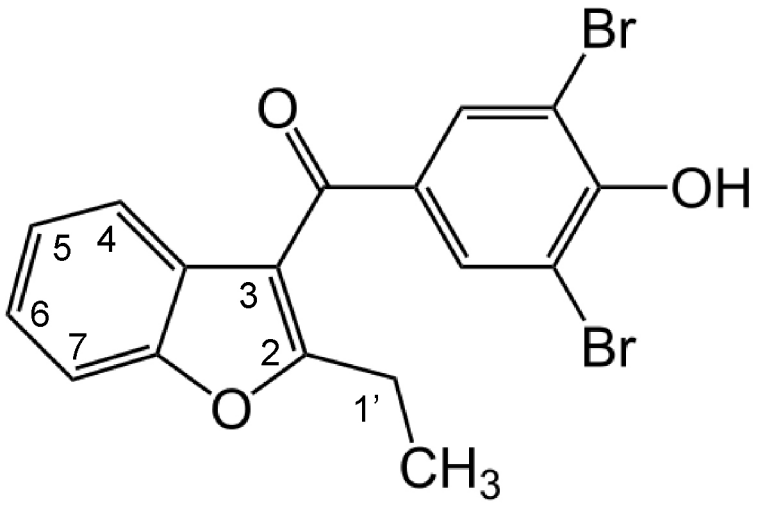

Fig. 1. Chemical structure of benzbromarone. Benzbromarone is a benzofurane derivative structurally related to amiodarone. Metabolism is mainly by hydroxylation in position 6 (CYP2C9) and 1′ (CYP3A4) (Kobayashi et al., 2012).