Toxicology 386 (2017) 40–48

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

8-Methoxypsoralen disrupts MDR3-mediated phospholipids efflux and bile acid homeostasis and its relevance to hepatotoxicity

Guolin Zhao[a], Dengqiu Xu[a], Ziqiao Yuan[a], Zhenzhou Jiang[a,b], Wang Zhou[a], Zhijian Li[a,e], Mengyue Yin[a], Zhixing Zhou[f], Luyong Zhang[a,c,d,*], Tao Wang[a,c,*]

[a] Jiangsu Key Laboratory of Drug Screening, China Pharmaceutical University, Nanjing 210009, China
[b] Key Laboratory of Drug Quality Control and Pharmacovigilance, China Pharmaceutical University, Nanjing 210009, China
[c] Jiangsu Center for Pharmacodynamics Research and Evaluation, China Pharmaceutical University, Nanjing 210009, China
[d] State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China
[e] Department of Pharmacology and Toxicology Laboratory, Xinjiang Institute of Traditional Uighur Medicine, Urumqi, Xinjiang 830049, China
[f] Tianjin Key Laboratory of Molecular Design and Drug Discovery, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China

ARTICLE INFO

Keywords:
Cholestasis
8-Methoxypsoralen
MDR3
Phospholipids

ABSTRACT

Since its discovery in 1987, multidrug resistance 3 P-glycoprotein (MDR3) had recognized to play a crucial role in the translocation of phospholipids from the inner to outer leaflets of bile canalicular membranes. An increasing number of reports suggest that drug-mediated functional disruption of MDR3 is responsible for drug-induced cholestasis. 8-Methoxypsoralen (8-MOP) is used clinically to treat psoriasis, vitiligo and other skin disorders. However, psoralens safety for long-term use is a concern. In the current study, we evaluate 8-MOP's potential hepatotoxicity and effects on bile formation. Sprague Dawley (SD) rats were treated daily 200 mg/kg or 400 mg/kg of 8-MOP orally for 28 days. The result showed a prominent decrease in biliary phospholipids output, which associated with the down-regulation of MDR3. Elevated bile acid serum level and increased biliary bile acid outputs were observed in 8-MOP-treated groups. The disturbance of bile acid homeostasis was associated with changes in enzymes and proteins involved in bile acid synthesis, regulation and transport. Human liver cell line L02 was used to determine on the mRNA and protein levels of MDR3. Cells treated with 8-MOP reveled a decrease in fluorescent PC (phosphatidylcholine) secretion into the pseudocanaliculi (formed between adjacent cells) compared with untreated cells. Our investigation represent the first evidence that 8-MOP can induce cholestatic liver injury by disturbing MDR3-mediated phospholipids efflux and bile acid homeostasis.

1. Introduction

8-MOP is a furanocoumarin found in herbs like *Radix Angelicae Biseratae* and a drug used clinically as a photochemotherapeutic agent to treat psoriasis and vitiligo (Bethea et al., 1999; Parrish et al., 1976). Chronic use of psoralens with UVA therapy (PUVA), is the standard therapy for the patients of psoriasis. However, long-term oral administration of 8-MOP with PUVA associated with several cases of liver injury, with marked increases in the levels of the serum liver enzymes alanine -aminotransferase (ALT) and aspartate -aminotransferase (AST) (Bjellerup et al., 1979; Pariser and Wyles 1980). In addition, in the 16th issue of *Adverse Drug Reaction Information Bulletin,* the State Food and Drug Administration of China reported 158 cases of adverse reaction with use of the Zhuangguguanjie pill (a traditional Chinese medicine including *Radix Angelicae Biseratae* and other 11 medicinal materials) characterized by cholestatic liver injury. 8-MOP listed as a possible toxic compound contributing to the hepatotoxity of the Zhuangguguanjie pill. Even though some cases have been reported, the underlying mechanisms of 8-MOP's hepatotoxicity have not been fully studied.

Multidrug resistance 3 P-glycoprotein (MDR3/ABCB4/MDR2 for

Abbreviations: MDR3, multidrug resistance 3 P glycoprotein; 8-MOP, 8-methoxypsoralen; SD, Sprague-Dawley; PC, phosphatidylcholine; PUVA, psoralens plus UVA therapy; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; ABC, ATP-binding cassette; PFIC3, progressive familial intrahepatic cholestasis type 3; ALP, alkaline phosphatase; γGGT, γ-glutamyl transpeptidase; TBIL, total bilirubin; TBA, total bile acid; NIH, National Institute of Health; DBIL, direct bilirubin; GSH, glutathione; H & E, hematoxylin and eosin; DAB, 3,3′-diaminobenzidine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; BCA, bicinchoninic acid; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; ANOVA, analysis of variance; PL, phospholipids; CYP7A1, cholesterol 7 alpha-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP8B1, sterol 12-alpha-hydroxylase; FXR, farnesoid X receptor; SHP, heterodimer partner; NTCP, Na^+/taurocholate cotransporting polypeptide; BSEP, bile salt export pump; MRP3, multidrug resistance-associated protein 3; PPARα, peroxisome proliferator-activated receptor α; VER, verapamil; PPREs, peroxisome proliferator response elements

* Corresponding authors at: Jiangsu Key Laboratory of Drug Screening, China Pharmaceutical University, Nanjing 210009, China.
E-mail addresses: lyzhang@cpu.edu.cn (L. Zhang), wangtao1331@126.com (T. Wang).

http://dx.doi.org/10.1016/j.tox.2017.05.011
Received 5 April 2017; Received in revised form 3 May 2017; Accepted 16 May 2017
Available online 24 May 2017
0300-483X/

humans, and often mdr2 for rats and mice), which is encoded by the ATP-binding cassette sub-family B member 4 (ABCB4) gene, is a 1279-amino acid transmembrane protein that belongs to the ATP-binding cassette (ABC) transporter family. Genetic mutations of human MDR3 can result in a wide spectrum of phenotypes from progressive familial intrahepatic cholestasis type 3 (PFIC3) to adult cholestatic liver disorders (Jacquemin 2001). One of the theories behind drug-induced liver injury (DILI) is through the functional disruption of MDR3 (He et al., 2015; Yoshikado et al., 2013, 2011). This type of drug-induced liver injury is characterized by elevations in alkaline phosphatase (ALP), serum γ-glutamyl transpeptidase (γ-GGT), and total bilirubin (TBIL) levels. Here, we first report the role of MDR3-mediated phospholipids efflux in 8-MOP-induced hepatotoxicity. Furthermore, alternations in biliary phospholipid levels are often accompanied by disturbances in bile acid homeostasis.

Given the available clinical reports, 8-MOP is one of the potential compounds that can induce cholestatic liver injury. In the present study, we investigated common toxicity criteria of SD rats administered with 8-MOP for 28 days. It was observed that 8-MOP administration induces cholestatic liver injury with marked increases in serum total bile acid (TBA), ALT, γGGT, and TBIL levels. A prominent decrease in biliary phospholipids output was also observed and was closely related with the down-regulation of MDR3. Knowing the physiological importance of billiary phospholipids, we further explored the role of MDR3-mediated phospholipids efflux due to 8-MOP *in vitro* experiments.

2. Materials and methods

2.1. Drugs, chemicals and antibodies

8-MOP (CAS number 298-81-7, purity > 98%) and Verapamil (CAS number 38176-02-2, purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were of high-analytical grade and commercially available. The following antibodies were used in this study: cytokeratin7 (CK7, ab181598) were purchased from Abcam, E-cadherin (sc-7870), PPARα (sc-9000), NTCP (sc-98485), CYP7A1 (sc-25536), BSEP (sc-74500) and β-actin (sc-69879) were purchased from Santa Cruz Biotechnology, FXR (bs-5528R) was purchased from Bioss Inc., MDR3 (LS-B5729) was purchased from LifeSpan Biosciences.

2.2. Animals and treatment

All experiments were approved by the Animal Ethics Committee of China Pharmaceutical University and the Laboratory Animal Management Committee of Jiangsu Province. Thirty female SD rats aged 8 weeks, weighing 200–220 g were purchased from the Laboratory Animal Center of Zhejiang University (Zhejiang, China). The animals were housed under standard condition and provided with food and water *ad libitum*. Animals were dosed orally with 200 or 400 mg/kg of 8-MOP for 28 consecutive days, while the control group was treated with 0.5% carboxymethylcellulose solution. The doses choice was based on a previous study (Lewis, 2004). 8-MOP was suspended in a 0.5% carboxymethylcellulose solution (control solution) before daily administration. Thirty female rats (each group with 10 animals) were dosed orally with 0, 200 or 400 mg/kg of 8-MOP for 28 consecutive days. All of the animals were acclimatized to the laboratory for 1 week before the experiments. On the 14th and 28th day, blood samples were collected for biological evaluation. After the last blood collection, rats were anesthetized with urethane (1 g/kg, ip) for bile collection for 2 h as described by Wang et al. (2014). Then, rats were sacrificed, and liver sections were collected. The study protocols were conducted in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

2.3. Biochemistry of serum and bile constituents

Serum ALT, γGGT, ALP, TBIL, direct bilirubin (DBIL) and TBA were determined using an HITACHI7080 Automatic Clinical Analyzer (Tokyo, Japan). The contents of biliary bile acids, TBIL and DBIL were determined by a method similar to that used for attaining the serum biochemistry. The concentration of glutathione (GSH) in the bile was analyzed using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The biliary phospholipids were measured by enzymatic methods using Phospholipids C test (Wako Pure Chemicals, Osaka, Japan).

2.4. Histological analysis

The liver samples were fixed with 10% formalin in phosphate-buffered saline for 24 h. After staining with hematoxylin and eosin (H & E), the sections were examined by a senior pathologist in a blind manner.

2.5. Immunohistochemistry

For immunohistochemistry, paraffin-embedded sections were used after a microwave-based antigen retrieval technique with a ChemMate Envision kit (Dako, Denmark). Sections were then incubated overnight at 4 °C with a primary antibody against E-cadherin or CK7 (dilution 1:200). Immunoperoxidase staining was developed using a 3,3′-diaminobenzidine (DAB) chromogen (Dako, Denmark). Slides were counterstained with hematoxylin, and the stained tissues were examined by light microscopy.

2.6. Cell culture and cell viability assay

The human liver cell line L02 is an immortalized non-tumor cell line derived from normal liver tissue and expressing a distinct ultrastructure compared to hepatic carcinoma cells, they are well-differentiated and considered to be an *in vitro* model of nonmalignant liver (Jin et al., 2009; Tian et al., 2012) (China Cell Culture Center, Shanghai) was cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 U/ml streptomycin in a humidified environment at 37 °C with 5% CO_2. For the cell viability assay, cells were seeded in 96-well plates at a density of 3000 cells/well. After treatment with 8-MOP (1–100 μM), DMSO or docetaxel (10 nM, positive control) for 48 h, cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.

2.7. Localization of fluorescent PC

L02 cells plated on glass-bottom dishes at 80% confluency (NEST Biotechnology, Nanjing, China) could variably form bile canaliculi-like structures between adjacent cells. After treatment with DMSO or 8-MOP (100 μM) for 48 h, L02 cells were labeled with 1 μM NBD-PC (Avanti Polar Lipids, USA) following a modified method (Ghonem et al., 2014). Fluorescent images were obtained by using laser scanning confocal microscopy (FV1000, Olympus, Japan) in a blind manner.

2.8. Real-time quantitative PCR

Total RNA was extracted using TRIzol (Vazyme Biotech, Nanjing, China) and the RNeasy kit according to the manufacturer's instructions, including a DNase digestion step. Total RNA was subjected to cDNA synthesis using PrimeScript RT Master Mix (Takara Biotechnology, Dalian, China). PCR was performed in a volume of 20 μL containing 10 μL of SYBRGreen Supermix (Vazyme Biotech, Nanjing, China), 1 μL of cDNA, 7 μL of RNase/DNase-free water and 500 nM each primer. The primer pairs used for PCR were listed in Table 1. Gene expression was evaluated by ΔΔCT method, using GAPDH (for rat genes) and β-actin