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Promising toxicological biomarkers for the diagnosis of liver injury types: Bile acid metabolic profiles and oxidative stress marker as screening tools in drug development

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ABSTRACT

Promising biomarkers were identified in adult male Crl:CD (SD) rats for the screening of new chemical entities for their potential to cause liver injury. We examined the serum biochemistry, liver histopathology, and bile acid profiles by LC-MS/MS, and the mRNA expression of transporters and CYPs by an RT-PCR after the following treatments to male Crl:CD (SD) rats: (a) bile duct ligation (BDL); (b) a single oral dose of 150 mg/kg α-naphthylisothiocyanate (ANIT); and (c) repeated oral doses of a novel pyrrolidinecarboxylic acid derivative (abbreviated as PCA) at 30, 300, and 1000 mg/kg. The serum total bile acid levels and bilirubin concentrations were found to be elevated in all of the groups. However, the bile acid component profiles of the PCA group differed significantly from BDL and ANIT models: deoxycholic acid, lithocholic acid, and sulfated bile acids were upregulated in a dose-dependent manner only in the PCA group. In addition, the PCA group demonstrated high levels of hepatic heme oxygenase-1 expression, whereas the profiles of the mRNA levels of the hepatic transporters and CYPs of all groups were found to be similar. The histopathological findings, for both the BDL and ANIT groups, were of bile duct hyperplasia, hepatocyte degeneration and necrosis. In contrast, only bile duct hyperplasia and hepatocyte degeneration were observed in the PCA group, even at a lethal dose. These results indicated that PCA induced a cholestatic condition and the increase of oxidative stress markers implies that this will also lead hepatocellular injury. In conclusion, the serum bile acid components and sulfated bile acid levels, and the expression of oxidative stress markers could provide information that aids in the diagnosis of liver injury type and helps to elucidate the mechanisms of hepatotoxicity. These findings can be extrapolated into our clinical investigation. The analysis of these crucial biomarkers is likely to be a useful screening tool in the lead optimization phase of drug discovery.

1. Introduction

Drug-induced liver injury has been one of the major causes of failure in the drug discovery stage and the withdrawal of drugs from the marketplace. There are two general types of liver injury: hepatocellular injury and cholestasis – a mixed type of both forms of injury also occurs. The hepatocellular injury type refers to a process that primarily involves the hepatocytes. A hepatocellular injury usually results in the elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with little or no elevation of alkaline phosphatase (ALP). Cholestasis is often divided into two categories: intrahepatic and extrahepatic. Extrahepatic cholestasis occurs outside the liver. It can be caused by gallstones or tumors of the common bile duct. Bile duct ligation (BDL) is often used as the rodent model for this condition. Intrahepatic cholestasis is caused by physiological and pathological factors. Alpha-naphthyl isothiocyanate (ANIT) is a chemical used in rodents to model human intrahepatic cholestasis. It is known to cause cholestasis by injuring the bile duct epithelial cells. The liver injuries caused by BDL and ANIT are evidenced by elevated serum

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total bile acid (TBA) levels, bilirubin (BIL), ALP, and finally through increased AST activity. Clinically, the levels of ALP and ALT have been used for the diagnosis of cholestasis, hepatocellular injury, and mixed-type damage [1]. While it seems possible to distinguish between hepatocellular injury and cholestasis using biochemistry and histopathology, difficulties are often encountered in the early stage of drug discovery in determining whether the liver damage caused by a new chemical entity should be classified as simple cholestasis or as mixed-type damage associated with hepatocellular injury. Aside from the basic biochemistry parameters, the bile acid components are one of the most sensitive markers of liver damage. They have been used for the clinical diagnosis of hepatobiliary diseases since the 1970s [2,3].

Bile acids are synthesized and conjugated by hepatic microsomal, mitochondrial, and lysosomal enzymes [4]. In humans, cholic acid (CA) and chenodeoxycholic acid (CDCA) are the major primary bile acids synthesized via the classic pathway. In rodents, CDCA is further converted to α- and β-muricholic acid (MCA) in the liver. Thus, MCA is also a primary bile acid in rodents. In the intestine, primary bile acids are deconjugated and converted by the microflora to secondary bile acids, mainly deoxycholic acid (DCA) and lithocholic acid (LCA). Physiologically, these bile acids are conjugated with taurine or glycine and are excreted into the bile. The sulfation of bile acid maintains bile acid homeostasis under pathological conditions. The formation of serum bile acid-sulfates (SSBA) increases during cholestatic diseases. Urine bile acid-sulfate quantification is used, in cases of biliary atresia, to reflect the degree of cholestasis in adults and newborns [5]. The measurement of changes in serum bile acids may therefore indicate the presence, and possibly the nature, of liver and biliary diseases. In clinical practice, both the concentration and composition ratios of serum bile acids are widely used for the diagnosis of hepatic disease [2,3]. We applied the LC-MS/MS method, to the determination of the primary and secondary bile acids and some of their conjugates – the perturbations of which are assumed to have toxicological implications.

Cholestasis induced alterations in hepatic uptake and in excretory transporter expression, which may contribute to the impaired excretion of biliary constituents in humans and animals. In humans, such alterations have been shown to reduce the basolateral uptake systems (NTCP and SLCO2), to preserve canalicular export pumps for bile salts and bilirubin (BSEP and MRP2), and to increase the levels of canalicular MDR P-glycoproteins (MDR1, MDR3) and the basolateral efflux pump, MRP3 [6]. Similar profiles have been demonstrated in a mouse BDL model [7], and in rat BDL and ANIT models [8]; however, other reports have demonstrated the elevation of Bsep and Mrp2 in a mouse ANIT model [9]. Farnesoid X receptor (FXR) and pregnane X receptor (PXR) are hepatic nuclear receptors that regulate gene transcription. Bile acids are endogeneous ligands of both FXR and PXR [10]. Once activated by bile acids, FXR downregulates Ntcp and the bile acid-synthesizing enzyme Cyp7a1 [11]; PXR increases the expression of Cyp enzymes and transporters involved in the metabolism and the elimination of potentially toxic chemicals from the body. PXR and FXR are two negative feedback mechanisms that reduce hepatic bile acid concentrations [9,10].

PCA, (2*S*,5*S*)-5-[(5-{[2-(difluoromethoxy)-4-isopropylbenzyl]oxy}-2,3-dihydro-1*H*-indol-1-yl)carbonyl]-1-methylpyrrolidine-2-carboxylic acid (Fig. 1), is a new chemical entity being investigated in relation to autoimmune diseases. The significant elevation of TBA and D-BIL levels was observed in rats, after the 4-day repeated administration of this compound at doses of up to 300 mg/kg/day. A histopathological analysis revealed that while there were no significant changes in the hepatocytes (including necrosis), there was slight proliferation of bile duct cells. In addition, the expressions of

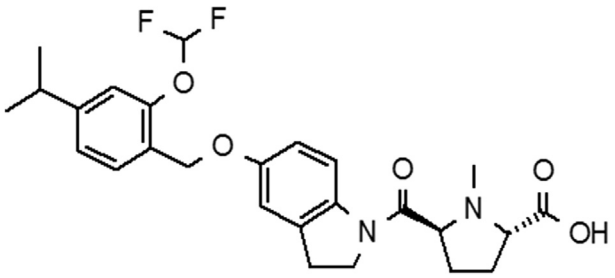

Fig. 1. The chemical structure of a novel pyrrolidinecarboxylic acid derivative (PCA).

Ho-1 and other oxidative stress marker genes were found to be markedly elevated in liver homogenate (unpublished data). Accordingly, some critical factors associated with oxidative stress, in addition to simple cholestasis, were suspected to have occurred in response to the administration of this compound and further investigation will be needed to characterize the types of liver injury that are associated. This may be achieved by an additional study involving the administration of higher doses of PCA. This study therefore aimed to determine the level of perturbation of the serum bile acid component profiles, the gene expression of hepatic enzymes and transporters by the contribution of PXR and FXR, in addition to serum biochemistry and liver histopathology. Consequently, we aimed to provide diagnostic information that would allow for the differentiation of the liver injury types, to identify the mechanisms of liver injury in response to PCA, and to compare these findings with the models of typical cholestasis. These investigations are expected identify the distinct biomarkers of hepatic injury and this approach could be utilized in the lead optimization phase as a screening tool for the evaluation of new chemical entities.

2. Experimental procedures

2.1. Chemicals and reagents

ANIT was purchased from Sigma–Aldrich (St. Louis, MO). PCA was synthesized in Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Reagents for measuring serum ALT, AST, ALP, TBA, T-BIL, D-BIL, lactate dehydrogenase (LDH), and serum sulfated bile acids (SSBA) were obtained from Kainos Laboratories Inc (Tokyo, Japan). CA, CDCA, DCA, LCA, tauro-cholic acid (TCA), and glyco-cholic acid (GCA) were purchased from Sigma–Aldrich. α-muricholic acid (α-MCA), β-muricholic acid (β-MCA), taouro-α-mulicholic acid (T-α-MCA), taouro-β-mulicholic acid (T-β-MCA) were purchased from Steraroids, Inc. (Newport, Rhode Island). Cholic-2,2,4,4-d4 acid (CA-d4), chenodeoxycholic-2,2,4,4-d4 acid (CDCA-d4), deoxycholic-2,2,4,4-d4 acid (DCA-d4), lithocholic-2,2,4,4-d4 acid (LCA-d4), taurocholic –2,2,4,4-d4 acid (TCA-d4), and glycocholic-2,2,4,4-d4 acid (GCA-d4) were purchased from Taiyo Nippon Sanso Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Animal treatment

Adult male Crl:CD (SD) rats weighing 180–200 g were purchased from Charles River Laboratories (Yokohama, Kanagawa, Japan). Three rats were housed per cage with *ad libitum* access to food and water. All animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society. The experimental protocols were approved by the Daiichi Sankyo Laboratory Animal Care and Use Committee. A single dose of ANIT