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Chemical characteristics for optimizing CYP2E1 inhibition

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ABSTRACT

Cytochrome P450 2E1 (CYP2E1) expression and activity in the liver is associated with the degree of liver damage in patients with alcoholic steatohepatitis (ASH) as well as non-alcoholic steatohepatitis (NASH). CYP2E1 is known to generate reactive oxygen species, which leads to oxidative stress, one of the hallmarks of both diseases. Apart from ROS, toxic metabolites can be formed by CYP2E1 metabolism, further potentiating liver injury. Therefore, CYP2E1 is implicated in the pathogenesis of ASH and NASH.

The aim of this study was to determine the chemical characteristics of compounds that are important to inhibit CYP2E1. To this end, structurally related analogs that differed in their lipophilic, steric and electronic properties were tested. In addition, homologues series of aliphatic primary alcohols, secondary alcohols, aldehydes, ketones and carboxylic acids were tested. It was found that inhibition of the CYP2E1 activity is primarily governed by lipophilicity. The optimal log $D_{7.4}$ (octanol/water distribution coefficient at pH 7.4) value for inhibition of CYP2E1 was approximately 2.4. In the carboxylic acids series the interaction of the carboxylate group with polar residues lining the CYP2E1 active site also has to be considered.

This study sketches the basic prerequisites in the search for inhibitors of CYP2E1, which would strengthen our therapeutic armamentarium against CYP2E1 associated diseases, such as ASH and NASH.

1. Introduction

Alcoholic steatohepatitis (ASH) is a common liver disorder characterized by steatosis and inflammation seen in patients that abuse alcohol. Its exact prevalence is unknown, but it is estimated to be present in 10–35% of hospitalized alcoholics [1]. Patients with ASH have a poor prognosis; up to 50% of patients develop cirrhosis. Abstinence from alcohol does not guarantee full recovery; only in 27% of patients histological normalization is found, whereas in 18% cirrhosis develops and in the remaining patients ASH persists [2].

Nonalcoholic steatohepatitis (NASH) is a liver disorder with histological similarity to ASH, seen in patients with no history of alcohol abuse (maximal 2 units per day for men and 1 unit per day for women). It is often associated with the metabolic syndrome and obesity [3–5]. NASH is diagnosed in 2–3% of the general adult population in developed countries [6]. In NASH, 15–25% of patients will eventually develop cirrhosis [7] due to persistent inflammation in which oxidative stress plays an important role [8].

Both ASH and NASH have in common that the expression and activity of the enzyme cytochrome P450 2E1 (CYP2E1) are up-regulated [9–12]. CYP2E1 is the main cytochrome P450 of the microsomal ethanol oxidizing system (MEOS) and has a role in the metabolism of xenobiotics. It accepts a broad variety of substrates, including ethanol, ketones and fatty acids [12]. These substrates accumulate in ASH and NASH, which could explain the induction of the enzyme [12]. Several studies have observed an association between the increase in CYP2E1 expression and activity in the liver and the degree of liver damage in patients with ASH and NASH [13–15].

CYP2E1 is known to generate reactive oxygen species (ROS) [12,16,17]. Superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are formed during the catalytic cycle of CYP2E1 [11,18,19]. In the pathogenesis of both ASH and NASH, there is a central role for the production of ROS and the induction of oxidative stress and subsequent liver damage. It was previously shown that CYP2E1 activity

Abbreviations: ASH, alcoholic steatohepatitis; NASH, nonalcoholic steatohepatitis; CYP2E1, cytochrome P450 2E1; NADP+, nicotinamide adenine dinucleotide phosphate; TCA, tri-chloro-acetic acid; NFDM, non-fat dry milk.

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correlates with ethanol induced liver damage and lipid peroxidation [20]. Therefore CYP2E1 activity is implicated in the pathogenesis of these diseases. Besides ROS, CYP2E1 can also form other toxic metabolites, further potentiating liver injury [21].

Because previous studies have suggested that the production of ROS and the formation of toxic metabolites by cytochrome P450 2E1 are related to its monooxygenase activity [11,18,19,22], inhibition of the CYP2E1 monooxygenase activity might reduce the oxidative stress caused by CYP2E1.

The aim of this study was to determine the impact of lipophilic, steric and electronic properties of compounds on the inhibition of CYP2E1 activity. This will give the basis prerequisites for inhibitors of CYP2E1, which might be used in the treatment of both ASH and NASH, diseases still lacking effective pharmacotherapeutic treatment.

2. Materials and methods

2.1. Chemicals

4-Nitrophenol, 4-nitrocatechol, phenol, 4-chlorophenol, 3,4-dichlorophenol, 2,4-dichlorophenol, 4-(trifluoromethyl)phenol, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP+), D-glucose-6-phosphate, tri-chloroacetic acid (TCA), primary alcohols, secondary alcohols, 2-ketones, aldehydes and carboxylic acids (all up to a chain length of eight carbon atoms) were obtained from Sigma–Aldrich (St. Louis, USA). Magnesiumchloride, di-potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Anti-CYP2E1 antibodies for Western Blot were obtained from Millipore (Temecula, USA), horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, USA) and non-fat dry milk (NFDM) was obtained from Biorad (Veenendaal, the Netherlands). Anti-CYP2E1 antibodies were kindly provided by Prof. Dr. M. Ingelman-Sundberg (Karolinska institute).

2.2. Preparation of rat liver microsomes

Lewis rats were treated to induce CYP2E1 in the liver. Therefore, the rats received drinking water that contained 1% (v/v) acetone ad libitum for seven days. Twenty four hours before sacrifice, the food was removed to further induce CYP2E1 in the liver [22]. The rats were anaesthetized with CO_2/O_2 (ratio 2/3) and decapitated. The study was approved by the institutional ethical review board on experiments with laboratory animals.

The livers were isolated and rinsed in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 22.8 mM potassium chloride and 3.4 mM EDTA. The livers were homogenized and centrifuged at 10,000× g for 20 min at 4 °C. The supernatant was centrifuged at 65,000× g for 60 min at 4 °C. The supernatant was removed and the remaining pellet was resuspended in buffer. This solution was centrifuged at 1,00,000× g for 50 min at 4 °C. The supernatant was removed and the pellet was resuspended in buffer to obtain 2 g liver per ml buffer. This corresponded to 15 mg protein/ml buffer. The microsomes that were obtained in this way were stored at −80 °C until use.

2.3. Western blot

Western Blot was performed to confirm the induction of CYP2E1. Equal amounts of protein from CYP2E1 induced samples and from control samples were separated by SDS-polyacrylamide gel electrophoresis (4–15% TGX gel, BioRad) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare) using the Criterion blotting system (Biorad). Protein concentrations were determined with the use of bicinchoninic acid according to Smith et al. [23]. Membranes were blocked with 5% NFDM. NFDM was diluted in washing buffer (0.05% Tween-20 in Tris Buffered Saline). After blocking, membranes were incubated overnight with anti-CYP2E1 antibody diluted 1:1000 in 5% NFDM in washing buffer. Horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 5% NFDM in washing buffer was used for detection of immunoreactive proteins by chemiluminescence (Thermo Scientific).

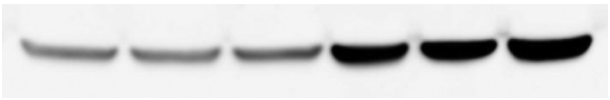

Fig. 1. Western blot of CYP2E1. Western blot of rat CYP2E1 in microsomes of rats that were treated to induce CYP2E1 (samples 1–3) compared to control (samples 4–6).

2.4. Determination of CYP2E1 activity

4-nitrophenol was used to determine CYP2E1 activity. This substrate is converted to 4-nitrocatechol by CYP2E1. Concentrations of 4-nitrocatechol formed and 4-nitrophenol remaining were measured by HPLC as described by Elbarbry et al. [24] with some modifications.

A reaction mixture of 175 μl was prepared containing a certain 4-nitrophenol concentration and a NADPH generating system (consisting of 3.3 mM $MgCl_2$, 3.3 mM glucose-6-phosphate, 1.3 mM NADP+ and 0.4 units/ml glucose-6-phosphate dehydrogenase) with or without an inhibitor dissolved in a 50 mM potassium-phosphate buffer (pH 7.4).

The reaction mixture and rat liver microsomes were pre-incubated in separate tubes at 37 °C for 5 min. After 5 min, 25 μl of the rat liver microsomes were added to the reaction mixture and the tube was incubated at 37 °C for 4 min. The reaction was stopped by adding 100 μl of 10% TCA to the tube and vortexing the tube. After centrifuging at 12,000× g for 4 min, the supernatant was injected into the HPLC (reversed phase Altima™ C18 column, mobile phase 30% acetonitrile and 70% milliQ with 0.1% TFA) to determine the concentrations 4-nitrophenol and 4-nitrocatechol. HPLC flow rate was 1 ml/min and the run time was 5 min. The UV-absorbance was measured at 350 nm CYP2E1 activity was also investigated in control samples and in CYP2E1 induced samples in

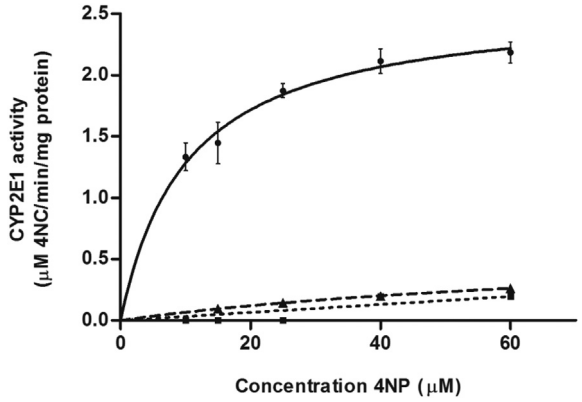

Fig. 2. CYP2E1 activity. Michaelis–Menten curve of CYP2E1 activity measured by the conversion of 4-nitrophenol (4NP) into 4-nitrocatechol (4NC) in rat microsomes treated to induce CYP2E1 (uninterrupted line), control microsomes (interrupted line) and CYP2E1 induced microsomes in the presence of CYP2E1 antibodies (dotted line). The results are expressed as mean ± SEM, n = 2.