Drug Metabolism and Pharmacokinetics 30 (2015) 82–88

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular article

UDP-glucuronosyltransferase (UGT) 1A1 mainly contributes to the glucuronidation of trovafloxacin☆

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ARTICLE INFO

Article history:
Received 23 June 2014
Received in revised form
2 September 2014
Accepted 4 September 2014
Available online 28 September 2014

Keywords:
Trovafloxacin
UDP-Glucuronosyltransferase
UGT1A1
Acyl-glucuronide
Hepatotoxicity

ABSTRACT

Identification of drug-metabolizing enzyme(s) responsible for the metabolism of drugs is an important step to understand not only interindividual variability in pharmacokinetics but also molecular mechanisms of metabolite-related toxicity. While it was reported that the major metabolic pathway of trovafloxacin, which is an antibiotic, was glucuronidation, the UDP-glucuronosyltransferase (UGT) isoform(s) responsible for the trovafloxacin glucuronidation has not been identified yet. In the present study, among the functional human UGT members, UGT1A1, UGT1A3, and UGT1A9 exhibited higher trovafloxacin acyl-glucuronidation activities. While other UGT members such as UGT1A8, UGT2B7, and UGT2B15 showed glucuronidation activity toward trovafloxacin, the metabolic velocity was extremely low. In human liver microsomes, trovafloxacin acyl-glucuronidation followed the Hill equation with S_{50} value of 95 μM, V_{max} value of 243 pmol/min per mg, and a Hill coefficient of 2.0, while the UGT1A1-expressing system displayed Michaelis–Menten kinetics with a substrate inhibition, with K_m value of 759 μM and V_{max} value of 1160 pmol/min per mg. In human liver microsomes prepared from poor metabolizers (UGT1A1*28/*28), significantly reduced trovafloxacin acyl-glucuronide formation activity was observed, indicating that UGT1A1 mainly, while other UGT members such as UGT1A3 and UGT1A9 partially, contributes to the glucuronidation of trovafloxacin.

1. Introduction

UDP-glucuronosyltransferases (UGTs) catalyze glucuronidation of endogenous and exogenous compounds by transferring glucuronic acid from a co-substrate, UDP-glucuronic acid, to substrates [1]. Human UGTs are super-family enzymes and are classified into four subfamilies, UGT1, UGT2, UGT3, and UGT8, on the basis of evolutionary divergence [2,3]. Glucuronidation of drugs is largely mediated by functional UGT1 and UGT2 members, such as UGT1A1, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 [4]. While the contribution of the recently identified UGT8 member to drug metabolism is limited [5], it has been reported that UGT3A1 and UGT3A2 can metabolize various endogenous and exogenous compounds using UDP *N*-acetylglucosamine, UDP-glucose, and UDP-xylose, rather than UDP-glucuronic acid, as a sugar donor [6,7].

Identification of drug-metabolizing enzyme(s) responsible for the metabolism of drugs is an important step to understand interindividual variability in pharmacokinetics. This is because inhibition of the enzyme by a co-administered drug or its genetic deficiency can cause an increased drug concentration that can further result in the development of adverse reactions. These alterations of drug concentration are especially significant when the drug is solely metabolized by a single drug-metabolizing enzyme. SN-38 is an active metabolite of an anti-cancer agent, irinotecan [8]. It has been reported that SN-38 is mainly metabolized by UGT1A1 and therefore, individuals who carry a genetic polymorphism of UGT1A1 have a higher risk of developing clinical toxicity due to increased blood concentration of SN-38 [9]. Thus, the Food and Drug Administration has strongly encouraged that drug-metabolizing enzyme(s) responsible for the metabolism of drug candidates be determined. As certain metabolites such as an acyl-glucuronide are reactive and are possibly associated with the development of immunotoxicity or cytotoxicity [10], identification of drug-metabolizing enzymes responsible for the metabolism of drugs is also important to understand molecular mechanisms of drug- and metabolite-induced toxicity.

Abbreviations: UGT, UDP-glucuronosyltransferase; HPLC, high performance liquid chromatography; AIC, Akaike's information criterion.

☆ Funding for this work was provided in part from a Kitasato University Research Grant for Young Researchers (R.F.).

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http://dx.doi.org/10.1016/j.dmpk.2014.09.003

Trovafloxacin is an antibiotic that was released on the market in 1998 [11]. This promising agent, however, was withdrawn from the market relatively soon after its release due to the risk of hepatotoxicity, including acute liver failure. Currently, the mechanism underlying trovafloxacin-induced hepatotoxicity still remains to be solved. While trovafloxacin was metabolized to its acyl-glucuronide, *N*-acetyltrovafloxacin, and trovafloxacin sulfate, the glucuronidation was the main metabolic pathway of trovafloxacin, as the majority of the metabolites in serum and urine were glucuronide [12]. Because accumulating evidence indicates that the chemical reactivity of acyl glucuronide can be associated with drug-induced toxicity [13], formation of trovafloxacin acyl-glucuronide might be related to the onset of trovafloxacin-induced hepatotoxicity. Meanwhile, the UGT isoforms responsible for trovafloxacin acyl-glucuronidation have not yet been determined. Identification of the UGT isoforms will further enable investigation of trovafloxacin-induced hepatotoxicity by UGT isoforms-expressed cell-based cytotoxic assays [14].

In the present study, trovafloxacin acyl-glucuronidation in human liver and small intestine microsomes, as well as in mouse liver microsomes, was analyzed to obtain the kinetic constants. The UGT isoforms responsible for the trovafloxacin acyl-glucuronidation were determined using UGT isoforms-expressing systems. To further analyze the contribution of UGT1A1 to the metabolism of trovafloxacin, trovafloxacin acyl-glucuronidation was determined in human liver microsomes derived from UGT1A1*1/*1, UGT1A1*1/*28, and UGT1A1*28/*28 carriers.

2. Materials & methods

2.1. Chemicals and reagents

UDP-glucuronic acid and alamethicin were purchased from Sigma–Aldrich (St Louis, MO, USA). Trovafloxacin was purchased from Wako Pure Chemical (Osaka, Japan). Human and mouse liver microsomes, human small intestine microsomes, and UGT Supersomes were obtained from BD Gentest (Woburn, MA, USA). Rabbit and rat liver microsomes were purchased from XenoTech (Kansas City, KS). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

2.2. Enzyme assays

Trovafloxacin acyl-glucuronide formation was determined as follows. A typical incubation mixture (200 μl of total volume) contained 50 mM Tris–HCl (pH 7.4), 4 mM $MgCl_2$, 5 mM UDPGA, 50 μg/ml alamethicin, 0.05–0.2 mg/ml proteins, and trovafloxacin. The reaction was initiated by the addition of UDPGA after a 3-min preincubation at 37 °C. After incubation at 37 °C for 60 min, the reaction was terminated by addition of 200 μl of cold acetonitrile. After removal of the protein by centrifugation at 12,000 g for 5 min, a 50-μl portion of the sample was subjected to HPLC.

2.3. HPLC conditions

Glucuronide was determined by the HPLC system with an LC-10AD pump (Shimadzu, Kyoto, Japan), an FP-2020 fluorescence detector (JASCO, Tokyo Japan), a SIL-10A autosampler (Shimadzu), an SLC-10A system controller (Shimadzu) and a Mightysil RP-18 GP column (4.6 × 150 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The mobile phases were 18% acetonitrile containing 20 mM phosphoric acid. The flow rate was 1.0 ml/min. Glucuronide was detected with a fluorescence detector at 280 nm excitation and 450 nm emission. Quantification of trovafloxacin glucuronide was carried out according to the method in our previous study [15]. Briefly, the incubation mixture including 2 μM trovafloxacin and 0.8 mg/ml rabbit liver microsomes, 50 mM Tris–HCl (pH 7.4), 4 mM $MgCl_2$, 5 mM UDPGA, and 50 μg/ml alamethicin was incubated at 37 °C for 0 h and 3 h. The increase of HPLC peak area of the trovafloxacin glucuronide was compared with decrease of the peak area of the parent compound. As the relationship between the peak area of trovafloxacin glucuronide and the amount of trovafloxacin glucuronide was determined, the trovafloxacin glucuronide in reaction mixture was quantified by measuring the peak area of glucuronide. As shown in Supplementary Fig. 1, we quantified the peak that was specifically observed when trovafloxacin was incubated with human liver microsomes in the presence of UDP-glucuronic acid. The retention times of trovafloxacin glucuronide and trovafloxacin were 2.4 and 14.1 min, respectively. When we treated the reaction mixture with beta-glucuronidase, we observed that the peak of trovafloxacin glucuronide in the HPLC analysis disappeared.

2.4. Data analysis

When kinetics of drug metabolism follows a simple Michaelis–Menten equation, the relationship between substrate concentration and velocity can be described by the following equation (eq. (1)):

$$V = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

where V is the initial velocity of the metabolic reaction, S is the substrate concentration, V_{max} is the maximum rate of metabolism, and K_m is the Michaelis constant, which is defined as the substrate concentration at 1/2 the maximum velocity. While the clearance is substrate concentration-dependent, the rate is constant when the substrate concentration is much smaller than K_m, providing the parameter, intrinsic clearance (CL_{int}) (eq. (2)):

$$CL_{int} = \frac{V_{max}}{K_m} \quad (2)$$

When substrate inhibition was observed, the data were analyzed by eq. (3):

$$V = \frac{V_{max}}{(1 + (K_m/S) + (S/K_{si}))} \quad (3)$$

where K_{si} is the constant describing the substrate inhibition interaction.

For sigmoidal kinetics, kinetic parameters were obtained by the Hill equation (eq. (4)):

$$V = \frac{V_{max} \times S^n}{{S_{50}}^n + S^n} \quad (4)$$

where S_{50} is the substrate concentration showing the 1/2 V_{max} and n is the Hill coefficient.

Kinetic data were also analyzed using Eadie–Hofstee plots. Furthermore, goodness of fit to kinetic models was assessed by calculation of Akaike's information criterion (AIC) values. AIC values of each model were compared and the best-fit model was determined.

3. Results

3.1. Trovafloxacin glucuronidation in human liver and small intestine microsomes

To determine the kinetic constants of trovafloxacin glucuronidation in human liver microsomes, trovafloxacin (2–500 μM)