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Functional characterization of human and cynomolgus monkey UDP-glucuronosyltransferase 1A1 enzymes

Nobumitsu Hanioka [a,*], Natsuko Tanabe [a], Hideto Jinno [b], Toshiko Tanaka-Kagawa [b], Kenjiro Nagaoka [a], Shinsaku Naito [c], Akiko Koeda [d], Shizuo Narimatsu [a]

[a] Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan
[b] Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[c] Research and Development Center, Otsuka Pharmaceutical Factory, Inc., 115 Kuguhara, Tateiwa, Muya-cho, Naruto, Tokushima 772-8601, Japan
[d] Department of Metabolism and Analytical Research, Ina Research Inc., 2148-188 Nishiminowa, Ina, Nagano 399-4501, Japan

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ABSTRACT

Aims: UDP-glucuronosyltransferase 1A1 (UGT1A1) plays important roles in the glucuronidation of various drugs and endogenous substances. Cynomolgus monkeys are regarded as experimental animals closer to humans in studies on safety evaluation and biotransformation for drug development. In this study, the similarities and differences in the enzymatic properties of UGT1A1 between humans and cynomolgus monkeys were precisely identified.
Main methods: Human and cynomolgus monkey UGT1A1s (humUGT1A1 and monUGT1A1, respectively) were cloned, and the corresponding proteins were heterologously expressed in insect cells. The enzymatic properties of UGT1A1 proteins were characterized by kinetic analysis of 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), estradiol at 3-hydroxy position (E-3OH) and 7-ethyl-10-hydroxycamptothecin (SN-38) glucuronidation.
Key findings: There were no significant differences in the levels of kinetic parameters for 7-HFC, E-3OH and SN-38 glucuronidation between humans and cynomolgus monkeys in both enzyme sources of liver microsomes and recombinant UGT1A1s. 7-HFC and E-3OH glucuronidation by human liver microsomes exhibited biphasic and sigmoidal kinetics, respectively, whereas the kinetics by cynomolgus monkey liver microsomes fitted the typical Michaelis–Menten model. SN-38 glucuronidation by human and cynomolgus monkey liver microsomes exhibited autoactivation kinetics. In recombinant UGT1A1 enzymes expressed in insect cells, the kinetics of 7-HFC, E-3OH and SN-38 glucuronidation fitted the substrate inhibition (7-HFC glucuronidation) or Hill equation (E-3OH and SN-38 glucuronidation), and each glucuronidation showed the same kinetic profile between humans and cynomolgus monkeys.
Significance: These findings suggest that the enzymatic properties of human and cynomolgus monkey UGT1A1 enzymes are very similar.

Introduction

UDP-glucuronosyltransferases (UGTs) are membrane-bound enzymes that are localized inside the endoplasmic reticulum, and catalyze the conjugation of various endogenous substances (e.g. bile acids, bilirubin and steroids) and xenobiotics (e.g. drugs and environmental toxicants) to glucuronide (Ritter 2000; Tukey and Strassburg 2000). Consistent with their broad substrate profiles, UGTs are known to exist as a superfamily of independently regulated enzymes. The UGT superfamily is divided into many subfamilies on the basis of evolutionary divergence. Among the human UGT superfamily, two families (UGT1 and UGT2) and three subfamilies (UGT1A, UGT2A and UGT2B) are predominantly involved in glucuronidation (Mackenzie et al. 1997, 2005). The *UGT1A* gene is localized on chromosome 2q37 and encodes proteins with unique amino-terminal domains and identical carboxyl-terminal domains, which are formed from the alternate mRNA splicing of unique first exons with common exons 2–5. In contrast, *UGT2A* and *UGT2B* genes are clustered on chromosome 4q13 and individual UGT proteins are encoded by unique genes with six exons (Mackenzie et al. 1997, 2003, 2005; Guillemette 2003). Each UGT exhibits unique substrate and tissue specificities (Ritter 2000; Tukey and Strassburg 2000; Kiang et al. 2005).

UGT1A1 is expressed in the liver, bile duct, stomach and colon, and plays an important role in the detoxification of neurotoxic bilirubin by conjugating it with glucuronic acid for excretion in bile (Iyanagi et al. 1998; Tukey and Strassburg 2000). A reduced level of UGT1A1 activity has been reported to be associated with unconjugated hyperbilirubinemia (Crigler–Najjar syndrome and Gilbert's syndrome) (Mackenzie et al.

* Corresponding author. Tel./fax: +81 86 251 7943.
E-mail address: hanioka@pharm.okayama-u.ac.jp (N. Hanioka).

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1997; Tukey and Strassburg 2000; Guillemette 2003). UGT1A1 also catalyzes the glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of an anticancer drug, irinotecan (CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy camptothecin), to form inactive SN-38 glucuronide (Iyer et al. 1998; Hanioka et al. 2001). Furthermore, wide interindividual variability in SN-38 glucuronide formation in hepatic tissues is known and has been shown to correlate with a *UGT1A1* genetic factor (Iyer et al. 1998, 1999; Guillemette 2003); therefore, *UGT1A1* polymorphisms have been regarded to be one of the most important factors for irinotecan efficacy and toxicity (Ando and Hasegawa 2005; Hasegawa et al. 2006; Ando et al. 2007).

Many UGT enzymes have been suggested to be expressed in hepatic and/or extrahepatic tissues of mammals, including humans, monkeys, rats and mice (Mackenzie et al. 1997, 2005; Tukey and Strassburg 2000), and the cDNAs of several isoforms have been cloned (http://som.flinders.edu.au/FUSA/ClinPharm/UGT/udgpa.html). In general, nonhuman primates, such as rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*), are regarded as experimental animals closer to humans in studies on safety evaluation and biotransformation for drug development; therefore, examination of the functional characterization of monkey UGT enzymes is an important aspect of drug metabolism research. Vallée et al. (2001) cloned cynomolgus monkey UGT1A1 (monUGT1A1) cDNA encoding an ortholog of human UGT1A1 (humUGT1A1), and qualitatively determined the catalytic activities toward estrogens of recombinant monUGT1A1 expressed in HEK293 cells; however, detailed kinetic analyses of xenobiotic glucuronidation by the recombinant enzyme were not included. Furthermore, there has been no report on the quantitative function characterization of human and cynomolgus monkey UGT1A1s using both enzyme sources of liver microsomes and recombinant enzymes.

The purpose of this study was to precisely identify the similarities and differences in the enzymatic properties of UGT1A1 between humans and cynomolgus monkeys. To achieve this, human and cynomolgus monkey UGT1A1 enzymes were heterologously expressed in insect cells, and the enzymatic properties were subsequently examined by kinetic analyses for the glucuronidation of 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), estradiol at 3-hydroxy position (E-3OH), and SN-38.

Materials and methods

Materials

Three individual human liver microsomes (two men, 41 and 55 years old; one woman, 56 years old) and rabbit anti-human UGT1A1 antibody were purchased from BD Biosciences (San Jose, CA, USA). Three male cynomolgus monkey livers (4 years old, 2.7–2.9 kg) were supplied by Ina Research Inc. (Nagano, Japan). Cynomolgus monkey liver microsomes were prepared as described previously (Hanioka et al. 2006). The use of human and cynomolgus monkey livers for this study was approved by the ethics review boards of Okayama University. *Hin*dIII was purchased from Takara Bio (Shiga, Japan); pGEM-T vector was from Promega (Madison, WI, USA); pFastBac1 vector, Bac-to-Bac Baculovirus Expression System and *Spodoptera frugiperda* (Sf9) cells were from Invitrogen (Carlsbad, CA, USA); 7-HFC, 7-HFC glucuronide and E-3OH glucuronide were from Sigma-Aldrich (St. Louis, MO, USA); and estradiol was from Wako Pure Chemical Industries (Osaka, Japan). SN-38 and SN-38 glucuronide were supplied by Yakult Honsha (Tokyo, Japan). UDP-glucuronic acid was purchased from Nacalai Tesque (Kyoto, Japan); peroxidase-conjugated goat anti-rabbit immunoglobulin was from Zymed Laboratories (South San Francisco, CA, USA); and Enhanced ChemiLuminescence Plus was from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other chemicals and reagents used were of the highest quality commercially available.

Expression of recombinant UGT1A1 enzymes

humUGT1A1 cDNA was amplified by polymerase chain reaction (PCR) from humUGT1A1 cDNA cloned into pcDNA3.1 vector (Jinno et al. 2003) as a template using the forward primer 5′-AAGCTTAAAAAAATGGCTGTGGAGTCCCA-3′ and the reverse primer 5′-AAGCTTTCAATGGGTCTTGGATTTGTGGG-3′. The *Hin*dIII sites (underlined letters) were introduced at the 5′-end of the start codon and the 3′-end of the stop codon to facilitate subcloning into pFastBac1 vector. The cDNA encoding monUGT1A1 was amplified by nested PCR from cynomolgus monkey liver single-strand cDNA, prepared as described previously (Hanioka et al. 2006). The nucleotide sequences of the forward and reverse primers used were 5′-ATGGCTGTGGAGTCCCAAGGCAGACATC-3′ and 5′-CTCGAGTCTCAATGGGTCTTGGATTTGTGG-3′ for first PCR, and AAGCTTAAAAAAATGGCTGTGGAGTCCCA-3′ and 5′-AAGCTTTCAATGGGTCTTGGATTTGTGGG-3′ for second PCR. The PCR products of hum UGT1A1 and monUGT1A1 cDNAs were directly introduced into pGEM-T vector using TA cloning, and sequenced in both forward and reverse directions to confirm that there were no PCR errors. The cDNA fragments corresponding of humUGT1A1 and monUGT1A1 were cut from the pGEM-T plasmids with *Hin*dIII, and were subsequently subcloned into pFastBac1 vector digested with *Hin*dIII. The expression plasmids were sequenced to verify the correct orientation with respect to the promoter (polyhedrin promoter) for pFastBac1 vector.

Recombinant baculovirus carrying humUGT1A1 or monUGT1A1 cDNA was generated using the Bac-to-Bac Baculovirus Expression System according to the manufacturer's protocol. For protein expression, Sf9 cells (2.0×10^8 cells/flask) were infected with recombinant baculoviruses at a multiplicity of infection of 1.0. The cells were harvested at 72 h post-infection, and suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose, 0.1 mM dithiothreitol, 0.1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The cell suspensions were sonicated 20 times with 10-s bursts, following by centrifugation at 105,000 *g* for 60 min to obtain membrane fractions. The resulting pellets were resuspended in 50 mM Tris–HCl (pH 7.4) containing 20% glycerol and stored at −80 °C until use. Total protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Recombinant UGT1A1s (1.0 μg protein) as well as liver microsomes (20 μg protein) of humans and cynomolgus monkeys were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970) and electrotransferred to a polyvinylidene fluoride sheet, as described by Towbin et al. (1979). The sheet was incubated with rabbit anti-human UGT1A1 antibody (diluted at 1:5000) as the primary antibody and then with peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted at 1:5000) as the secondary antibody. Immunoreactive proteins were visualized with chemifluorescence (Enhanced ChemiLuminescence Plus), and band densities were relatively determined with ImageJ v1.42 (National Institute of Health Sciences, Bethesda, MD, USA).

Assay for UGT1A1-dependent enzymatic activities

Glucuronidation activities toward 7-HFC, E-3OH and SN-38 were determined by high-performance liquid chromatography as described previously with some modifications (Hanioka et al. 2001; Alkharfy and Frye 2002; Brill et al. 2006). The incubation mixture contained 7-HFC (1.0–200 μM), estradiol (0.5–100 μM) or SN-38 (0.5–100 μM) as a substrate, liver microsomes or recombinant UGT1A1s, 10 mM $MgCl_2$ and 5 mM UDP-glucuronic acid in a final volume of 500 μL of 50 mM Tris–HCl buffer (pH 7.4). The protein concentrations of liver microsomes and recombinant UGT1A1s were 10 and 50 μg/mL for 7-HFC and SN-38 glucuronidation assays, and 50 and 50 μg/mL for the E-3OH glucuronidation assay. The substrates were dissolved in