Toxicology in Vitro 26 (2012) 1286–1293

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

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

Highly variable pH effects on the interaction of diclofenac and indomethacin with human UDP-glucuronosyltransferases ☆

Hongbo Zhang [a,b], Anne Soikkeli [c], Ari Tolonen [d,1], Timo Rousu [d,2], Jouni Hirvonen [c], Moshe Finel [a,*]

[a] Center for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland
[b] Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, Finland
[c] Division of Pharmaceutical Technology, Faculty of Pharmacy, University of Helsinki, Finland
[d] Novamass Ltd., Medipolis Center, Kiviharjuntie 11, FI-90220, Oulu, Finland

ARTICLE INFO

Article history:
Available online 14 January 2012

Keywords:
Acyl migration
Diclofenac
Indomethacin
UDP-glucuronosyltransferase

ABSTRACT

In vitro glucuronidation assays of diclofenac and indomethacin at pH 7.4 are biased by the instability of the glucuronides due to acyl migration. The extent of this acyl migration may be reduced significantly by performing the glucuronidation reaction at pH 6.0. Testing the human UDP-glucuronosyltransferases (UGTs) of subfamilies 1A, 2A and 2B at pH 7.4 revealed that UGT1A10, UGT2B7 and UGT2B17 are the most active enzymes in diclofenac glucuronidation, while the highest indomethacin glucuronidation rates (corrected for relative expression levels) were exhibited by UGT2A1, UGT1A10 and UGT2B7. Interestingly, lowering the reaction pH to 6.0 increased the activity of many UGTs, particularly UGT1A10, toward both drugs, even if the rate of 4-methylumbelliferone glucuronidation by UGT1A10 at pH 6.0 was significantly lower than at pH 7.4. On the other hand, UGT2B15 lost activity upon lowering the reaction pH to 6.0. UGT1A6 does not glucuronidate diclofenac and indomethacin. Nevertheless, both drugs inhibit the 1-naphthol glucuronidation activity of UGT1A6 and their inhibition was stimulated by lowering the reaction pH, yielding significantly lower IC_{50} values at pH 6.0 than at pH 7.4. In conclusion, glucuronidation reactions pH affects their outcome in variable ways and could increase the toxicity of drugs that carry a carboxylic acid.

1. Introduction

The UDP-glucuronosyltransferases (UGTs) form a large and important family within the phase II drug metabolism enzymes. UGTs are membrane proteins of the endoplasmic reticulum that catalyze the conjugation of suitable substrates with glucuronic acid from UDP-glucuronic acid (UDPGA) (Wells et al., 2004). The 19 functional human UGTs of subfamilies 1A, 2A and 2B (Mackenzie et al., 2005) are expressed in a tissue-dependent mode. Most of them are mainly expressed in the liver, and part of the hepatic UGTs, such as UGTs 1A6, 1A9 and 2B7, are also expressed in other tissues, including intestine, kidney and other organs (Ohno and Nakajin, 2009; Court et al., 2012). In addition, there are extrahepatic UGTs that are mainly expressed along the intestine, like UGT1A10 (Ohno and Nakajin, 2009; Itäaho et al., 2009), or in the airways, like UGT2A1 (Sneitz et al., 2009; Bushey et al., 2011). Many UGTs can glucuronidate various different compounds with diverse chemical structures, a feature that lead to some overlap in substrate selectivity between individual UGTs. Nevertheless, the individual UGTs clearly differ from each other in the full spectrum of their substrate selectivity.

Glucuronidation frequently eliminates the biological activity of drugs, but acyl glucuronides are known to be reactive and may lead to drug toxicity (Shipkova et al., 2003). These glucuronides are often instable and easily isomerize in a process called "acyl migration" (Faed, 1984), and may cause adverse drug effects, such as drug hypersensitivity reactions, cellular toxicity and hepatotoxicity (Ritter, 2000; Shipkova et al., 2003). Diclofenac and indomethacin are non steroidal anti-inflammatory drugs (NSAIDs) that bear a carboxyl group (Fig. 1). They are eliminated from the body

Abbreviations: 4-MU, 4-methylumbelliferone; ACN, acetonitrile; DMSO, dimethyl sulfoxide; HIM, human intestinal microsomes; HLM, human liver microsomes; LC, liquid chromatography; LC–MS, LC coupled to mass spectrometry; NSAIDs, non-steroidal anti-inflammatory drugs; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

☆ This study was financially supported by the EU FP6 (LIINTOP # LSH-2005-1.2.3-4), by a personal stipend to H.Z. from the University of Helsinki (Grants for Young Researchers 2010) and by the Sigrid Juselius Foundation.

* Corresponding author. Address: Centre for Drug Research, Faculty of Pharmacy, P.O. Box 56, University of Helsinki, FIN-00014 Helsinki, Finland. Tel.: +358 919159193; fax: +358 919159556.
E-mail address: moshe.finel@helsinki.fi (M. Finel).
[1] Current address: Admescope Ltd., Kaitoväylä 1F2, 90570 Oulu, Finland.
[2] Current address: Department of Chemistry, P.O.Box 3000, FIN-90014, University of Oulu, Finland.

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2012.01.005

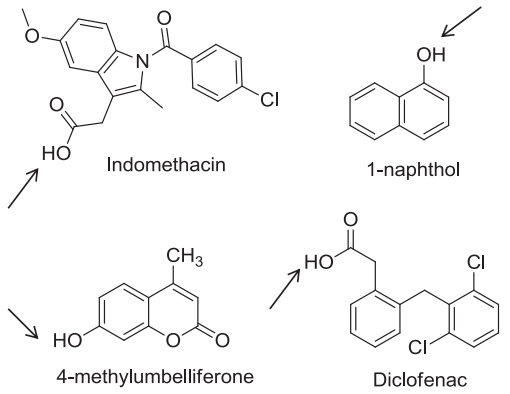

Fig. 1. Structures of the UGT substrates and inhibitors that were used in this study. The arrows indicate the glucuronidation site in each substrate.

through several metabolizing pathways, among which acyl glucuronidation, namely enzymatic conjugation of the carboxylic acid with glucuronic acid, is a major pathway (Tang, 2003 and references therein; Vree et al., 1993). Following acyl glucuronidation, acyl migration may occur in both drugs under physiological conditions (Spahn-Langguth and Benet, 1992), increasing the risk of toxic effects by either drug. For example, it was reported that diclofenac is associated with hepatotoxicity (Walker, 1997) that is likely to be caused by its acyl glucuronide (Tang, 2003).

Acyl migration also complicates the interpretation of glucuronidation analyses, particularly in drugs like diclofenac or indomethacin that also contain amines that might serve as alternative glucuronidation sites (Fig. 1). Hence, it is beneficial to find experimental conditions that stabilize the original acyl glucuronide, but do not lower enzyme activity. Reaction pH in *in vitro* assays may contribute to such analyses since it was previously known that lower pH stabilizes acyl glucuronides (Walker et al., 2007). On the other hand, the reaction pH may affect the protonation state of important amino acids in the enzyme catalytic site (Basu et al., 2004), and/or functional groups on the substrate molecule (Chang et al., 2009). In either way, it can affect the binding affinity of the aglycone to the UGT, leading to changes in the glucuronidation rate or inhibition efficiency of the tested compound.

The glucuronidation of NSAIDs, including diclofenac and indomethacin, by many individual UGTs, has already been tested before and the results demonstrated that several different UGTs can catalyze these reactions (Kuehl et al., 2005; Mano et al., 2007). However, the number of different UGT isoforms that were included in the previous studies have mostly been limited to the commercially-available UGTs and never included all the human UGTs of subfamilies 1A, 2A and 2B.

In addition to being substrates for different UGTs, indomethacin and diclofenac may inhibit some UGTs, even those that do not glucuronidate them and, therefore, are not generally regarded as enzymes that interact with these NSAIDs. UGT1A6 provides a good example for such an enzyme since it does not catalyze diclofenac glucuronidation (Kuehl et al., 2005), but is partly inhibited by it (Uchaipichat et al., 2004; Soikkeli et al., 2011). The inhibition of UGT1A6 by indomethacin and the effect of reaction pH on its inhibition by diclofenac and indomethacin have not been tested before, however.

In this study we have investigated the diclofenac and indomethacin glucuronidation by the 19 individual human UGTs at both pH 6.0 and 7.4. In addition, we have examined the inhibitory effect of both drugs on an enzyme that does not catalyze their glucuronidation, UGT1A6, but appears to bind them. The results reveal interesting findings on both the UGTs and the substrates.

2. Materials and methods

2.1. Materials

1-Naphthol, 4-methylumbelliferone (4-MU), 4-methylumbelliferone glucuronide, diclofenac, indomethacin, alamethicin and UDPGA (uridine-5-diphosphoglucuronic acid, triammonium salt) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pooled human liver microsomes (HLM), human intestinal microsomes (HIM) and recombinant UGT2B15 "supersomes" were bought from BD Biosciences (Franklin Lakes, NJ, USA). The other 18 recombinant human UGTs were produced in our laboratory using baculovirus-infected insect cells (Kurkela et al., 2007; Sneitz et al., 2009). The tetra-His antibodies, from QIAGEN (Hilden, Germany), were used to determine the relative expression levels of individual recombinant UGTs (Kurkela et al., 2007). All the solvents were HPLC grade.

2.2. Glucuronidation assays

Aliquots of HIM, HLM and UGTs were thawed on the day of the assays. The reaction mixture, total volume of 100 µl, contained 50 mM phosphate buffer (pH 5.5–8.0), 10 mM $MgCl_2$, 5 mM UDPGA, UGT-enriched insect membrane, HIM or HLM, and dimethylsulfoxide (DMSO) for the glucuronidation assay, depending on the compound solubility (Zhang et al., 2011). In the experiments with recombinant UGTs, 1% DMSO was used for 4-MU glucuronidation assays, whereas 5% DMSO was used for diclofenac and indomethacin glucuronidation assays. In the case of HIM and HLM, the DMSO concentration was 1% in all the assays, and the reaction mixtures also included 10 µg/ml alamethicin (5% of the total protein concentration in the reaction mixture, Zhang et al., 2011). The reaction conditions for the glucuronidation of 1-naphthol by recombinant UGT1A6 were as recently described (Soikkeli et al., 2011), including the presence of 5 µM 1-naphthol, 2 mM UDPGA and 5% DMSO in the reaction mixture. All the glucuronidation reactions in the present study were carried out in 1.5 ml plastic tubes and no multi-well plates were used.

The enzyme source, substrates and inhibitors, were pre-incubated at 37 °C for 5 min in the reaction mixture in the absence of UDPGA, followed by reaction initiation with the addition of UDPGA. The glucuronidation reactions were carried out at 37 °C and the incubation times were 60 min when the substrate was 4-MU, diclofenac and indomethacin, but 30 min when 1-naphthol glucuronidation was assayed. The reactions were terminated by adding 10 µl of 4 M perchloric acid followed by cooling on ice for 10 min and subsequent centrifugation at 13,000 rpm for 10 min in a bench top centrifuge. The supernatants from the latter centrifugations were subjected to analyses by either LC–MS, or LC with UV absorbance or LC with fluorescence detection.

The effect of pH on UGT1A10 and UGT2B15-catalyzed glucuronidation of 100 µM indomethacin, diclofenac and 4-MU (0.2 mg/ml protein in assays of indomethacin and diclofenac, and 0.05 mg/ml protein in the case of 4-MU) was first tested at six pH values (5.5, 6.0, 6.5, 7.0, 7.4 and 8.0). Since UGT2B15 did not exhibit indomethacin glucuronidation activity, it was mainly tested with diclofenac and 4-MU. Subsequently, the glucuronidation of indomethacin and diclofenac (100 µM substrate concentration) was examined by all the 19 UGTs of subfamilies 1A, 2A and 2B, as well as HLM and HIM, at both pH 6.0 and 7.4. The protein concentration in the recombinant UGTs tests was 0.5 mg/ml, while in the HIM and HLM assays it was 0.2 mg/ml. To compare individual UGTs more meaningfully, the glucuronidation rates were also corrected (normalized) according to the enzyme expression level in all the samples of the recombinant UGTs (with the exception of UGT2B15), using UGT1A10 as a reference. The relative expression