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SHORT COMMUNICATION

Inhibitory potential of nonsteroidal anti-inflammatory drugs on UDP-glucuronosyltransferase 2B7 in human liver microsomes

Yuji Mano · Takashi Usui · Hidetaka Kamimura

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Abstract
Objective A number of nonsteroidal anti-inflammatory drugs (NSAIDs) are subject to glucuronidation in humans, and UDP-glucuronosyltransferase (UGT) 2B7 is involved in the glucuronidation of many NSAIDs. The objective of this study was to identify a NSAID with potent inhibitory potential against UGT2B7 using liquid chromatography with tandem mass spectrometry (LC-MS/MS).
Methods A rapid screening method for detecting the inhibitory potential of various drugs against UGT2B7 was established using a LC-MS/MS system. The effects of nine NSAIDs (acetaminophen, diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and salicylic acid) against UGT2B7-catalyzed 3′-azido-3′-deoxythymidine glucuronidation (AZTG) were investigated in human liver microsomes (HLM) and recombinant human UGT2B7.
Results Mefenamic acid inhibited AZTG most potently, with an IC_{50} value of 0.3 μM, and its inhibition type was not competitive. The IC_{50} values for diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and niflumic acid against AZTG were 6.8, 178, 51, 40, 23, and 83 μM, respectively, while those for acetaminophen and salicylic acid were >100 μM. The IC_{50} values for NSAIDs against AZTG in recombinant human UGT2B7 were similar to those obtained in HLM.
Conclusion The method established in this study is useful for identifying drugs with inhibitory potential against human UGT2B7. Among the nine NSAIDs investigated, mefenamic acid had the strongest inhibitory effect on UGT2B7-catalyzed AZTG in HLM. Thus, caution might be exercised when mefenamic acid is coadministered with drugs possessing UGT2B7 as a main elimination pathway.

Keywords AZT · UGT2B7 · Inhibition · NSAIDs · Human

Y. Mano (✉) · T. Usui · H. Kamimura
Drug Metabolism Research Laboratories, Astellas Pharma Inc.,
1-8, Azusawa 1-Chome, Itabashi-ku,
Tokyo 174-8511, Japan
e-mail: yuuji.mano@jp.astellas.com

Introduction

Glucuronidation catalyzed by UDP-glucuronosyltransferase (UGT) is one of the major steps in the metabolism of endogenous substances and xenobiotics. It is generally acknowledged that UGT2B7 is an important isozyme in the glucuronidation of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) [1]. In fact, pharmacokinetics of drugs were altered by inhibition of glucuronidation. Diflunisal caused a 2- to 5-fold increase in indomethacin concentration in plasma, which led to side effects in the central nervous system and gastrointestinal tract [2], and UGT2B7 is partly involved in the glucuronidation of indomethacin [3]. In addition, plasma levels of 3′-azido-3′-deoxythymidine (AZT) were elevated with concomitant administration of valproic acid and fluconazole [4, 5]. When a drug with a narrow therapeutic window whose main elimination pathway is UGT2B7 is concomitantly dosed with drugs with strong potential to inhibit UGT2B7, inhibition of UGT2B7 may lead to an adverse reaction. Thus, the search for drugs that potently inhibit UGT2B7 is important from a clinical point of view.

A number of NSAIDs are metabolized into glucuronides in humans. Although UGT isozymes generally show relatively low substrate specificity, UGT2B7 is known to be responsible for the glucuronidation of naproxen [6],

diclofenac [7], and salicylic acid [8]. Other than these, UGT2B7 also glucuronidates diflunisal [1], ketoprofen [3], and indomethacin [3]. In contrast, UGT1A1, 1A6, and 1A9 are responsible for glucuronidation of acetaminophen [9], and UGT1A1 is mainly involved in that of niflumic acid [10].

A large fraction of NSAIDs are excreted as glucuronides in humans. In the case of diflunisal, ketoprofen, and naproxen, for example, approximately 40%, 74%, and 57% of the doses, respectively, are excreted as glucuronides [11]. Further, respective ratios for indomethacin, mefenamic acid, niflumic acid, and salicylic acid are at least 60%, 30%, 35%, and 30%, respectively [11–14]. Given this, it is likely that relatively high doses of NSAIDs that result in systemic exposure may inhibit UGT2B7 activity.

Although the glucuronidation of morphine-3OH and 6OH is mainly catalyzed by UGT2B7, it has also been shown that UGT2B7 catalyzes AZT glucuronidation (AZTG) more selectively [15]. This is supported by the fact that UGT2B7 protein levels are more closely correlated with AZTG in individual human liver microsomes (HLM) and the fact that the K_m value against AZTG in recombinant human UGT2B7 is similar to that in HLM [15]. In addition, AZT is eliminated primarily via glucuronidation, and some drugs are known to inhibit AZTG in vivo [4, 5]. Thus, AZTG is a more useful model for evaluating drug interaction via UGT2B7.

In this study, the inhibitory potencies of nine NSAIDs, including acetaminophen, diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and salicylic acid against UGT2B7-catalyzed AZTG in HLM were investigated using a liquid chromatography with tandem mass spectrometry (LC-MS/MS) method. As a large number of NSAIDs have an affinity for UGT2B7, the identification of drugs inducing potent inhibition of this enzyme is clinically important.

Materials and methods

Chemicals and reagents

Acetaminophen, AZT, diclofenac, indomethacin, ketoprofen, mefenamic acid, naproxen, and niflumic acid were purchased from Sigma (St. Louis, MO, USA). Diflunisal and salicylic acid were purchased from ICN Biomedicals (Aurora, OH, USA) and Wako Pure Chemicals (Osaka, Japan), respectively. AZT glucuronide was obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Pooled and individual HLM were purchased from Xenotech (Kansas City, KS, USA) and the baculovirus-infected insect cells expressing human UGT2B7 (recombinant UGT2B7) were from BD Sciences (Bedford, MA, USA). All other chemicals were of analytical grade.

In vitro glucuronidation

First, in order to evaluate the correlation between the AZTG and UGT2B7-catalyzed morphine glucuronidations, AZT (500 μM) was incubated with microsomes from 16 individual human livers (0.1 mg protein/ml) for 20 min in a final volume of 0.25 ml Tris-HCl buffer (50 mM, pH 7.5) containing 8 mM $MgCl_2$, 25 μg/ml alamethicin, 10 mM saccharic acid 1,4-lactone, and 5 mM uridine diphosphate glucuronic acid (UDPGA). Next, to evaluate the inhibitory potential of the NSAIDs, reaction mixtures containing AZT were incubated with pooled HLM in the presence and absence of nine NSAIDs (acetaminophen, diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and salicylic acid). The concentrations of the NSAIDs were as follows: 1–50, 50–1,000, 20–500, 10–400, 0.1–10, 20–500, and 20–1,000 μM for diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, naproxen, and niflumic acid, respectively. Each reaction mixture contained HLM (0.1 mg protein/ml), 500 μM AZT, and the inhibitors in a final volume of 0.25 ml Tris-HCl buffer (50 mM, pH 7.5). The AZT concentration (500 μM) was below its K_m value (1.4 mM; [5]). In a separate study, the ways mefenamic acid inhibits AZTG were also evaluated in HLM. AZT (100–3,000 μM) was incubated with pooled HLM under the same conditions mentioned above in the absence and presence of mefenamic acid (0.25 μM). Finally, the inhibitory potential of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid against AZTG was also assessed with recombinant UGT2B7. The protein and substrate concentrations were 0.1 mg protein/ml and 500 μM, respectively. The microsomal protein concentration and incubation time were set to yield linear glucuronide formation. Incubation time (10–30 min) and protein content (0.05–0.1 mg protein/ml) were investigated in order to optimize conditions for AZTG in HLM and recombinant UGT2B7.

After preincubating the reaction mixture at 37°C for 5 min, the reaction was started by adding UDPGA (5 mM). After 20- and 30-min incubation periods in HLM and recombinant UGT2B7, respectively, the reaction was terminated by adding 0.05 ml acetonitrile to precipitate the protein. Next, 10% formic acid (0.01 ml) and 0.02 ml of the 5-μg/ml deuterium isotope solution of AZT glucuronide (used as an internal standard) were added. The samples were centrifuged at 1,870× g for 5 min to obtain the supernatant. Aliquots (25 μl) were injected into the LC-MS/MS system. The substrate and inhibitors were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1% (v/v).

Assay

The peak areas of AZT glucuronide and its internal standard were analyzed using the LC-MS/MS system. A TSQ7000 triple quadrupole mass spectrometer with an atmosphere pressure ionization (API) source (Thermo Finnigan, San Jose, CA, USA) was used. The API source was fitted with an electrospray ionization inlet for ionizing the analytes. Nitrogen was employed as the sheath and auxiliary gases, with pressures of 80 psi and 40 U, respectively. The electron spray voltage was set at 4.5 kV, and the heated capillary temperature was maintained at 350°C. In the negative ion mode, AZT glucuronide and its internal standard were subjected to selected reaction monitoring, which transmits molecular ions at m/z 442 and 445, respectively. These ions were subjected to collision-activated dissociation with argon (2.0 mtorr) at 20 eV, then the product ions were monitored at m/z 442 and 445 for AZT glucuronide and the internal standard, respectively. Chromatographic separation was achieved using a Capcellpak UG80 column (4.6 mm×150 mm, 5 μm, Shiseido, Tokyo, Japan), the mobile phase was composed of 0.1% formic acid:acetonitrile (7:3, v/v), and the flow rate was 0.5 ml/min. The standard curve for the AZT glucuronide was linear from 0.1 to 10 μM, and the correlation coefficient was >0.99. The accuracy and precision of the back-calculated values at each concentration were less than 15%.

Data analysis

The correlation between AZTG and morphine glucuronidation at the 3OH and 6OH positions was determined using the Pearson's moment method and Prism Ver. 3.02 (Graph Pad Software, San Diego, CA, USA). Morphine-3OH and 6OH glucuronidation activity in individual human liver microsomes was obtained from the manufacturer. The IC_{50} value of each inhibitor, estimated from the inhibition of AZTG in the presence of inhibitors at respective concentrations, was estimated by fitting Eq. 1 to the data using Prism Ver. 3.02, where I is the inhibitor concentration.

$$\%\,\text{of control} = 100 \times \frac{IC_{50}}{I + IC_{50}} \quad (1)$$

The K_i value of mefenamic acid was determined using Eq. 2.

$$\frac{CL_{int}}{CL_{int}'} = 1 + \frac{I}{K_i} \quad (2)$$

where CL_{int} and CL_{int}' represent the intrinsic clearance of AZTG in HLM in the absence and presence of mefenamic acid, respectively.

Results

Correlation study

The glucuronidation velocity of AZT in microsomes from individual human livers significantly correlated with that of morphine at both the 3OH and 6OH positions (p <0.0001). The coefficient of determination (r^2) was 0.81 and 0.86 for morphine-3OH and 6OH glucuronidation, respectively (Fig. 1).

Inhibition of NSAIDs in HLM

The inhibitory effects of acetaminophen, diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and salicylic acid against UGT2B7-catalyzed

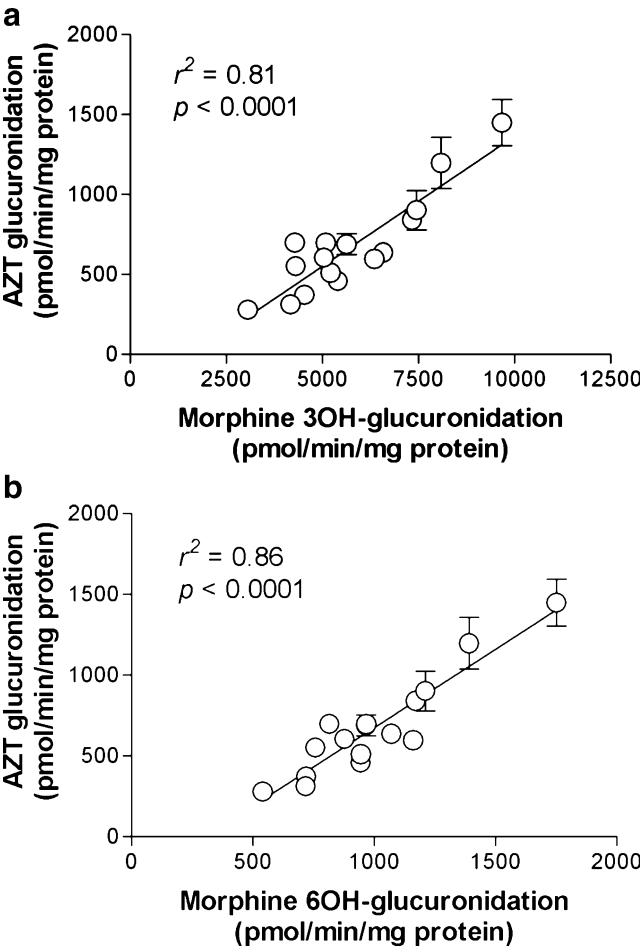

Fig. 1 Correlation analysis between 3′-azido-3′-deoxythymidine glucuronidation (AZTG) and morphine glucuronidation at the 3OH and 6OH positions in microsomes from 16 individual human livers. AZT (500 μM) was incubated with microsomes (0.1 mg protein/ml) for 20 min. The x axis represents the activity for morphine glucuronidation at the 3OH (**a**) and 6OH (**b**) positions, and the y axis represents AZT glucuronidation activity. Each incubation was performed in triplicate, and the data represent the mean ± standard deviation (SD)

AZTG were investigated in HLM. Mefenamic acid had the strongest inhibitory effect against AZTG, with an IC_{50} value of 0.3±0.04 μM [mean ± computer-calculated standard error (SE)]. The IC_{50} values of diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and niflumic acid were 6.8±0.5, 178±37, 51±2.2, 40±1.1, 23±1.6, and 83±8.4 μM, respectively, while acetaminophen (1,000 μM) and salicylic acid (100 μM) showed no inhibition. The type of inhibition exerted by mefenamic acid against AZTG in HLM was investigated. In the absence of mefenamic acid, AZTG exhibited Michaelis-Menten kinetics with K_m and V_{max} values of 2,225± 212 μM and 2,155±110 pmol/min/mg protein, respectively. In the presence of mefenamic acid, the estimated K_m and V_{max} values were altered to 3,094±425 μM and 1,675± 138 pmol/min/mg protein, respectively. The inset graph in Fig. 2 illustrates that the inhibition exerted by mefenamic acid was not competitive, and the K_i value estimate was 0.3 μM.

Inhibition of NSAIDs in recombinant UGT2B7

The inhibitory effect of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid against AZTG was also assessed using recombinant UGT2B7. Diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid inhibited AZTG with IC_{50} values of 3.4±0.18, 122±27, 34±2.9, 24±0.39, 0.093± 0.0088, and 61±4.6 μM, respectively (Fig. 3).

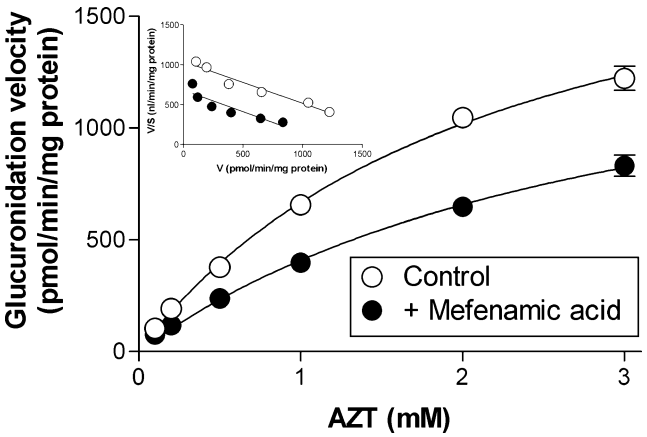

Fig. 2 Inhibitory effects of mefenamic acid on 3′-azido-3′-deoxythymidine glucuronidation (AZTG) in human liver microsomes (HLM). The Eadie-Hofstee plot is represented as an inset. AZT (100–3,000 μM) was incubated in the presence and absence of mefenamic acid (0.25 μM) in HLM (0.1 mg protein/ml) for 20 min. The *x* and *y* axes represent the substrate concentration and AZTG velocity, respectively. Each incubation was performed in triplicate, and the data represent the mean ± standard deviation (SD)

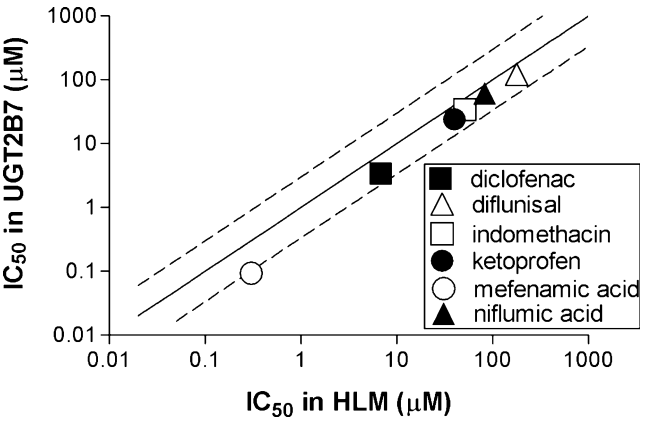

Fig. 3 Comparison of the inhibitory potentials of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid against UDP-glucuronosyltransferase (UGT) 2B7 activity in human liver microsomes (HLM) and recombinant human UGT2B7. 3′-azido-3′-deoxythymidine (AZT) (500 μM) was incubated with HLM and UGT2B7 (0.1 mg protein/ml) for 20 and 30 min, respectively, in the presence or absence of inhibitors. The *x* and *y* axes represent the IC_{50} values in HLM and those in recombinant UGT2B7, respectively. The *solid line* represents a line of unity, whereas *dashed lines* represent ones with a three-fold difference in unity

Discussion

This paper describes the inhibitory potentials of nine NSAIDs against UGT2B7-catalyzed AZTG in HLM. The glucuronidation velocity of AZT in HLM significantly correlated with that of morphine at both the 3OH and 6OH positions (Fig. 1). In addition, a significant correlation between AZTG and the amount of UGT2B7 has been previously described, and only UGT2B4, 2B7, and 2B17 are known to glucuronidate AZT [15]. These findings strongly indicate that AZTG is a probe reaction for UGT2B7.

Among the NSAIDs investigated, mefenamic acid showed the strongest inhibition against UGT2B7, with an IC_{50} value of 0.3 μM. Along with inhibition of UGT2B7, it has been reported that mefenamic acid also inhibits UGT1A1 and 1A9 [16]. The IC_{50} values of diclofenac, diflunisal, indomethacin, ketoprofen, and naproxen against AZTG in HLM were 6.8, 178, 51, 40, and 23 μM, respectively. These values are somewhat lower than those previously reported (59, 200, and 172 μM for diclofenac, indomethacin, and naproxen, respectively) [17–19]. Although the reason for the discrepancy in the IC_{50} values remains to be determined, one possible explanation is the difference in the incubation conditions. Relatively high microsomal concentrations (2.5 and 4.5–9.5 mg protein/ml) were used in the previous reports, while 0.1 mg protein/ml was used in the present study. In addition, incubation times in the previous report were longer than those in this study. High microsomal protein content may increase the nonspecific binding of inhibitors [20], and longer incubation times

may significantly reduce inhibitors' concentration during incubation, both of which may lead to the underestimation of their inhibitory potencies.

The NSAIDs tested in this study also inhibited the glucuronidation of estradiol 3β-glucuronidation in HLM, which is catalyzed mainly by UGT1A1 [21], and the 4-methylumbelliferone glucuronidation in recombinant human UGT1A9 [22]. Niflumic acid, which is mainly glucuronidated by UGT1A1 [10], had the strongest inhibitory effect on UGT1A1 and 1A9 [21, 22]. Despite potent inhibition of UGT1A1 and 1A9, the inhibitory potential of niflumic acid against UGT2B7 in HLM was relatively weak (IC_{50}:83 μM). The IC_{50} values of diclofenac, diflunisal, indomethacin, and niflumic acid on UGT1A1-catalyzed estradiol 3β-glucuronidation were 60.9, 37.8, 51.5, and 22.2 μM, respectively, and those of ketoprofen and naproxen were >100 μM. Comparison with the results of the present study suggests that the affinity of diclofenac, ketoprofen, and naproxen for UGT2B7 is higher than that for UGT1A1, while that of diflunisal and niflumic acid is lower for UGT2B7 than for UGT1A1. As few probe reactions for respective UGT isozymes in HLM have been reported, additional inhibition study on UGT isozymes other than UGT2B7 was not available in this study.

In order to compare the inhibitory potentials of NSAIDs against AZTG in HLM with those on glucuronidation in recombinant UGT2B7, the IC_{50} values of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid in recombinant UGT2B7 were also determined. The IC_{50} values of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid in recombinant UGT2B7 were 3.4, 122, 34, 24, 0.093, and 61 μM, respectively, which are similar to those in HLM (Fig. 3). There are some reports that compare the IC_{50} values for UGT2B7 between HLM and recombinant UGT2B7 [23, 24]. For example, the IC_{50} values of S-flurbiprofen against UGT2B7-catalyzed gemcabene glucuronidation were found to be 60.6 μM in HLM and 27.4 μM in recombinant UGT2B7, and diclofenac was found to inhibit UGT2B7-catalyzed denopamine glucuronidation with IC_{50} values of 41.5 and 24.0 μM in HLM and UGT2B7, respectively.

The IC_{50} values for the NSAIDs investigated in this study are compared with their therapeutic concentrations in order to assess the possible clinical inhibitory potentials against drugs catalyzed mainly by UGT2B7. It has been reported that the therapeutic concentrations of diclofenac, diflunisal, indomethacin, ketoprofen, mefenamic acid, and niflumic acid are 3.6, 36–360, 0.84–8.4, 1.2, 28, and 68 μM, respectively [25, 26]. The percent unbound in plasma is <0.5% in diclofenac, 0.1% in diflunisal, 10% in indomethacin, and 0.8% in ketoprofen, while those in mefenamic acid and niflumic acid have not been reported [26]. Thus, the unbound plasma concentrations of these NSAIDs were calculated as follows: <0.018 μM for diclofenac, 0.036–0.36 μM for diflunisal, 0.084–0.84 μM for indomethacin, and 0.0096 μM for ketoprofen, which are lower than the corresponding IC_{50} values for UGT2B7 in HLM. Although the plasma unbound concentrations of mefenamic acid remain to be determined, it may be possible that given its total plasma levels and the IC_{50} value, mefenamic acid would inhibit UGT2B7 in clinical use.

To summarize, among the nine NSAIDs investigated, mefenamic acid had the strongest inhibitory effects. Thus, caution might be exercised when mefenamic acid is coadministered with drugs possessing UGT2B7 as a main elimination pathway.

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