INHIBITION AND ACTIVE SITES OF UDP-GLUCURONOSYLTRANSFERASES 2B7 AND 1A1.

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(Received June 11, 2002; accepted August 27, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
Two human UDP-glucuronosyltransferases (UGTs), UGT2B7 and UGT1A1, catalyze the glucuronidation of many endo- and xenobiotics. Although UGT1A1 uniquely catalyzes the glucuronidation of the endobiotic, bilirubin, and UGT2B7 uniquely catalyzes the glucuronidation of morphine to both the 3-O glucuronide and the 6-O glucuronide, both catalyze the glucuronidation of the mixed opioid agonist/antagonist buprenorphine with high efficiency. Etonitazenyln, a μ opioid receptor antagonist, was found to inhibit competitively opioid, steroid, and other substrate glucuronidation reactions catalyzed by UGT2B7. Data showing several benzodiazepines and alternative substrates interacting competitively support previous work, which indicates a single binding domain within UGT2B7.

The glucuronidation of xenobiotics and endobiotics represents a major route of disposition for these agents in animals. In this process, UDP-glucuronosyltransferases (UGTs), residing in the endoplasmic reticulum and nuclear membrane of cells, catalyze the conjugation of exogenous and endogenous lipophilic substrates creating metabolites that are more water soluble and readily excreted. Although the role of glucuronidation has generally been considered to be a dominant feature of detoxification, recent studies have shown that certain glucuronide metabolites may display more potent pharmacologic or toxicological activity than the parent compound. For example, morphine-6-glucuronide is a more active analgesic agent than morphine (Shimomura et al., 1971; Osborne et al., 1988; Osborne et al., 1992), and certain estrogen glucuronides induce hepatic toxicity (Vore et al., 1997). Glucuronides may also serve as part of a therapeutic regimen (Gunning et al., 1994; Nolen et al., 1995).

Two UGT gene families have been identified in humans which encode for proteins that catalyze the glucuronidation of xenobiotics and endobiotics (Mackenzie et al., 1997). The human UGT1 gene family is a complex of 12 unique first exons coding for 9 isoforms, each of which share common second through fifth exons encoding for an identical carboxyl terminal moiety for each protein (Ritter et al., 1992). These isoforms, with the exception of UGT1A5, are important in the metabolism of endobiotics (bilirubin and estrogens), tertiary amines, phenols, and flavonoids (King et al., 2000). It is interesting to note that UGT1A1 catalyzes bilirubin glucuronidation, a critical physiological metabolic reaction, and the glucuronidation of many other xenobiotics such as buprenorphine, an opioid agonist/antagonist, and endobiotics such as estrone and 2-hydroxyestrogen catechols.

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This research was supported by National Institute of Health Grant GM26221.

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Chemicals. Losartan was a gift from Merck Laboratories (Rahway, NJ). Morphine, dextrorphan, naltriben, etonitazenyln isothiocyanate, and UDP-[U-14C] glucuronic acid were purchased from Merck Laboratories, Hoffman

1 Abbreviations used are: UGT, UDP-glucuronosyltransferases; zidovudine, 3’-azido-3’-deoxythymidine; HK293 cells, human embryonic kidney 293 cells.
LaRoche (Nutly, NJ), Research Biochemicals International (Natick, MA), TOCRIS Chemicals (Ballwin, MO), and ICN Radiochemicals (Costa Mesa, CA). All other aglycone substrates and inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). The protein assay reagents were purchased from Bio-Rad Labs (Hercules, CA). All other reagents were of the highest purity.

Expression of UGTs in Human Embryonic Kidney 293 Cells. The expression of human UGTs 2B7 (Coffman et al., 1997), 1A1 (King et al., 1996), and 1A6 (King et al., 1999) in HK293 cells has been previously described.

Glucuronidation Assays. The membrane preparation for each of the UGTs expressed in HK293 cells and stored at −80°C was previously described (Battaglia et al., 1994). These preparations were thawed and resuspended in 10 mM Tris-buffered saline (pH 7.4) containing 0.5 mM dithiothreitol. Protein concentrations were determined by the Bio-Rad protein assay.

The glucuronidation of steroids and bilirubin was analyzed using the extraction method previously described (Matern et al., 1994). Opioid glucuronidation was measured using the SepPak method (Puig and Tephly, 1986). Zidovudine glucuronidation was determined by the Pacifici method (Pacifici et al., 1996). All of the other assays were determined by the chromatography method described by Green et al. (1994). Except for the zidovudine glucuronidation assay, all enzyme assay mixtures contained 50 mM Tris buffer, 10 mM MgCl₂, 8.5 mM saccharalactone, 2 mM [¹⁴C]UDP-glucuronic acid (0.1 μCi), the aglycone substrate, and the inhibitor. The zidovudine glucuronidation assay mixtures had all of the same ingredients but included 5 mM UDP-glucuronic acid and 3'-azido-3'-deoxythymidine-2-[¹⁴C] (0.04 μCi). Reaction time and pH were optimized in each case to produce linear and maximal product formation.

The kinetic parameters were calculated by the use of the Enzyme Kinetics program (Trinity Software, Plymouth, NH) and the inhibition constant (Kᵢ) was derived by the Dixon method.

Results

Inhibition of UGT2B7 Activities. UGT2B7 is an important catalyst for the glucuronidation of many important therapeutic agents of diverse chemical structure. Although the substrate reactivity is quite diverse, a previous study using the NMR spectroscopy of an aglycone active site-fusion protein pointed to only one active site (Coffman et al., 2001) for UGT2B7. Studies were performed to explore this further and to determine whether drug-drug interactions were possible by using inhibitors and alternate substrates for UGT2B7. Morphine, one of the morphinan class of opioid substrates, and androsterone, a steroid, were chosen as representative substrates since their glucuronidation is catalyzed by UGT2B7. A number of potential inhibitors of UGT2B7 were tested. One such inhibitor, etonitazenyi, is known as a selective μ opioid antagonist (Burke et al., 1984), which is not glucuronidated by UGT2B7. Figures 1 and 2 show the competitive inhibition of morphine and androsterone by etonitazenyi. The inhibitor constants (Kᵢ) for etonitazenyi were 71 and 141 μM when studied with morphine and androsterone, respectively.

Flunitrazepam and oxazepam are benzodiazepines, which are examples of either a nonsubstrate or poor substrate for UGT2B7 (Cheng et al., 1998; Coffman et al., 1998). Both flunitrazepam and oxazepam inhibited, competitively, morphine glucuronidation with low Kᵢ values of 25 and 63 μM, respectively. Flunitrazepam had previously been shown to inhibit morphine glucuronidation in rat liver microsomes (Thomassin and Tephly, 1990) and catechol estrogen glucuronidation catalyzed by recombinant UGT2B7 (Cheng et al., 1998).

Alternate opioid substrates for UGT2B7 might be expected to inhibit morphine glucuronidation competitively due to their similarity in structure and reactivity with UGT2B7. Naltrexib, a substrate whose Kᵢ and Vₘₐₓ values are low, 25 μM and 460 pmol/min/mg of protein, respectively, inhibited competitively morphine glucuronidation with a Kᵢ value of 13 μM.

Another important substrate for UGT2B7 is zidovudine, a mainstay in the treatment of human immunodeficiency virus and acquired immunodeficiency syndrome infection. This compound is an human immunodeficiency virus-reverse transcriptase inhibitor, which is clinically effective and is converted to 3'-azido-3'-deoxy-5'-glucouronyl-thymidine via the catalytic action of UGT2B7 (Barbier et al., 2000). Competitive inhibition was seen with flunitrazepam, oxazepam, morphee, and naltrexib. The Kᵢ values obtained for flunitrazepam, oxazepam, morphine, and naltrexib were 26, 31, 405, and 9 μM, respectively. The Kᵢ values for naltrexib and morphine were similar to their Kᵢ values when used as substrates, 20 and 300 μM, respectively.

Losartan, an angiotensin II receptor antagonist and another substrate for UGT2B7 with a Kᵢ of 80 μM, was also inhibited competitively by flunitrazepam (Kᵢ, 55 μM).

Inhibition of UGT1A1. UGT 1A1 is considered to be the most important enzyme involved in the disposition of bilirubin. However, UGT1A1 also catalyzes the glucuronidation of a number of other endobiotic and xenobiotic substrates which include the catechol estrogren, 2-hydroxyestrone; the antraquinone, antraflavic acid; and the opioid, buprenorphine. Since the opioid substrate, buprenorphine, is an excellent substrate for both UGT1A1 and UGT2B7, and since etonitazenyi was an effective competitive inhibitor of morphine glucuronidation catalyzed by UGT2B7, it was of interest to determine whether etonitazenyi might inhibit the UGT1A1-catalyzed glucuronidation of buprenorphine. Etonitazenyi competitively inhibited buprenorphine glucuronidation catalyzed by UGT1A1 (Fig. 3) with a Kᵢ value of 5.5 μM, a value approximately 20-fold lower than that found when used as an inhibitor of UGT2B7. In spite of its profound inhibitory effect on buprenorphine glucuronidation, etonitazenyi concentrations above 100 μM were necessary to show any inhibition with 2-hydroxyestrone and antraflavic acid (Table 1) and had little to no effect on bilirubin glucuronidation (Fig. 4).
The assays were performed under the following optimal conditions for androsterone glucuronidation: 37°C for 15 min at pH 7.4. The concentration of UDP-glucuronic acid was 2.0 mM, and the aglycone substrate concentrations are from 5 to 50 μM. The competitive inhibition of androsterone glucuronidation is denoted by the addition of etonitazenyl to the reaction. The data are shown by □, control; ⋄, 50 μM etonitazenyl; and ■, 100 μM etonitazenyl.

Given these results, studies were conducted to test the effect of buprenorphine on bilirubin, 2-hydroxyestrone, and anthraflavic acid glucuronidation. Buprenorphine had little to no inhibitory effect on either bilirubin (Fig. 4) or 2-hydroxyestrone and anthraflavic acid glucuronidation (Table 1).

**Studies on UGT1A6.** Given results obtained with UGT2B7 and UGT1A1, the etonitazenyl inhibition of glucuronidation was considered to be due to interaction with enzyme sites only related to opioid binding or substrates using sites proximate to opioid binding. Inhibition studies were performed with the recombinant human UGT1A6, which does not accept opioid substrates. Etonitazenyl was used as a possible inhibitor with 1-naphthol and 4-methylumbelliferone, two excellent substrates for UGT1A6. As expected, inhibition was not observed with the glucuronidation of either 1-naphthol or 4-methylumbelliferone (data not shown).

**Discussion**

A previous study from this laboratory suggested that the aglycone binding region of UGT2B7 possessed a single site, which could accept a number of chemically divergent substrates (Coffman et al., 2001). The current study supports this idea. $K_i$ values determined for UGT2B7 with substrates such as naloxone, zidovudine, androsterone, and buprenorphine were essentially identical to $K_i$ values obtained for these compounds when they were used to displace morphine binding to the aglycone binding region of the fusion protein used in that study. Furthermore, etonitazenyl inhibition of morphine, androsterone, losartan, and zidovudine glucuronidation was competitive, and $K_i$ values, obtained for etonitazenyl, were comparable irrespective of the substrate studied. Studies using other inhibitors or alternate substrates of UGT2B7 also support the notion that there is one binding domain in UGT2B7. Alternate substrates such as naltriben and morphine, when used as inhibitors, display $K_i$ values similar to their $K_m$ values. Due to the many and diverse substrates for UGT2B7, a major question remains as to whether there might be potential drug-drug interactions, in vivo, involving this protein. This has yet to be answered, but it would seem unlikely, based on the high capacity of UGT2B7 to serve as a catalyst in the glucuronidation of these substrates. Furthermore, no clinical studies have suggested that drug-drug or drug-endobiotic interaction exists.

On the other hand, inhibition studies carried out with recombinant human UGT1A1 suggest that this protein possesses more than one active site. Buprenorphine and bilirubin are two substrates that react with human UGT1A1 with high efficiency. Etonitazenyl, a $μ$-specific opioid antagonist and an inhibitor of UGT2B7, was chosen to see if it inhibited opioid glucuronidation catalyzed by UGT1A1. Etonitazenyl inhibited the UGT1A1-catalyzed glucuronidation of buprenorphine competitively and with high potency ($K_i$, 5.5 μM), a value 20-fold lower than that found for the $K_i$ of etonitazenyl on morphine glucuronidation.
The assays were performed under the following optimal conditions for bilirubin glucuronidation: 37°C for 60 min at pH 7.4. The concentration of UDP-glucuronic acid was 2.0 mM, and the aglycone substrate concentration was 20 μM. The open bars indicate assays performed with etonitazenyl, and the hatched bars indicate assays performed with buprenorphine. The values ± S.D. are from three experiments.

ronidation catalyzed by UGT2B7. Suprisingly, etonitazenyl had no effect on bilirubin glucuronidation at concentrations up to 25 μM and required 100 μM or higher to effect a modest inhibition. To test the idea that UGT1A1 had separate active sites for bilirubin and buprenorphine, buprenorphine glucuronidation was used as an inhibitor of bilirubin glucuronidation. Buprenorphine had no significant inhibitory effect on bilirubin glucuronidation except at extraordinary concentrations. Therefore, it appears that distinct binding regions are involved in the reaction of these substrates with UGT1A1. In related studies, neither etonitazenyl nor buprenorphine significantly inhibited the UGT1A1-mediated glucuronidation of estrogen catechols, 17α-ethinylestradiol, or anthralfavic acid. Previously, we postulated that patients treated with buprenorphine, possibly for opioid dependence, might experience jaundice due to the possible inhibition of bilirubin glucuronidation (King et al., 2000). It is important to point out that the current study suggests that this would be unlikely because of different binding domains within UGT1A1 for buprenorphine and bilirubin.

Etonitazenyl has been shown here to inhibit a wide range of glucuronidation reactions mediated by UGT2B7 and opioid glucuronidation by UGT1A1. It had no effect on UGT1A6-catalyzed glucuronidation of phenols (1-naphthol) or coumarins (4-methylumbelliflorene). Further studies need to be performed with this compound to test its effect on other UGTs, such as UGT1A3 and UGT1A4, which are known to react with amine substrates such as the tertiary amines which are converted to quaternary amine glucuronides (Green and Tephly, 1998).

Acknowledgments. We acknowledge Erik Twait for his valuable assistance in these studies. We also thank Birgit Coffman for her advice in the preparation of this manuscript.

References


