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

GLADYS R. RIOS AND THOMAS R. TEPHLY

Department of Pharmacology, University of Iowa, Iowa City, Iowa

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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 3-0 glucuronide and the 6-0 glucuronide, both catalyze the glucuronidation of the mixed opioid agonist/antagonist buprenorphine with high efficiency. Etonitazenyl, 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. Etonitazenyl also competitively inhibited the glucuronidation of buprenorphine catalyzed by UGT1A1. However, neither etonitazenyl nor buprenorphine inhibited bilirubin glucuronidation except at very high concentrations. Therefore, it is unlikely that buprenorphine therapy for opioid or other drug addiction would influence bilirubin glucuronidation and lead to hyperbilirubinemia. Anthralfavic acid and catechol estrogen glucuronidation, catalyzed by UGT1A1, was also not inhibited by etonitazenyl or buprenorphine. Reactions catalyzed by UGT1A6 were not affected by etonitazenyl. These studies indicate that UGT2B7 has one binding site and that UGT1A1 has two or more binding sites for xenobiotics and endobiotics.

The glucuronidation of xenobiotics and endobiotics represents a major route of disposition for these agents in animals. In this process, UDP-glucuronosyltransferases (UGTs1), 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 pharmacological or toxicological activity than the parent compound. For example, morphine-6-glucuronide is a more active analgesic agent than morphine, and alternative substrates interacting competitively support previous work, which indicates a single binding domain within UGT2B7.

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. The UGT2 gene family codes for proteins which are products of separate genes (Mackenzie and Rodbourn, 1990; Haque et al., 1991). These enzymes are very important in drug metabolism, especially UGT2B7. UGT2B7 has a wide distribution in the human organism and reacts with a wide variety of chemical agents and drugs (King et al., 2000) such as morphine, zidovudine, and nonsteroid anti-inflammatory drugs. It has been shown to exist in many extrahepatic tissues, including brain, and to catalyze the glucuronidation of the neurotransmitter serotonin (King et al., 1999), as well as many opioid drugs. The existence of UGT2B7 in the brain is of great importance in the conversion of morphine to the morphine 6-glucuronide, making the requirement for the transport of this conjugate across the blood-brain barrier and into the brain unnecessary.

The current study was undertaken to study the effects of certain potential inhibitors and alternate substrates on UGTs, especially those known to react with the opioid substrates. Several inhibitors and alternate substrates for UGT2B7 were then studied with UGTs 1A1 and 1A6. The differential inhibition of UGT1A1 catalyzed glucuronidation by these compounds provides evidence for more than one active site in this isoform.

Materials and Methods

Chemicals. Losartan was a gift from Merck Laboratories (Rahway, NJ). Morphine, dextrophan, naltriben, etonitazenyl isothiocyanate, and UDP-[U-14C] glucuronic acid were purchased from Merck Laboratories, Hoffman...
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., 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 (0.1μCi), 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, etonitazenyl, 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 etonitazenyl. The inhibitor constants (Kᵢ) for etonitazenyl 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'-glucuronylthymidine via the catalytic action of UGT2B7 (Barbier et al., 2000). Competitive inhibition was seen with flunitrazepam, oxazepam, morphine, 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 are 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 estrogen, 2-hydroxyestrone; the anthraquinone, anthraflavic acid; and the opioid, buprenorphine. Since the opioid substrate, buprenorphine, is an excellent substrate for both UGT1A1 and UGT2B7, and since etonitazenyl was an effective competitive inhibitor of morphine glucuronidation catalyzed by UGT2B7, it was of interest to determine whether etonitazenyl might inhibit the UGT1A1-catalyzed glucuronidation of buprenorphine. Etonitazenyl 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, etonitazenyl concentrations above 100 μM were necessary to show any inhibition with 2-hydroxyestrone and anthraflavic acid (Table 1) and had little to no effect on bilirubin glucuronidation (Fig. 4).


Ketone, losartan, and zidovudine glucuronidation was competitive, and that study. Furthermore, etonitazenyl inhibition of morphine, androsterone binding to the aglycone binding region of the fusion protein used in for these compounds when they were used to displace morphine and buprenorphine were essentially identical to UGT2B7 with substrates such as naloxone, zidovudine, androsterone, the addition of etonitazenyl to the reaction. The data are shown by /H18554/H9262/M 50 domain in UGT2B7. Alternate substrates such as naltriben and morphine glucuronidation by etonitazenyl.

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 /H18554/H9262/M, 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 values determined for UGT2B7 with substrates such as naloxone, zidovudine, androsterone, and buprenorphine were essentially identical to /K 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 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 values similar to their /K 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, 5.5 µM), a value 20-fold lower than that found for the /K of etonitazenyl on morphine glucuronic acid was 2.0 mM, and the aglycone substrate concentrations are from 5 to 50 µM. The competitive inhibition of androsterone glucuronidation was shown by the addition of etonitazenyl to the reaction. The data are shown by /H18554/H9262/M, 100 µM etonitazenyl.

The competitive inhibition of UGT1A1-mediated buprenorphine glucuronidation by etonitazenyl.

The assays were performed under the following optimal conditions for buprenorphine glucuronidation: 37°C for 20 min at pH 7.0. The concentration of UDP-glucuronic acid was 2.0 mM, and the aglycone substrate concentrations were from 10 to 100 µM. The competitive inhibition of buprenorphine glucuronidation was shown by the addition of etonitazenyl to the reaction. The data are shown by /H18554/H9262/M, 3.125 µM etonitazenyl; /M, 6.25 µM etonitazenyl; /A, 12.5 µM etonitazenyl; and /C, 25 µM etonitazenyl.

TABLE 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>Etonitazenyl (100 µM)</th>
<th>Buprenorphine (100 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aglycone</td>
<td></td>
<td>pmol/min/mg protein</td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>Anthraflavic acid (20 µM)</td>
<td>222 ± 4</td>
<td>204 ± 21</td>
<td>234 ± 6</td>
</tr>
<tr>
<td>2-Hydroxyestrone (25 µM)</td>
<td>197 ± 10</td>
<td>133 ± 13</td>
<td>178 ± 8</td>
</tr>
</tbody>
</table>
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.

Figure 4. The effect of buprenorphine and etonitazenyl on UGT1A1-mediated bilirubin glucuronidation.

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References
Mackenzie PI and Rodbourn L (1990) Organization of the rat UDP-glucuronosyltransferase complex locus UGT1 which encodes human bilirubin, phenol and other UDP-glucuronosyltransferase.