Short Communication

NMR CHARACTERIZATION OF AN S-LINKED GLUCURONIDE METABOLITE OF THE POTENT, NOVEL, NONSTEROIDAL PROGESTERONE AGONIST TANAPROGET

Received December 5, 2005; accepted May 5, 2006

ABSTRACT:
Tanaproget is a potent, first-in-class nonsteroidal progesterone receptor agonist being investigated at Wyeth for use in contraception. A major in vitro and in vivo metabolite of tanaproget formed in humans was initially characterized as a glucuronide of tanaproget. However, whether the glucuronide was linked to the nitrogen or sulfur of the benzoxazine-2-thione group in tanaproget could not be determined by liquid chromatography/mass spectrometry (LC/MS) and LC-tandem mass spectrometry analysis. To obtain additional structural details for this metabolite, additional quantities were generated from rat liver microsomal incubations and purified by high-performance liquid chromatography (HPLC) for NMR analysis. The NMR data for the metabolite confirmed that the glucuronide was covalently bound to either the sulfur or the nitrogen of the benzoxazine-2-thione moiety. The lack of key through-bond (scalar) and through-space (dipolar) one-dimensional (1D) and two-dimensional (2D) NMR couplings and correlations in the metabolite spectra (due primarily to low sample concentration) precluded an unambiguous structure elucidation. Subsequent synthesis of the S- and N-glucuronides of tanaproget from tanaproget facilitated the unambiguous regio- and stereochernical assignment of the metabolite by comparison of 1D NMR chemical shifts and scalar coupling constants, 2D NMR correlations, and HPLC and LC/MS characteristics between the synthetic compounds and the metabolite. From extensive comparison of the spectral and chromatographic data of the microsomal metabolite and the synthetic compounds, the metabolite has been determined to be the S-(β)-o-glucuronide of tanaproget.

Materials and Methods

Chemicals. Chemicals used in the model compound syntheses, and deuterated NMR solvents were obtained from Aldrich (Milwaukee, WI). Ammonium acetate, magnesium chloride, and UDPGA were obtained from Sigma (St. Louis, MO). Solvents for extractions and chromatography were HPLC grade or deuterated dimethyl sulfoxide (DMSO-d$_6$). Acetone, sodium hydride, and dimethyl formamide (DMF) were obtained from Acros Organics (Morris Plains, NJ).

Synthesis of N- and S-Glucuronic Acid Derivatives of Tanaproget. A 0.5-mmol solution of tanaproget in anhydrous dimethyl formamide (5 ml) was added dropwise under a nitrogen atmosphere to a solution of sodium hydride (25 mmol) that was cooled to $-70^\circ$C (dry ice). After stirring for 10 min, a 0.5-mmol solution of acetobromo-$\alpha$-glucuronic acid methyl ester in dimethyl formamide (5 ml) was added dropwise. The reaction solution was then warmed to room temperature and stirred for a total of 8 h. The reaction solution was partitioned between water (100 ml) and ethyl acetate (100 ml). The aqueous layer was extracted again with ethyl acetate (100 ml). The combined organic layers were washed with saturated sodium chloride solution (100 ml) and dried (magnesium sulfate), and solvent was removed in vacuo to yield 0.350 g of crude reaction material. To a portion of this material (0.210 g) was added a solution

ABBREVIATIONS: LC/MS, liquid chromatography/mass spectrometry; 1D, one-dimensional; 2D, two-dimensional; gHSQC, gradient heteronuclear single quantum coherence; gHMBC, gradient heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; UDPGA, uridine diphosphoglucuronic acid; DMSO-d$_6$, deuterated dimethyl sulfoxide; UGT, UDP-glucuronosyltransferase.
of methanol/Hunig’s base ((iso-Pr)2NEt)/water (5 ml/2 ml/2 ml), and the solution was stirred for 8.5 h at room temperature. The reaction solution was then adjusted to pH 2.5 using HCl (conc., approximately 1.5 ml) and chromatographed by reversed-phase HPLC (YMC-Pack CN, 150 × 20 mm i.d., 5-μm particle size) using a gradient of 15 to 35% acetonitrile/10 mM (aqueous) ammonium acetate. From repeated injections, approximately 5 mg of tanaproget were purified to >98% purity based on NMR analysis.

Preparation of Tanaproget Glucuronide Conjugate from Rat Liver Microsomes for NMR. Liver microsomes from Sprague-Dawley rats were prepared in-house using a differential ultracentrifugation method (Lake, 1987) with slight modifications. Microsomal protein and cytochrome P450 were prepared in-house using a differential ultracentrifugation method (Lake, 1987) with slight modifications. Microsomal protein and cytochrome P450 were purified to 98% based on NMR analysis.

Results and Discussion

Both the N-glucuronide, 2, and the S-glucuronide, 3, forms of tanaproget were synthesized starting from tanaproget and aceto-bromo-o-glucuronic acid methyl ester (Scheme 1). The purity was excellent (>98%), and a sufficient amount of each (at least 5 mg) was obtained, enabling complete NMR assignments of each. Table 1 summarizes the NMR results for 2 and 3.

The protonated and deprotonated LC/MS spectra (not shown) of the S-glucuronide 3 gave an [M + H]+ ion at m/z 474 and an [M − H]− ion at m/z 472, respectively (as was also the case for 2; spectra not shown), indicating glucuronidation of tanaproget. Also observed for 3 in the positive and negative ionization mode, respectively, are the ions m/z 298 and m/z 296, assigned as tanaproget; that is, the loss of glucuronic acid. In addition, a fragment at m/z 175, assigned as glucuronic acid, was detected in the negative ionization spectrum.

Metabolite M1 LC/MS spectra (not shown) gave protonated and deprotonated molecular ions [M + H]+ and [M − H]− at m/z 474 and
472, respectively, which indicated a molecular mass of 473 Da. This was 176 Da larger than tanaproget. Loss of 176 Da from m/z 474 in the positive ionization mass spectrum and from m/z 472 in the negative ionization mass spectrum generated the fragment ions at m/z 298 and 296, respectively, which are assigned as tanaproget. A glucuronic acid ion fragment was observed at m/z 175 in negative ionization mode. These data were consistent with glucuronic acid conjugation of tanaproget.

A 1H NMR spectrum for M1 is shown in Fig. 1A, and a compilation of the results from NMR experiments on M1 is given in Table 1. Despite several attempts at using freshly isolated M1 samples, the purity and concentration of the samples were typically low (<70% pure and an estimated total amount of 20 μg of M1 in solution), which hampered acquisition of complete 2D NMR datasets. We note that during the process of the microsomal incubation and subsequent isolation steps, the glucuronide solution tended to readily undergo oxidative transformation to the carbamoylglucuronide. Key 1H and 13C NMR correlations to H-17, H-18, and H-19.

is in a position peri- to the N-glucuronide has moved downfield to 7.80 ppm, compared with 7.13 ppm in the parent molecule, indicating the proximity of the glucuronic acid group (and possibly the carboxylic acid moiety) to this proton. 1H-15N gHMBC experiments were run on all compounds studied, but signals from the key nitrogen N-5 did not rearrange to a thionocarbamate (N(C=S)O) group before glucuronidation. Key 1H and 13C NMR chemical shift data reported above for the derivatized benzoxazine-2-thione carbon (C-6) of M1 are observed at 161.3 ppm, quite upfield-shifted from the observed thiocarbonyl carbon chemical shift (182.8 ppm) of the benzoxazine-2-thione group observed in tanaproget. These two types of reversed-phase HPLC columns, as described under Materials and Methods, were each used under two different mobile phase gradient conditions. One mobile phase was a gradient of 10 mM ammonium acetate in water/acetonitrile (90:10 to 10:90 in 20 min), and the other mobile phase was a gradient of 10 mM formic acid in water/acetonitrile (90:10 to 10:90 in 20 min). These HPLC conditions covered a wide range of selectivity as evidenced by the change of elution order of the major and minor components in the samples. To compare and match the retention time of the major peaks in the two samples, spiking experiments were per-
formed. The synthetic glucuronide 3 that was determined to be S-glucuronide was found to have retention times identical to that of metabolite M1 under all 10 HPLC conditions.

Literature reports of glucuronide conjugation through an S-linkage are rare (Jeffcoat et al., 1980; Nakaoka et al., 1989; Smith et al., 1992; Ethell et al., 2003; Martin et al., 2003), with little data available on their structural characterization or the UDP-glucuronosyltransferases (UGTs) catalyzing their formation. N- and O-glucuronides are more commonly observed (Green and Tephly, 1998; Hawes, 1998; Kuehl and Murphy, 2003; Tricker, 2003). We recently reported that UGT1A9 and 2B7 were the major isoforms involved in the formation of tanaproget S-glucuronide (Elmarakby et al., 2005).

Direct NMR structure elucidation of the metabolite M1 generated from rat liver microsomal incubation was challenging because of the lack of protons proximal to the glucuronic acid group attached to the benzoxazine-2-thione moiety of tanaproget which, therefore, precluded homonuclear correlations to the attached sugar group. The lack of nuclear Overhauser effects from the sugar to the remainder of the molecule constituted only negative proof that M1 was an S-glucuronide and not an N-glucuronide. Furthermore, chemical shift arguments for the benzoxazine-2-thione carbonyl and the glucuronic acid group were also insufficient evidence for an unambiguous structure proof. Synthetic compounds turned out to be a more straightforward path to structure elucidation, since they could be made in relatively pure, concentrated amounts.

In summary, from extensive chromatographic and spectral comparison of the microsomally derived metabolite M1 and the synthetic compounds 2 and 3, the glucuronide metabolite of tanaproget has been determined to be the S-β-(O)-glucuronide conjugate.

Acknowledgments. We thank Andrew Fensome of Wyeth Medicinal Chemistry for providing us with the tanaproget samples and for invaluable discussions during the project. We also thank Syed Ahmad of Wyeth Drug Safety and Metabolism for technical assistance with the microsomal incubations.
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