In Vitro Metabolic Characterization, Phenotyping, and Kinetic Studies of 9cUAB30, a Retinoid X Receptor-Specific Retinoid

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ABSTRACT:

The present study was conducted to compare the in vitro phase I and phase II metabolic profiles of (2E,4E,6Z,8E)-8-(3′,4′-dihydro-1′(2′-f)-naphthalen-1′-ylidene)-3,7-dimethyl-2,4,6-octatrienoic acid (9cUAB30) in human, rat, and dog microsomes and to characterize and identify the associated metabolic kinetics and specific isozymes from human liver microsomes (HLM) responsible for metabolism, respectively. Data from these experiments revealed that nine (M1–M9) phase I metabolites along with a single glucuronide conjugate were observed across the species investigated. With the exception of glucuronidation, no evidence of metabolism was detected for phase II enzymes (data not shown). Significant differences between species with regard to metabolic profile, stability, and gender were noted. For the eight phase I metabolites detected in HLM, the specific isozymes responsible for the biotransformations were CYP2C8, CYP2C9, and CYP2C19, with minor contributions from CYP1A2 and CYP2B6. For the glucuronide conjugate, UGT1A9 was the major catalyzing enzyme, with a minor contribution from UGT1A3. Kinetic analysis of eight of the detected metabolites indicated that four seemed to follow classical hyperbolic kinetics, whereas the remaining four showed evidence of either autoactivation or substrate inhibition.

Retinoids belong to a class of compounds chemically related to vitamin A and have been shown to be effective in vitro against many types of cancer, including breast cancer (Crouch and Helman, 1991; Delia et al., 1993; Rubin et al., 1994; Sun et al., 1997; Wu et al., 1997). Biological activities of retinoids are mediated through retinoid X receptors (RXR), which belong to a family of transcription factors that can modulate several signaling pathways. When activated by retinoids, RXR can modulate several pathways involved in the expression of genes associated with the development and progression of cancer.

Materials and Methods

Chemical and Biological Reagents. 9cUAB30 was supplied by the National Cancer Institute (Bethesda, MD), and α-phenylcinnamic acid (97%) as the internal standard, 3′-p-hydroxy-paclitaxel, paclitaxel, acetylaminophen-glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium acetate, acetonitrile, and methanol (high-performance liquid chromatography grade) were obtained from Fisher Scientific (Atlanta, GA). The NADPH regenerating system (solution A and B), UGT assay mix (solution A and B), and 6α-hydroxy-paclitaxel were obtained from BD Biosciences (Bedford, MA).

Microsomes and P450 Enzymes. All the species of hepatic microsomes were obtained from XenoTech, LLC (Lenexa, KS). Isozymes CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 (Bactosomes) obtained from Escherichia coli-expressed recombinant enzymes were purchased from Xenotech, LLC. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 were purchased from BD Biosciences.

Metabolic Incubations. The incubations for metabolite identifications were conducted with a 10 µM concentration of the substrate (9cUAB30) in a total...
reaction volume of 0.5 ml at 37°C in a shaking water bath. A 20 μM concentration was used for phenotyping studies, whereas 0.2, 0.5, 1, 5, 10, 50, and 100 μM concentrations were used in the kinetic studies. The composition of the reaction mixtures consisted of a pH 7.4 phosphate buffer solution containing an NADPH regenerating solution consisting of 1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 120 μM sodium citrate buffer, 3.3 mM magnesium chloride, and liver microsomes (1 mg/ml total protein) or isozymes (2.2–13.3 nmol/ml P450). The total organic solvent concentration in the reaction mixture was approximately 0.6% (v/v) and determined not to interfere with metabolic activity. For the glucuronidation reactions, 9cUAB30 (10 μM) was incubated with liver microsomes, UDP-glucuronic acid cofactor at 2 mM in deionized water, along with 50 mM Tris-HCl, 8 mM MgCl₂, and 25 μg of alamethicin in deionized water. The reaction mixture also contained the NADPH regenerating solution as described previously. Aliquots (50 μl) from the reaction mixtures used to determine metabolic stability were sampled at 5, 15, 30, 60, and 120 min, and the reaction was quenched by the addition of an equal volume of acetonitrile. Reactions for the kinetic and isozyme phenotyping studies were quenched after 15 min and 1 h of incubation time, respectively, with a volume of acetonitrile equal to that of the reaction mixture. All the samples from the various reaction mixtures were centrifuged at 11,000 rpm for 5 min, and the supernatant was analyzed directly by liquid chromatography/tandem mass spectrometry (LC/MS/MS). A series of positive and negative controls were simultaneously incubated under identical conditions. The positive control substrate/metabolite pairs consisted of paclitaxel/6α-hydroxy-paclitaxel and 3'-p-hydroxy-paclitaxel for oxidation reactions along with acetaminophen/acetaminophen-glucuronide for glucuronidation. Negative control reactions consisted of a reaction mixture with no substrate and a separate reaction with no microsomes where quenched microsomes were back-added to the reaction mixture after incubation.

**Analytical Method.** Chromatographic separation of the metabolites was achieved using a PerkinElmer Series 200 high-performance liquid chromatography system (Norwalk, CT) with a 100 × 2 mm, Aquasil C₁₈ reverse-phase column (Thermo Electron, Bellefonte, PA). A mobile phase consisting of 5 mM ammonium acetate and methanol was optimized for the separation of the metabolites. A gradient profile was used where the methanol concentration was increased from 40% to 90% at a linear rate over 11.5 min and then back to 40% in 2.5 min at a constant flow rate of 400 μl/min. Mass detection was accomplished from 10-μl injections with an Applied Biosystems (Foster City, CA) 4000 QTRAP triple quadrupole ion trap mass spectrometer equipped with an electrospray ionization source operated at a potential of 5 kV at 450°C. Data were collected from the instrument using Analyst 1.4.1 (Applied Biosystems) in both Q1 mass scan mode and independent data acquisition mode using enhanced product ion scans in the ion trap mode for both polarities.

**Results**

**Metabolism by HLM.** Five metabolites (M1–M5) in the positive ion mode, three metabolites (M6–M8) in the negative ion mode, and one glucuronide conjugate were detected by LC/MS/MS analysis from the in vitro reaction medium after incubation of 9cUAB30 with gender-specific HLM for 2 h at 37°C. Peaks corresponding to these metabolites (Figs. 2–4) were absent in the negative control reactions that were generated by adding solvent inactivated microsomes to the reaction mixture after incubation. Metabolites M1 through M4 are consistent with oxidized metabolites (M + 16), whereas M5 was found to be consistent with dehydrogenation/oxidation of the substrate (M + 14). Metabolites M6 through M8 were found to have masses consistent with dehydrogenation (M – 2). There was no evidence of any conjugative phase II metabolism observed (data not shown).

**Metabolism by Rat Liver Microsomes.** In vitro reactions conducted in rat liver microsomes (RLM) produced metabolites M1 through M8 and a single glucuronide conjugate as previously observed in the HLM reactions. In addition to these, a new metabolite, M9, corresponding to doubly oxidized species (M + 32) was also detected. There was no evidence of any conjugative phase II metab-
olism observed (data not shown). Peaks corresponding to these metabolites (Figs. 2–4) were absent in the negative control reactions.

**Metabolism by Dog Liver Microsomes.** In vitro reactions in dog liver microsomes (DLM) resulted in detection of six previously observed metabolites, M2 through M4 and M6 through M8, and a single glucuronide conjugate as observed for both HLM and RLM. No evidence for metabolites M1, M5, or M9 was found in reactions with DLM. There was no evidence of any conjugative phase II metabolism observed (data not shown). All the metabolites detected in these reactions (Fig. 2–4) were absent in the negative control reactions.

**Metabolic Stability.** Metabolic stability of 9cUAB30 was determined in duplicate at five time points between 5 and 120 min in gender-specific HLM, RLM, and DLM for both oxidative and glucuronide-conjugate reactions. Figure 5, A through C, shows the phase I metabolic stability for 9cUAB30 in both genders for rat, dog, and human, respectively. Figure 6, A through C, shows the corresponding stability of 9cUAB30 for the glucuronidation reactions. The error bars reflect the difference of the individual values from the mean. These data suggest that 9cUAB30 undergoes a significant amount of oxidative metabolism in both genders of RLM with less than 15% of the starting concentration (10\(10^6\)M) remaining after 120 min. Less significant losses of 9cUAB30 were observed in HLM and DLM. Under the same incubation conditions, about 50% of the starting concentration of 9cUAB30 remained in each gender of HLM, whereas 50 and 71% remained in male and female DLM, respectively. For the glucuronidation reactions, loss of 9cUAB30 was found to be rapid and extensive in both genders of all three species such that 10% or less was found to be remaining after 120 min of incubation.
Identification of Metabolites. Metabolite identification was based on characteristic mass shifts of the molecular ions \([\text{M+H}]^{+}\), \([\text{M-H}]^{-}\) in combination with the product ions obtained from the enhanced product ion mass spectrum of each peak in both polarity modes as compared with an authentic 9cUAB30 standard. Characteristic ions observed in the positive ion spectrum (Fig. 7A) for 9cUAB30 include \(m/z\) 277 (loss of \(\text{H}_2\text{O}\)), \(m/z\) 249 (loss of \(\text{HCOOH}\)), and \(m/z\) 235 (loss of \(\text{CH}_3\text{COOH}\)). Other abundant fragment ions include \(m/z\) 209 [M-86] 

\(m/z\) 195 [M-100] 

\(m/z\) 169 [M-126] 

which likely result from fragmentation of the alkene chain and contain the dihydronaphthalen-1'-ylidene functionality. Fragment ions observed in the negative ion spectrum (Fig. 7B) include \(m/z\) 249 (loss of \(\text{CO}_2\)), \(m/z\) 181 [M-112] 

\(m/z\) 141 [M-152] 

\(m/z\) 119 [M-174]. In the negative ion mode, M1 through M4 \((m/z\) 309), M5 \((m/z\) 307), and M9 \((m/z\) 325) all had characteristic mass shifts of 16 Da (oxidation), 14 Da (oxidation/dehydrogenation), and 32 Da two degrees of oxidation, respectively, for the parent and characteristic fragment ions. Because of the limited amount of fragment ions detected for metabolites M1 through M5, the precise location of each biotransformation on the parent could not be made. In the positive ion mode, metabolites M6 through M8 displayed mass shifts of \(-2\) Da (dehydrogenation) for the parent and characteristic fragment ions. Figure 8 shows the representative MS/MS spectra for M4, M5, and M8. The single glucuronide metabolite was characterized by the M + 176 ion and subsequent loss of 176 Da as detected in a neutral loss scan (Fig. 9).

Isozyme Mapping. Commercially available human P450 and UGT isoforms were evaluated for their ability to metabolize 9cUAB30. The experiments were conducted by incubating the substrate with individual isozymes or UGT and monitoring the reaction mixture for the previously described metabolites. The results for metabolites M1 through M5, M6 through M8, and the UGT are shown in Fig. 10. In general, CYP2C8, CYP2C9, and CYP2C19 were determined to be responsible for the majority of the observed biotransformations for the oxidative metabolites M1 through M5, along with minor contributions from CYP1A2. In particular, CYP2C9 was found to be the largest...
single contributor to M1, whereas CYP2C8's largest contribution was to M3. CY2C19 contributed most significantly to M3 and M4, whereas CYP1A2's largest contribution was to M4. The only significant contribution to M5 appeared to come from CYP2C8. The group of isozymes as found with M1 through M5 metabolites was also determined to be the most active in the formation of the dehydrogenated metabolites M6 through M8. The formation of M6 was found to be primarily from CYP2C9 with small contributions from CYP2C8, CYP2C19, and CYP2B6, whereas M7 was produced almost equally by CYP2C8 and CYP2C19. In the formation of M8, CYP2C19 was observed as the single largest contributor; however, other contributions were also made by CYP2C8 and CYP2C9, along with minor contributions from CYP1A2, CYP2B6, and CYP2D6. For the glucuronide conjugate detected, UGT1A9 was found to be the single largest contributor, with smaller contributions noted from UGT1A3 and UGT1A8.

**Kinetic Analysis.** The concentration-dependent human microsomal metabolism for each of the identified metabolites, M1 through M8 (excluding M6), was evaluated. Figure 11 shows the graph for M1, which was found to be representative for the other measured metabolites except for M2, which is also shown. Under the conditions described above, M6 was not generated in sufficient quantities to conduct kinetic analysis. The relationships between relative formation rates of metabolites and substrate concentration showed hyperbolic saturation kinetics for each metabolite. The Michaelis constants for the oxidative metabolites (M1–M5) and the dehydrogenated metabolites (M7, M8) extend over a broad range from 0.97 to 25.2 μM and 3.8 to 16.5 μM, respectively (Table 1). Eadie-Hofstee plots (insets in Fig. 11) for each of the metabolites were constructed and used in a diagnostic manner to identify atypical metabolic kinetic behavior (data shown only for M1 and M2). Metabolites M1, M4, and M5 show monophasic kinetics consistent with standard hyperbolic Michaelis-Menten kinetics. The Eadie-Hofstee plots for M2 and M7 exhibited a convex component that suggests substrate inhibition. For M3 and M8, the majority of the data points are consistent with Michaelis-Menten kinetics; however, there is an indication of autoactivation (sigmoidal response) at the lower concentrations tested. Because authentic standards for each of the metabolites measured were not available, absolute determinations for the rate of formation (V) were not made.

**FIG. 7.** A and B, product ion spectrum of 9cUAB30 in the positive (A) and negative (B) ion mode.

**FIG. 8.** A–C, negative ion mode product ion spectra of oxidized metabolite (M4) (A) and an oxidized plus dehydrogenated metabolite (M5) (B). C, positive ion mode spectrum of a dehydrogenated metabolite (M8).

**FIG. 9.** Negative ion mode product ion spectrum from a neutral loss scan of a glucuronidated metabolite of 9cUAB30.
Discussion

This work represents the first investigation of 9cUAB30 metabolism in a P450 enzyme system. Our experimental data show that 9cUAB30 is metabolized to nine phase I oxidative or dehydrogenated metabolites and one glucuronide conjugate in gender-specific HLM, RLM, and DLM (Fig. 12). Characterization of the metabolites, determination of their associated kinetics, and enzyme mapping were accomplished using LC/MS/MS. Characteristic mass shifts combined with product ion scans (MS/MS) were used in all the experiments.

Variation in the metabolic profile as a function of gender was only observed in RLM. This was also the only species observed to produce M9, identified as a metabolite with two degrees of oxidation. The presence of M9 as observed in this species at the 2-h time point is likely a result of excessive oxidation based on the metabolic half-life of 9cUAB30 in RLM (Fig. 5). This is supported by analysis of the 1-h time point, which shows the presence of M9 but at a lower relative concentration (data not shown). The oxidative metabolite M4 was detected in readily measurable quantities in male RLM yet was barely detectable above background in the female RLM. No substantial gender differences were noted in the other species tested; however, differences in the metabolic profile were noted between species.

Whereas the M1 oxidative metabolite was found to be the most abundant in RLM, it was among the smallest in HLM and DLM. Additionally, the ratio of M6/M7 in the RLM was inversely proportional to that found in the HLM. The smallest quantity of these two metabolites was found in reactions with the DLM, where approximately equal amounts were measured. The third dehydrogenated metabolite, M8, was the most abundant in all three species tested. The presence of three dehydrogenated metabolites each chromatographically separated and derived from a molecule where only two dehydrogenated tetralin ring products are possible may be explained by cis-trans isomerization of the polyene chain. Conversion between the cis- and trans-isomeric forms of the parent would allow for more than the three observed products. Comparable biotransformation for a structurally related analog, retinoic acid has previously been reported (Chen and Juchau, 1998). Evidence for P450-catalyzed dehydrogenation of tetralin rings has been shown with testosterone (Nagata et al., 1986; Korzekwa et al., 1990) and in aliphatic compounds (Rettie et al., 1995). The presence of the small peak in the ion chromatograms, which elutes just after 9cUAB30, produced identical mass spectra for both Q1 mass scans and product ion (MS/MS) scans as compared with 9cUAB30 (data not shown). Mass spectra were also obtained for authentic standards of cis- and trans-retinoic acid, which showed MS or MS/MS alone was incapable of differentiating between the two isomeric forms (data not shown).

Metabolic stability for phase I metabolism exhibited a detectable difference between genders for both rats and dogs in terms of amount of substrate remaining after 2 h of incubation. 9cUAB30 was found to
be the least stable in RLM, yet exhibited comparable stability in DLM and HLM. No statistical significant difference was noted between genders in HLM. The amount of observed glucuronidation for 9cUAB30 was found to be much more extensive compared with oxidation for all three species tested. Statistically significant differences in the amount of glucuronidation between genders were noted for both rats and dogs but not in humans.

Metabolism of xenobiotics can be affected by many parameters, including genetic polymorphisms, high interindividual variability, and gender differences. Because variable expressions of individual isozymes can affect the metabolic fate of a drug candidate, it is important to determine which P450 enzymes contribute to the metabolic process (Shapiro et al., 1995; Cai et al., 2003). The individual isozymes responsible for the biotransformation of 9cUAB30 into the observed metabolites were determined to be primarily in the CYP2C family with a small contribution from CYP1A2 for the phase I and from the UGT1A family for glucuronidation based on the groups tested. The CYP2C family belongs to a group of isozymes that are involved in metabolizing drugs for which specific isozymes have been identified (Parkinson, 1996). Surprisingly, there was essentially no contribution from the CYP3A4 isozyme, which is generally believed to be an important enzyme in xenobiotic metabolism, as well as being involved in the oxidation of a broad range of substrates. This enzyme also typically represents a much higher percentage (20–30%) of the P450 content in human liver (Shimada et al., 1994). As a result of the low contribution to the overall metabolism of 9cUAB30, a shared identity (83%) in amino acid sequence (Aoyama et al., 1989), and similarity in substrate specificity (Thummel and Wilkinson, 1998), CYP3A5 was not evaluated. Previous work using human P450 isozymes for both a functional and structurally related compound, retinoic acid, showed the CYP2C family to be the primary P450 enzymes involved in oxidative metabolism with minor contributions from CYP3A (Shirley et al., 1996; Nadin and Murray, 1999; McSor-

TABLE 1

Michaelis constants and peak area ratios for 9cUAB30 metabolites

<table>
<thead>
<tr>
<th>Parameters</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M7</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>0.19</td>
<td>0.14</td>
<td>0.49</td>
<td>0.40</td>
<td>0.04</td>
<td>0.008</td>
<td>0.08</td>
</tr>
<tr>
<td>$K_{m}$ (μM)</td>
<td>25.2 ± 6.1</td>
<td>0.97 ± 0.4</td>
<td>9.7 ± 3.2</td>
<td>15.1 ± 6.3</td>
<td>9.7 ± 2.6</td>
<td>3.8 ± 1.8</td>
<td>16.5 ± 7.8</td>
</tr>
</tbody>
</table>
Chen H and Juchau MR (1998) Biotransformation of 13-cis- and 9-cis-retinoic acid to all-trans-
retinoic acid in rat conceptual homogenates. Evidence for catalysis by a conceptual isomerase.
Drug Metab Dispos 26:222–229.
Chithalen JV, Liu L, Petkovich M, and Jones G (2002) HPLC-MS/MS analysis of the products
generated from all-trans-retinoic acid using recombinant human CYP26A. J Lipid Res 43:
1133–1142.
Crouch GD and Helman LJ (1991) All-trans-retinoic acid inhibits the growth of human rhab-
hematopoietic cell lines including those unresponsive to retinoid acid. Cancer Res 55:6036–
6041.
Ebner T and Burchell B (1993) Substrate specificities of two stably expressed human liver
Green MD, King CD, Mojarrabi B, Mackenzie PT, and Tephy TR (1998) Glucuronidation of
amines and other xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase
WL, and Muccio DD (2003) 9cUB30, an RXR specific retinoid, and/or tamoxifen in the
Han SB and Choi H (1996) Human and dog liver cytochrome P450 enzyme systems. Drug Metab
on the mechanism of the cytochrome P-450IA1-catalyzed formation of 4-hydroxysterol from
Lin JH and Ly AY (1997) Role of pharmacokinetics and metabolism in drug discovery and
between distal retinoic acid response elements in regulation of Cyp26A1 inducibility.
McDonald LC and Daly AK (2000) Identification of human cytochrome P450 isoforms that
Nadin L and Murray M (1999) Participation of CYP2C8 in retinoic acid 4-hydroxylation in
Nagata K, Liberalo DJ, Gillette JR, and Susama HA (1986) An unusual metabolite of testoster-
one. Drug Metab Dispos 14:559–565.
metabolism and interactions on the basis of in vitro investigation. Basic Clin Pharmacol
Toxicol 96:167–175.
isozyme specificity and the relationship between ω-hydroxylation and terminal desaturation
Rubin M, Fenig E, Rosenauer A, Menendez-Botet C, Acktar C, Bentel JM, Yahalom J,
Mendelsohn J, and Miller WH Jr (1994) 9-Cis retinoic acid inhibits growth of breast cancer
carboxylic acid containing compounds by UDP-glucuronosyltransferase isoforms. Arch Bio-
in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and
toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Phar-
macol Exp Ther 270:414–423.
and reductive metabolism of 9cUB30 in human liver microsomes. Drug Metabol Dispos 24:
293–302.
and rapid phase II metabolisation by identification mass spectrometry. Drug Metab Dispos
Sonneller E, van den Brink CE, van der Leeze BM, Schuilkes RK, Petkovich M, van der Burg
B, and van der Saag PT (1998) Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly
specific for all-trans-RA and can be induced through RA receptors in human breast and colon
9cUB30, an RXR specific retinoid, and/or tamoxifen in the prevention of methylnitrosourea-induced mammary cancers. Drug Metab Rev 35:83–90.
et al. (1997) Inhibition of trans-retinoic acid-resistant human breast cancer cell growth by retinoid

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