It is suggested that formation of more polar metabolites of all-trans-retinoic acid (atRA) via oxidative pathways limits its biological activity. In this report, we investigated the biotransformation of oxidized products of atRA via glucuronidation. For this purpose, we synthesized 4-hydroxy-RA (4-OH-RA) in radioactive and nonradioactive form, 4-hydroxy-retinyl acetate (4-OH-RAC), and 5,6-epoxy-RA, all of which are major products of atRA oxidation. Glucuronidation of these retinoids by human liver microsomes and human recombinant UDP-glucuronosyltransferase(s) (UGTs) was characterized and compared with the glucuronidation of atRA. The human liver microsomes glucuronidated 4-OH-RA and 4-OH-RAC with 6- and 3-fold higher activity than atRA, respectively. Analysis of the glucuronidation products showed that the hydroxyl-linked glucuronides of 4-OH-RA and 4-OH-RAC were the major products, as opposed to the formation of the carboxyl-linked glucuronide with atRA, 4-oxo-RA, and 5,6-epoxy-RA. We have also determined that human recombinant UGT2B7 can glucuronidate atRA, 4-OH-RA, and 4-OH-RAC with activities similar to those found in human liver microsomes. We therefore postulate that this human isozyme, which is expressed in human liver, kidney, and intestine, plays a key role in the biological fate of atRA. We also propose that atRA induces its own oxidative metabolism via a cytochrome P450 (CYP450) and is further biotransformed into glucuronides via UGT-mediated pathways.

atRA is a major metabolite of vitamin A (all-trans-retinol) that undergoes isomerization and metabolism in vivo, yielding 13-cis-retinoic acid (13-cis-RA), 9-cis-retinoic acid (9-cis-RA) (1–3), 5,6-epoxy-RA, 4-oxo-RA (4), 3,4-didehydro-RA, 4- and 18-hydroxy-RA (4-OH-, 18-OH-RA) (5–9), and all-trans-retinol-β-glucuronide (RAG) (10–12). These metabolites are thought to be involved in mediating atRA function. It has been well established that atRA and some of its metabolites modulate expression of a set of genes involved in apoptosis, cellular growth and differentiation, and embryonic development (13, 14). Retinoids are also effective in preventing carcinogenesis and can inhibit proliferation of a large variety of normal and neoplastic cells. In addition, atRA is currently used as a chemotherapeutic agent against promyelocytic leukemia (15), various endothelial cancers (16), breast cancer (17), and endometrial cancer (18). Retinoid metabolism and atRA-induced growth inhibition in head and neck squamous cell carcinoma cell lines has also been documented (16). Furthermore, the glucuronidated atRA derivative, RAG, has been shown to be less cytotoxic than the parent atRA, while retaining its potency for driving cell growth, differentiation, and proliferation (12, 19). This highlights the importance of elucidating the physiological role of glucuronide conjugates of atRA and oxidized derivatives of atRA.

Recently, a novel atRA-inducible cytochrome P450 (CYP26) that specifically metabolizes atRA to 18-OH-RA and 4-OH-RA has been identified (5–9). The 4-OH-RA is subsequently oxidized to 4-oxo-RA by an alcohol dehydrogenase(s) (20). Such polar metabolites of atRA are thought to limit its biological activity (5–9). Characterization of the enzymatic metabolism of atRA will provide significant insight into the regulation of retinoid homeostasis.

The purpose of the present work was to examine phase II metabolism of atRA and identify the human UGTs (EC 2.4.1.17) involved in the glucuronidation of atRA, 4-OH-RA, 4-oxo-RA, 5,6-epoxy-RA, and 4-OH-RAC (structures shown in Fig. 1). UGTs catalyze the conjugation of endogenous and xenobiotic compounds to α-glucuronic acid derived from UDP-glucuronic acid (UDP-GlcUA). This converts the metabolites into a more polar form, promoting their excretion in urine and/or bile. However, several examples of bioactivation of parent compounds via glucuronidation have been reported (21–25). UGTs can be divided into two families, UGT1A and UGT2B, based on their sequences. In this work, human liver microsomal UGTs and recombinant isoforms from both families were evaluated for their ability to catalyze glucuronidation of retinoids in various stages of oxidation.

To obtain the appropriate substrates for these studies, we synthesized 4-OH-RAC, 5,6-epoxy-RA, and both radioactive and nonradioactive forms of 4-OH-RA. The studies in this report center on examining the glucuronidation of atRA, 4-OH-RA,
4-oxo-RA, 5,6-epoxy-RA, and 4-OH-RAc by human liver microsomes and the human recombinant UGTs, 1A3 and 2B7. Human liver UGT2B7 is involved in the glucuronidation of a large variety of endogenous and exogenous substrates including bile acids, steroids, and a variety of xenobiotics (26–30). This report identifies UGT2B7 as the major isoform catalyzing the formation of carboxyl- and hydroxyl-linked glucuronides of retinoids at high levels of activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

[14C]UDP-GlcUA was purchased from American Radiolabeled Chemicals (St. Louis, MO). atRA; 9-cis-RA, 13-cis-RA, retinol, 13-cis-retinol, BrJ5, UDP-GlcUA, and sarcosaccharo were purchased from Sigma. (E)-Retinoid acetic acid (atRA) and RAc were purchased from ACROS Organics (Pittsburgh, PA), and (E)-[11,12-3H]RA, (4H)RA, specific activity 35.8 Ci/mmol, and sodium borohydride (NaBH4; 204 mCi/mmol), were purchased from NEN Life Science Products. All other reagents were of the highest commercially available quality.

**Synthesis of Radioactive and Nonradioactive Retinoid Substrates**

**Synthesis of (±)-5,6-Epoxide-(E)-retinoic Acid (Scheme 1)—**Racemic 5,6-epoxy-RA (2) was synthesized by epoxidation of atRA (31) with monoperophthalic acid, which was synthesized by a reaction of phthalic anhydride with alkaline H2O2 as described elsewhere (32) and quantitated iodometrically. 1.0 g (3.3 mmol) was reacted with a 15-fold molar excess of monoperophthalic acid in diethyl ether (50 ml) for 1 h at room temperature. The formation of the oxirane was quantitative as evidenced by TLC analysis using ethyl acetate-hexane (40:60 or 50:50, v/v) as the mobile phase. The reaction mixture was washed with saturated sodium bicarbonate, water, and saturated NaCl. The ether layer was dried over anhydrous MgSO4, and solvent was removed in vacuo. The oxirane was recrystallized from diethyl ether/hexane (84% yield). Mass and 1H NMR spectra were obtained for the corresponding methyl ester, which was synthesized by reaction of the free acid with ethereal diazomethane prepared as described below: EI-MS m/z 330 (M+), 315 (M – CH3)+, 299 (M – OCH3)+, 271 (M – CO2CH3)+; 1H NMR (500 MHz, MeSO4-d4) d (ppm) 6.97 (d, J = 15.0, 15.3 Hz, 1H), 6.42 (d, J = 15.1 Hz, 1H), 6.25 (d, J = 6.5, 11.1 Hz, 2H), 6.04 (d, J = 15.7 Hz, 1H), 5.78 (s, 1H), 2.26 (s, 4H), 1.95 (s, 3H), 1.73 (brs, 2H), 1.37 (brs, 2H), 1.09 (s, 3H), 0.88 (s, 3H), 0.85 (s, 3H).

**Synthesis of 4-(E)-Retinyl Ester (Acetate) (Scheme 2)—**Acetate (4) (1.13 g, 3.44 mmol) (3) was stirred with 30 g of MnO2 in 120 ml of CH2Cl2 for 90 h at room temperature. The MnO2 was removed by filtration, and the solvent was removed in vacuo. The product was purified by silica gel chromatography using ethyl acetate-hexane (10:90, v/v) as the mobile phase, which afforded a yield of 75%: EI-MS m/z 342 (M+), 327 (M – CH3)+, 298 (M – CO2)+, 282, 267, 249, 145 (base peak); IR (cm−1) 1685 (α,β-unsaturated carbonyl), 1242, 1475 (ester), 1380–1365 (CH3 [acetate]); 1H NMR (300 MHz, CDCl3) d (ppm) 1.99 (s, 6H), 1.86 (s, 3H), 1.84 (m, 2H), 1.90 (s, 3H), 1.99 (s, 3H), 2.07 (s, 3H), 2.51 (t, 2H), 4.74 (d, 2H), 5.66 (t, 1H), 6.22 (d, 1H), 6.27 (d, 1H), 6.34 (d, 1H), 6.39 (d, 1H), 6.40 (dd, 1H).

**Synthesis of (±)-4-Hydroxy-(E)-retinyl acetate (Scheme 3)—**A solution of 7 (0.5 g, 1.5 mmol) in methanol (40 ml) was reacted with aqueous NaOH (6 N, 40 ml) for 4 h at room temperature and then added to aqueous H2SO4 (500 ml, 1 N). The mixture was extracted with ethyl acetate, and the pooled ethyl acetate extracts were washed with water and saturated NaCl, dried over MgSO4, and solvent was removed in vacuo. 10 was subsequently purified by recrystallization from diethyl ether/hexane (86% yield), and its purity was verified by analytical reverse phase HPLC analysis and UV spectroscopy as described previously (31).
allowed to proceed at 4 °C for 15 min and then for 1 h at room temperature. The reaction mixture was neutralized with phosphate buffer and extracted with ethyl acetate. The pooled ethyl acetate extracts were washed with water and subsequently dried over MgSO4, and the solvent was removed. The corresponding free acid (9) was obtained by hydrolysis of 8. A solution of 8 (0.35 g, 1.1 mmol) in methanol (40 ml) was reacted with aqueous NaOH (6 N, 40 ml) for 4 h at room temperature and then acidified to pH 5.0 with citrate buffer (3 M). This buffer was used for the incubation mixtures and the samples were incubated for an additional 30 min at room temperature to hydrolyze the carboxyl glucuronide. Controls without NaOH were run concurrently. Hydroxyl-linked glucuronides are stable under these experimental conditions. This procedure allowed clear determination of the formation of hydroxy- and/or carboxyl-linked glucuronides (structures shown in Fig. 2). After development in chloroform-methanol-glacial acetic acid-water (25:25:4:2, v/v/v/v) to hydrolyze carboxyl glucuronides in the course of TLC. Alternatively, after completion of the incubations, 8 N NaOH was added to the incubation mixtures and the samples were incubated for an additional 30 min at room temperature to hydrolyze the carboxyl glucuronide. Controls without 8 N NaOH were run concurrently. Hydroxyl-linked glucuronides are stable under these experimental conditions. The TLC conditions allowed for separation of carboxyl- and hydroxyl-linked glucuronides. After development, the plates were dried and subjected to autoradiography for 3–7 days at -80 °C.

**RESULTS**

**Synthesis of Retinoid Substrates**—Many of the retinoids used in the current study were synthesized because they are not commercially available. These include compounds 2-7 and 9-11 (Schemes 1–3). Racemic 5,6-epoxyretinoic acid (2) was synthesized from a 56-year-old man who had died of cerebral bleeding (HLM15) and from a 13-year-old girl who died from brain damage (HLM18). These samples were obtained from the University of Groningen, Groningen, The Netherlands. The HLM served as a control for the glucuronidation assays by providing a basis of comparison for recombinant UGTs. Human Recombinant UGTs—Human recombinant UGT1A3 was expressed in a mammalian expression system as described previously (34). UGT2B7 was expressed in human embryonic kidney (HEK293) cells as reported previously (28, 29). Enriched endoplasmic reticulum membrane fractions were prepared as described previously (35). The membrane fractions were stored at -80 °C in 5 mM HEPES, 0.25 mM sucrose, 20 mM MgCl2 (pH 7.4). The enzymatic activity of the recombinant UGT proteins was sustained for up to 6 months under these conditions. Enzyme Assays—UGT activity was measured with both radioactive and unlabeled forms of atRA and 4-OH-RA as the aglycons with UDP-GlcUA serving as the sugar donor (retinoid structures shown in Fig. 1). All retinoid substrates were prepared in the form of mixed micelles with Brij 58 (0.12%). The Brij 58 micelles both activated the enzyme and solubilized the retinoids. Human liver microsomes and recombinant UGTs (50 μg of protein) were used in the assays. All enzymatic assays were performed on a microplate reader. The amount of product formed was less than 10% of total substrate added and was linearly proportional to the amount of microsomal protein added. The retinoid derivatives (0.10 mM final concentration) were incubated in 100 mM HEPES-NaOH, pH 7.5, 5 mM MgCl2, 5 mM saccharolactone, and 0.05% Brij 58 in a final volume of 60 μl. The reaction mixture was preincubated with the proteins at room temperature for 10 min before starting the reaction with the addition of either 50 mM UDP-GlcUA for radioactive retinoids (4.17 mM final concentration) or 20 mM [14C]-UDP-GlcUA (3.33 mM final concentration) for unlabeled retinoids. The reactions were incubated for 30 min at 37 °C. Reactions were stopped with 20 μl of ethanol, vortexed and placed on ice. For TLC, 60 μl of the reaction mix was applied to the preadsorbent layer of a 19-channeled silica gel TLC plate (Baker Sil250-PA (19C); VWR Scientific) after which the plates were dried and developed twice in chloroform-methanol-glacial acetic acid-water (65:20:5:10, v/v/v/v) to hydrolyze carboxyl glucuronides in the course of TLC. Alternatively, after completion of the incubations, 8 N NaOH was added to the incubation mixtures and the samples were incubated for an additional 30 min at room temperature to hydrolyze the carboxyl glucuronide. Controls without 8 N NaOH were run concurrently. Hydroxyl-linked glucuronides are stable under these experimental conditions. The TLC conditions allowed for separation of carboxyl- and hydroxyl-linked glucuronides. After development, the plates were dried and subjected to autoradiography for 3–7 days at -80 °C.

**TLC Identification of the Type of Glucuronide Biosynthesized**—For the identification of the type of glucuronide biosynthesized (structures shown in Fig. 2), two methods were used. First, in addition to TLC in the acidic system described above, samples were chromatographed in an acidic system described above. Retinoids were extracted with ethanol-acetone/dichloromethanol-acetone (5:2:4, v/v) to hydrolyze carboxyl glucuronides in the course of TLC. After development in chloroform-methanol-glacial acetic acid-water (25:25:4:2, v/v/v/v) to hydrolyze carboxyl glucuronides in the course of TLC. Additionally, after completion of the incubations, 8 N NaOH was added to the incubation mixtures and the samples were incubated for an additional 30 min at room temperature to hydrolyze the carboxyl glucuronide. Controls without 8 N NaOH were run concurrently. Hydroxyl-linked glucuronides are stable under these experimental conditions. Additional TLC parameters were determined using EnzymeKinetics software (Trinity Software, Campton, NH).
studies. atRA was glucuronidated by UGT2B7. UGT2B7 glucuronidated the retinoid substrates on 4-OH-RAc being the optimal substrates for glucuronidation by UGT2B7. The enzymatic activities of HLM15 and the recombinant UGTs were summarized in Table I. UGT2B7 had high activity toward the retinoid substrates toward all retinoid substrates used are summarized in Table I. UGT2B7. This indicated that both microsomal and recombinant UGTs have a much higher affinity for free atRA than for the 4-hydroxylated derivative.

### Product Identification

Fig. 3 is an autoradiogram of a TLC plate from a representative assay of glucuronidation of [3H]atRA, and unlabeled 4-OH-RA and 4-OH-RAc by HLM15 and UGT2B7. The metabolites formed by glucuronidation of retinoids containing carboxyl and/or hydroxyl functional groups were either carboxyl- or hydroxyl-linked glucuronidates (Fig. 2). Biosynthesis of double glucuronidates was not observed. Double development of the TLC plate with chloroform-methanol-glacial acetic acid-water 65:25:2:4 (v/v) solvent effectively separated the mixture of the two glucuronidates (Fig. 3). An unidentified 14C-labeled endogenous product was detected (Fig. 3, lanes 1 and 4) when radiolabeled UDP-GlcUA was used.

To identify which glucuronidates were produced with a given retinoid substrate, an alkaline solvent system was used to develop the TLC plate. Under these conditions, carboxyl-linked glucuronidates were hydrolyzed as has been described previously (36), whereas the 4-OH-RA glucuronide was resistant to alkaline hydrolysis (data not shown). Additional proof of the identity of the glucuronide biosynthesized was achieved by alkaline hydrolysis (8 • NaOH, 30 min room temperature) performed prior to TLC separation.

The TLC analysis demonstrated that free atRA and the cis-isomers, as expected, exclusively formed carboxyl-linked glucuronide. 4-OH-RA or 4-OH-RAc were glucuronidated exclusively at the hydroxyl function, producing alkaline-resistant ether glucuronides. This indicated that the presence of other UGT isoforms might be involved in the glucuronidation of this retinoid.

In separate studies designed to compare the rates of glucuronidation for isomeric forms of atRA, the substrates atRA, 9-cis-RA, and 13-cis-RA were incubated with HLM18. Although, this microsomal preparation had much lower activity toward isomeric forms of atRA than did HLM15, the limited amount of the latter preparation necessitated its use. The specific activities indicated that 9-cis-RA was the best substrate for human liver microsomes, followed by atRA and 13-cis RA. The activities were 590 ± 102, 110 ± 9, and 88 ± 31 pmol/mg protein × min, respectively. All three substrates were glucuronidated at their carboxyl function.

### Kinetic Analysis

Kinetic parameters were determined for the glucuronidation of the carboxyl-function of atRA and the hydroxyl-function of 4-OH-RA using HLM15 and recombinant UGT2B7 (Table II). The K_m for atRA was 182-fold lower than for 4-OH-atRA with both microsomes and recombinant UGT2B7. This indicated that both microsomal and recombinant UGTs have a much higher affinity for free atRA than for the 4-hydroxylated derivative.

### Table I

| Substrate         | HLM  | UGT1A3 | UGT2B7 |
|-------------------|------|--------|--------|
| atRA              | 227 ± 126^a | 13 ± 2^a | 275 ± 24^a |
| 4-Oxo-RA          | 200 ± 51^a  | 10 ± 1^a  | 21 ± 5^a  |
| 5,6-Epoxy-RA      | 571 ± 67^a  | 17 ± 1^a  | 172 ± 20^a |
| 4-OH-RA           | 1375 ± 330^b | 23 ± 7^a  | 1236 ± 293^a |
| 4-OH-RAc          | 821 ± 71^b  | ND^a     | 564 ± 187^b  |

^a Carboxyl-linked glucuronides.
^b Hydroxyl-linked glucuronides.
^c Not determined.

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**Fig. 1. Structures of the retinoid substrates used in these studies.**

sized by reacting atRA (1) with monoper oxyphthalic acid (Scheme 1). (±)-4-Hydroxyretinyl acetate (5) was synthesized by first oxidizing retinyl acetate (3) with manganese dioxide to afford 4-oxoretinyl acetate (4) and reduction of the latter with sodium borohydride to yield (5) (Scheme 2). atRA (1) was esterified to the corresponding methyl ester (6) using ethereal diazomethane which was subsequently oxidized with manganese dioxide to produce 4-oxomethylretinoate (7) (Scheme 3). The free acid (10) was prepared by saponification of the ester. Racemic 4-hydroxy methylretinoate (8) was prepared by reduction of (7) with sodium borohydride and (±)-4-hydroxyretinonic acid (9) was prepared by hydrolysis of (8). Trilated (±)-4-hydroxy retinoic acid (11) was synthesized by reduction of (10) with NaB[3H]_4.

**Enzymatic Glucuronidation—** Human liver microsomes and human recombinant UGTs were assayed for glucuronidation activity toward atRA, 9-cis-RA, 13-cis-RA, 4-OH-RA, 4-OH-RAc, 5,6-epoxy-RA, and 4-oxo-RA (structures shown in Fig. 1). Two human recombinant UGTs were available; UGT1A3 and UGT2B7. The enzymatic activities of HLM15 and the recombinant UGTs have a much higher affinity for free atRA than for the 4-hydroxylated derivative.
The glucuronidation of \([\text{3H}]\text{RA}\) with unlabeled UDP-GlcUA by human liver microsomes, two products were obtained: upper band—carboxyl-linked glucuronide of atRA; lower band—unidentified glucuronidated product of atRA biotransformation. With human liver microsomes (HLM15) and UGT2B7 membranes, Lanes 1 and 4 represent incubations where UDP-GlcUA was omitted. Lanes 2 and 3 and lanes 5 and 6 represent duplicate determinations of atRA glucuronidation as described under “Experimental Procedures.” With human liver microsomes, two products were obtained: upper band—carboxyl-linked glucurononide of atRA; lower band—unidentified glucuronidated product of atRA biotransformation. The third through sixth panels (lanes 7–18) represent glucuronidation of 4-OH-RA and 4-OH-RAc with \([^{14}\text{C}]\text{UDP-GlcUA}\). Lanes 7 and 13 show an unidentified glucuronide of an endogenous substrate biosynthesized under these experimental conditions. Double development of the TLC plate is necessary to achieve separation of all biosynthesized retinoid glucuronides.

Retinyl β-glucuronide of atRA (carboxyl-linked)

4-Oβ-glucuronide of 4-OH-atRA (hydroxyl-linked)

**Discussion**

The important roles that atRA and RAG play in cellular differentiation, proliferation, apoptosis, and other cellular events necessitate studies directed at understanding the metabolism of atRA. Additionally, atRA is an important chemotherapeutic agent for skin, breast, endometrial, and hematopoietic cancers. Therefore, knowledge of atRA metabolism is essential to understand its limitations as an anti-cancer compound.

Isomers of atRA, such as 9-cis-RA and 13-cis-RA, are considered to be as biologically active as atRA (37). Experimental results from in vitro and in vivo systems indicate that atRA oxidative metabolism involves both cytochrome P450 and prostaglandin H synthase-mediated processes. We have previously demonstrated that atRA and 13-cis-RA are hydroxylated at the C-4 position by prostaglandin H synthase (33, 38, 39). Recent reports suggest that the generation of 4-OH- and 18-OH-RA relies on a cytochrome P450-dependent process. Members of a new cytochrome P450 family, CYP26, can be induced by atRA and can also inactivate biologically active atRA (5–9).

A major unanswered question is whether oxidized retinoic acid metabolites are only intermediates in the catabolic degradation of atRA, or if these oxidized metabolites possess biological functions similar to those of atRA. If the formation of more polar metabolites of atRA via hydroxylation is indeed deleterious to its biological activity, then the role of atRA and RAG will not be completely understood without identifying the sequence of events in the metabolism of atRA. To gain insight into these questions, two major issues must be addressed: 1) the physiological mechanisms that promote atRA-induced RA hydroxylation by CYP26, and 2) the metabolic fate of these oxidized-derivatives. In this report, we focused our investigation on the glucuronidation by UGTs of oxidized retinoids in comparison to atRA.

Several commercially unavailable compounds needed for these studies, such as 4-OH-RA, 4-OH-RAc, and 5,6-epoxy-RA, were synthesized for use as substrates for human UGTs. Metabolites such as these are involved in mediating atRA function. For example, 4-oxo-RA binds to retinoid receptors with an affinity comparable to that of atRA and can regulate the expression of genes (4). 4-oxo-RA can also bind to cellular atRA binding proteins (40). All of this suggests that this derivative possesses biological activity similar to that of atRA.

Since limited information exists on the biosynthesis and biotransformation of 5,6-epoxy-RA, we were particularly interested in characterizing the metabolism of this important compound. 5,6-Epoxy-RA has been identified as a major in vivo metabolite of atRA (as well as retinol and RAc) in rodents (12, 41–43). We have recently identified 5,6-epoxy-RA as a major in vivo metabolite of 13-cis RA in skin, and we have detected the glucuronide of 5,6-epoxy-RA in human bile. 5,6-Epoxy-RA also exhibits potent activity in various biological assays. For example, 5,6-epoxy-RA was more effective than RAc at producing potent growth effects in vitamin A-deficient rats (44). In addition, the oxirane exhibited inhibitory effects similar to those of atRA on 12-O-tetradecanoylphorbol-13-acetate-dependent tu-

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**Table II**

Kinetics of glucuronidation of atRA and 4-OH-RA using human liver microsomes (HLM15) and recombinant UGT2B7

| Substrate | \(K_m\) \(\mu M\) | \(V_{max}\) pmol/mg × min | \(V_{max}/K_m\) µl/min × mg |
|-----------|-----------------|---------------------------|--------------------------|
| atRA      | 1.5 ± 1         | 764 ± 17                  | 509                      |
| 4-OH-RA   | 273 ± 17        | 2176 ± 97                 | 8                        |

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\(^2\) V. M. Samokyszyn, unpublished data.
Retinoid Glucuronidation by Human Liver Microsomes and UGTs

mor promotion in the two-stage (initiation-promotion) mouse skin carcinogenesis assay (45). Additionally, 5,6-epoxy-RA is more potent than atRA in opposing the effects of 12-O-tetradecanoylphorbol-13-acetate on the induction of tumor promotional markers in bovine lymphocytes (31) and polymorphonuclear leukocytes (46).

These previous findings imply that the retinoid response may involve metabolic activation of atRA to the 5,6-epoxide with the oxirane representing the pharmacologically active agent. Epoxidation of these retinoids does not appear to occur through a cytochrome P450-catalyzed mechanism (47). We have unequivocally demonstrated that the 5,6-epoxides of atRA and 13-cis-RA are generated via peroxyl radical-dependent mechanisms (33, 38, 39, 48–50). In addition, we have recently demonstrated that 5,6-epoxy-RA is generated during prostaglandin H synthase-catalyzed oxidation of atRA involving a direct oxene transfer mechanism analogous to cytochrome P450.8

Table I shows the rates of glucuronidation and identification of the type of glucuronide formed for various retinoids. These data demonstrate that glucuronidation of atRA, 4-oxo-RA, and 5,6-epoxy RA is directed toward the carboxyl function of these substrates, whereas the 4-hydroxylated derivatives are glucuronidated almost exclusively at the 4-0H position. This gives rise to three observations. 1) 4-OH-derivatives of atRA and RAc are glucuronidated at their hydroxyl function by human liver microsomes and UGT2B7 with significantly higher activities than those observed for the formation of carboxyl-linked glucuronides of atRA and the 4-oxo- and 5,6-epoxy derivatives of atRA. A comparison of hydroxylated versus non-hydroxylated compounds suggests that the 4-hydroxy group directs glucuronidation to the hydroxyl function and that the carboxyl group is not required for glucuronidation to occur at the 4-OH position. However, the significantly lower enzymatic activity toward 4-OH-RA indicates that a free carboxyl function is required for optimal glucuronidation. 2) atRA, 5,6-epoxy RA, and 4-oxo-RA are glucuronidated exclusively at the carboxyl function. 3) Although the glucuronidation of 4-OH substrates by UGT2B7 is similar to that found in microsomes, the significantly lower activity of the recombinant protein toward 4-oxo-RA and 5,6-epoxy-RA might suggest the involvement of other UGT isoforms in the glucuronidation of these retinoids. Other isoformic forms of atRA, such as 9-cis- and 13-cis-RA, were also actively glucuronidated by human liver microsomes. Of the three compounds, atRA, 9-cis-RA, and 13-cis-RA, the 9-cis derivative is the best substrate for microsomal glucuronidation at the carboxyl position and, in comparison to the other retinoids studied, is second only to 5,6-epoxy-RA.

The Km values for the formation of the carboxyl-linked glucuronide of atRA is in the low micromolar range (1.3–1.5 m) both for HLM15 and recombinant UGT2B7. The maximal catalytic rates (Vmax) for atRA, as determined with HLM15 and recombinant UGT2B7, are 764 and 523 pmol glucuronidated × min−1 × mg protein−1, respectively. These correspond to catalytic efficiencies (Vmax/ Km) of 509 µl × min−1 × mg−1 for HLM15 and 402 µl × min−1 × mg−1 for recombinant UGT2B7, revealing significant efficiency of formation of atRA carboxyl-linked glucuronide. The Km values for 4-OH-directed glucuronidation are 273 and 221 µM for HLM15 and recombinant UGT2B7, respectively. Typical Vmax values were determined to be in the low nanomolar range (2176 and 1709 pmol × min−1 × mg−1 for HLM15 and recombinant 2B7, respectively), leading to a much lower efficiency of formation for the hydroxyl-linked glucuronides, as shown by a Vmax/ Km of 8 µl × min−1 × mg−1 for both HLM15 and recombinant UGT2B7. In general, the presence of the hydroxyl group in the retinoid moiety switches the site of glucuronidation from carboxyl to hydroxyl and reverses the affinity of the UGT(s) involved. The corresponding catalytic efficiencies (Vmax/ Km) were several hundredfold higher for glucuronidation of the carboxyl function of atRA than for glucuronidation of the 4-OH moiety of the hydroxylated retinoid.

Of the UGT isoforms investigated to date, human recombinant UGT2B7 has the highest capacity to glucuronidate atRA and 4-OH-RA. UGT2B7 activities toward atRA and 4-OH-RA are similar to the reported activities in human liver microsomes, suggesting that UGT2B7 plays a key role in metabolizing atRA and 4-OH-RA to the carboxyl-linked RAG and the hydroxy-linked 4-OH-RAG. UGT2B7 is capable of catalyzing the biosynthesis of the hydroxyl-linked glucuronide when 4-OH-RA is the substrate or the carboxyl-linked glucuronide when atRA is the substrate. Recent studies on glucuronidation of steroid hormones and fatty acids by UGT2B7 have shown that this isoform is actively involved in the formation of both hydroxyl- and carboxyl-linked glucuronides of those lipophilic substrates (30). Taken collectively, retinoids, steroid hormones, and fatty acids are important ligands involved in initiating cellular signaling events. We postulate that UGT2B7 may be involved in controlling intracellular levels of ligands, such as steroids and atRA. If this is the case, it may also be involved in a feedback loop that controls the amounts of ligands available for steroid and retinoid receptors.

In summary, we speculate that atRA induces its own oxidative metabolism via a cytochrome P450, CYP26, mechanism followed by a UGT-dependent mechanism. The hydroxyl-linked glucuronide of 4-OH-RA is the directed product of atRA metabolism by CYP26. Thus, 4-OH glucuronidation of 4-OH-RA terminates the biological activity of atRA, while the carboxyl-linked glucuronide of atRA might be a biologically active compound involved in cellular processes. Thus, CYP26 and UGT2B7 may together play a crucial role in the metabolism and biological fate of atRA.

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