Tazarotene (Fig. 1), a novel acetylenic retinoid, is known to be effective in the topical treatment of psoriasis and acne (Weinstein, 1997; Shalita et al., 1999). Tazarotene, a prodrug, was designed to undergo rapid and complete metabolism to its active metabolite tazarotenic acid (Fig. 1). The exact mechanism of action of tazarotenic acid may have significant effects on its systemic exposure. The objective of this study was to identify the human liver microsomal enzyme(s) responsible for the in vitro metabolism of tazarotenic acid. Tazarotenic acid was incubated with 1 mg/ml pooled human liver microsomes, in 100 mM potassium phosphate buffer (pH 7.4), at 37°C, over a period of 30 min. The microsomal enzymes that may be involved in tazarotenic acid metabolism were identified through incubation with microsomes containing cDNA-expressed human microsomal isozymes. Chemical inhibition studies were then conducted to confirm the identity of the enzymes potentially involved in tazarotenic acid metabolism. Reversed-phase high performance liquid chromatography was used to quantify the sulfoxide metabolite, the major metabolite of tazarotenic acid. Upon incubation of tazarotenic acid with microsomes expressing CYP2C8, flavin-containing monoxygenase 1 (FMO1), or FMO3, marked formation of the sulfoxide metabolite was observed. The involvement of these isozymes in tazarotenic acid metabolism was further confirmed by inhibition of metabolite formation in pooled human liver microsomes by specific inhibitors of CYP2C8 or FMO. In conclusion, the in vitro metabolism of tazarotenic acid to its sulfoxide metabolite in human liver microsomes is mediated by CYP2C8 and FMO.

Materials and Methods

Chemicals and Reagents. Tazarotenic acid, the sulfoxide metabolite of tazarotenic acid, the sulfone metabolite of tazarotenic acid and internal standard 4-(4,4-dimethylchroman-6-ylethynyl)-benzoic acid (AGN 190252) were provided by Allergan, Inc. (Irvine, CA). The following chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO): paclitaxel, 9-cis-retinoic acid, 13-cis-retinoic acid, quercetin, potassium phosphate monobasic, potassium phosphate dibasic, β-NAD, and glucose 6-phosphate monosodium salt. Glucose-6-phosphate dehydrogenase was purchased from Roche.
The incubation mixture was vortexed and centrifuged at 1000 g for 10 min to remove protein. The supernatant (100 µl) was injected onto the HPLC system.

**Metabolism of tazarotenic acid by cDNA-expressed P450 isozymes.** Microsomes (1 mg/ml) prepared from human B-lymphoblastoid cell lines containing specific human cytochrome P-450 isozymes were incubated with 10 µM tazarotenic acid, for 30 min, at 37°C, in the presence of an NADPH-regenerating system, in a final volume of 1 ml. The microsomes contained CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11. All incubations were performed in duplicate on two separate occasions.

**Chemical inhibition of CYP2C8 in pooled human liver microsomes.** Inhibition experiments using pacthaxel, 9-cis-retinoic acid, 13-cis-retinoic acid and quercetin as inhibitors of CYP2C8, were conducted in a similar manner. CYP2C8 is believed to mediate the metabolism of pacthaxel (Taxol), 9-cis-retinoic acid (Panretin), and 13-cis-retinoic acid (Accutane) (Parkinson, 1996; Mugford and Kedderis, 1998; Marill et al., 2002). Quercetin is a specific inhibitor of CYP2C8 (Parkinson, 1996). Both the inhibitor and tazarotenic acid were added to pooled human liver microsomes (1 mg/ml) before addition of cofactors in a total volume of 0.5 ml and incubated for 30 min, at 37°C.

For all IC50 experiments, the concentration of tazarotenic acid was 10 µM. The inhibitor concentration varied from 0.1 up to 1000 µM. Control incubations containing no inhibitor were performed during each IC50 experiment. K_i experiments were performed at concentrations of tazarotenic acid from 10 to 200 µM. At least four different substrate concentrations were used in each K_i experiment to ensure substrate concentrations ranged from below K_m to above K_m. Inhibitor concentrations used were 0, 1, 5, 25, 100 µM for each K_i experiment. All incubations were performed in duplicate on two separate occasions. K_i values were used to calculate the in vivo predicted fractional inhibition (i) values. The i value approximates the ratio of the anticipated human plasma concentration of a drug to the K_i of that drug on the oxidation of tazarotenic acid to the sulfoxide metabolite.

**Chemical inhibition of FMO in pooled human liver microsomes.** Inhibition experiments to determine the IC50 of methimazole as an inhibitor of FMO activity were conducted by adding both inhibitor and tazarotenic acid to 0.25 mg/ml prepared from baculovirus insect cell lines engineered to stably express specific human liver microsomal isoforms were obtained from BD Gentest Corporation (Woburn, MA).

**Experimental Protocols. Incubation method optimization.** The incubation conditions were first optimized in preliminary experiments using pooled human liver microsomes by varying the substrate concentration (1 to 200 µM), the incubation time (20 to 90 min) and the microsomal protein content (0.25 to 1 mg/ml). The optimized incubation conditions were chosen based on the formation of the sulfoxide metabolite from tazarotenic acid in human liver microsomes. The microsomal reaction mixture contained 10 µM tazarotenic acid, 1 mg/ml human liver microsomal protein, NADPH-regenerating system (0.4 mM NADP, 4 mM glucose 6-phosphate, 2 mM MgCl2, 0.6 units/ml glucose-6-phosphate dehydrogenase and 1 mM NAD) and 0.1 M potassium phosphate buffer (pH 7.4). Incubations were conducted in final volume of 2 ml, in a water bath shaker (Precision, Chicago, IL), at 37°C, for 30 min. The reaction was terminated by transferring a 400-µl aliquot of microsomal reaction mixture to an Eppendorf tube containing 400 µl of ice-cold acetonitrile and 10 µl of 100 µg/ml internal standard. The resulting sample (0.8 ml) was vortexed and centrifuged at 1000g for 10 min to remove protein. The supernatant was evaporated to dryness under nitrogen; the dried residue was reconstituted in 0.4 ml of mobile phase (20% ACN, 80% 10 mM ammonium acetate/0.5% acetic acid, pH 3.4) and centrifuged at 1000g for 10 min. The supernatant (100 µl) was injected onto the HPLC system.

**Metabolism of tazarotenic acid by cDNA-expressed FMO enzymes.** Microsomes (0.25 mg/ml) prepared from baculovirus insect cell lines containing human flavin-containing monoxygenase (FMO) isozyme were incubated with 10 µM tazarotenic acid, for 30 min, at 37°C, in the presence of an NADPH-regenerating system, in a final volume of 1 ml. The microsomes contained FMO1 (found in human fetal livers) or FMO3 (found in adult human livers) (Cashman, 1995). All incubations were performed in duplicate on two separate occasions. A Hewlett Packard model 1100 solvent (Agilent Technologies, Palo Alto, CA) equipped with a Hewlett Packard model 1100 UV-visible detector (Agilent Technologies) was employed. Separation of tazarotenic acid and its metabolites was achieved on a Phenomenex Luna C18 column (3.0 mm x 150 mm, 5 µm particle size) (Phenomenex, Torrance, CA). Mobile phase A was 100% acetonitrile and mobile phase B consisted of 10 mM ammonium acetate/0.5% acetic acid, pH 3.4. The following gradient program with a flow rate of 0.6 ml/min was used. Zero to fifteen minute mobile phase A 20 to 80%, 15 to 18.5 min mobile phase A 80%, and 18.5-20 min mobile phase A 80–20%. Tazarotenic acid and its metabolites were detected using a UV-visible detector.
Erand Hewlett Packard Data Acquisition System 1.0a (Agilent Technologies) was employed for peak integration and data analysis.

**Mass spectral analyses** were performed on a PE-Sciex API 365 tandem mass spectrometer (Applied Biosystems, Foster City, CA). The liquid chromatographic separation was performed on a reversed-phase HPLC column (LUNA C-18, 2.0 mm x 30 mm, 3 μm particle size; Phenomenex Torrance, CA) eluted at 0.2 ml/min using a similar HPLC gradient system. The mass spectrometer was operated in positive ion mode of electrospray method with full scan from m/z 67 to 350.

**Data Analysis.** GraFit Version 4.0 (Erithacus Software Limited, London, UK) was used for determining $K_m$, $V_{max}$, $IC_{50}$ and $K_i$ values. Depending on the study type, each data set obtained from an incubation experiment was fitted to the appropriate enzyme kinetics equation by nonlinear regression analysis. Each data set included mean values collected from two separate determinations performed in duplicate each time (i.e., for a total of four replicates). To ensure these parameters were determined during linear enzyme kinetics, assays were performed under initial rate conditions (Fig. 3).

**Results**

**Incubation Method Optimization.** Tazarotenic acid was metabolized by human liver microsomes to one major peak, labeled sulfoxide metabolite (Fig. 2). The Michaelis constant ($K_m$) for the sulfoxidation of tazarotenic acid, under these conditions, was 24.9 ± 8.0 μM, and the maximum velocity of the reaction ($V_{max}$) was 210 ± 38 pmol/mg/min (Fig. 3).

**Identification of the Metabolite in Human Liver Microsomes.** Preliminary characterization of the major metabolite peak, the sulfoxide metabolite of tazarotenic acid, and parent compound peak (tazarotenic acid) in the incubated human liver microsomal sample was performed by comparing the HPLC retention times with those of the standard sulfoxide metabolite, tazarotenic acid and internal standard as illustrated in Fig. 2. The characterization of the sulfoxide metabolite was also carried out by LC/MS/MS. The mass spectral data of the sulfoxide metabolite were compared with the mass spectral data of standard metabolite (Fig. 4). From the LC/MS/MS spectra of sulfoxide metabolite standard, it was found that this metabolite produced the protonated molecular ion [M + H]$^+$ at an orifice voltage of 41 V.

The positive-ion mode of the sulfoxide metabolite exhibited a major protonated molecular ion at m/z 340. The mass spectrum also showed at least three characteristic fragments at m/z 323, 308, and...
280. The resultant fragments may represent the loss of the hydroxy group (m/z 323), the combined loss of the hydroxy group and a methyl group (m/z 308), and the combined loss of a methyl group and carboxyl group (m/z 280) from the parent ion. The fragmentation pattern produced by standard sulfoxide metabolite was identical to that produced by the metabolite formed after incubation with human liver microsomes.

Metabolism of Tazarotenic Acid by cDNA-expressed P450 Isozymes. Involvement of CYP2C8 in tazarotenic acid metabolism was determined using cloned human cytochrome P450 isozymes. When tazarotenic acid was incubated with microsomes containing individually expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP4A11 at 1 mg/ml for 30 min, marked formation of the sulfoxide metabolite was only detected in the microsomes containing CYP2C8 (Fig. 5).

Effect of Chemical Inhibition on CYP2C8-mediated Metabolism of Tazarotenic Acid. Involvement of CYP2C8 in tazarotenic acid metabolism was further confirmed by inhibition of the formation of the sulfoxide metabolite by paclitaxel, 9-cis-retinoic acid, 13-cis-retinoic acid, and quercetin. Paclitaxel, a potential CYP2C8 substrate (Parkinson, 1996) inhibited the formation of the sulfoxide metabolite from tazarotenic acid in a concentration-dependent manner with an IC_{50} of 14.9 ± 6.2 μM (Fig. 6) and a K_i of 30.0 ± 7.0 μM. 9-cis-retinoic acid and 13-cis-retinoic acid, both potential CYP2C8 substrates (Mugford and Kedderis, 1998; Marill et al., 2002) inhibited the formation of the sulfoxide metabolite from tazarotenic acid in a concentration-dependent manner. The IC_{50} values determined for 9-cis-retinoic acid and 13-cis-retinoic acid were 17.6 ± 2.8 μM and 15.1 ± 2.5 μM, respectively, and their respective K_i values were determined to be 20.2 ± 8.7 and 66.2 ± 14.3 μM. Quercetin, a specific inhibitor of CYP2C8 inhibited the formation of the sulfoxide metabolite from tazarotenic acid in a concentration-dependent manner with an IC_{50} of 4.07 ± 2.69 μM and a K_i of 19.7 ± 8.2 μM. The results of these chemical inhibition studies are summarized in Table 1.

Metabolism of Tazarotenic Acid by cDNA-expressed FMO Isozymes. It was determined that both FMO1 and FMO3 could mediate the metabolism of tazarotenic acid to its sulfoxide metabolite using microsomes containing cloned human FMO. When tazarotenic acid was incubated with microsomes expressing FMO1 or FMO3 at 0.25 mg/ml for 30 min at 37°C, a marked increase in the formation of the sulfoxide metabolite was only detected in the microsomes containing FMO1 or FMO3 (Fig. 7). Concurrent to these experiments tazarotenic acid was incubated with CYP3A4 as a negative control.

Effect of Chemical Inhibition on FMO-mediated Metabolism of Tazarotenic Acid. Methimazole is a known substrate of FMO and is commonly used as a competitive inhibitor (Rettie and Fisher, 1999). The involvement of FMO in tazarotenic acid metabolism was supported by the inhibition of sulfoxide metabolite formation by methimazole in a concentration-dependent manner with an IC_{50} of 60.2 ± 16.2 μM (Fig. 8).
Effect of CYP2C8 substrates and inhibitors on the metabolism of tazarotenic acid to the sulfoxide metabolite

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 ± S.E. (μM)</th>
<th>Ki ± S.E. (μM)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel (Taxol)</td>
<td>14.9 ± 6.2</td>
<td>30.0 ± 7.0</td>
<td>0.00743–0.123</td>
</tr>
<tr>
<td>9-cis-Retinoic acid (Panretin)</td>
<td>17.6 ± 2.8</td>
<td>20.2 ± 8.7</td>
<td>0.00483–0.0242</td>
</tr>
<tr>
<td>13-cis-Retinoic acid (Accutane)</td>
<td>15.1 ± 2.5</td>
<td>66.2 ± 14.3</td>
<td>0.0178–0.0474</td>
</tr>
<tr>
<td>Quercetin</td>
<td>4.07 ± 2.69</td>
<td>19.7 ± 8.2</td>
<td>0.00108–0.0207</td>
</tr>
</tbody>
</table>

* i = Predicted fractional inhibition in vivo based on anticipated human plasma concentration following oral administration of tazarotenic acid (0.6 μM), paclitaxel (Taxol) (0.23–4.3 μM) (PDR entry), 13-cis-retinoic acid (Accutane) (0.556–1.53 μM) (PDR entry), 9-cis-retinoic acid (0.149–0.762 μM) (Razvi et al., 1998), quercetin (0.00331–0.648 μM) (Hollman et al., 1996), and a Kᵢ value of 25 μM under these tazarotenic acid incubation conditions.

**Discussion**

These studies demonstrated that the in vitro metabolism of tazarotenic acid in human liver microsomes results in the production of one major metabolite, namely the sulfoxide metabolite of tazarotenic acid. The metabolite structure was confirmed by LC/MS/MS.

Initially, preliminary correlation analysis studies were conducted using individual liver microsomal preparations from human donors phenotyped by the supplier for the following P450-specific activities: CYP1A2, CYP2A6, CYP2C, CYP2D, CYP2E1, CYP3A, and CYP4A. The correlation analysis results demonstrated that correlation coefficient (r) between the tested P450-specific activities with the oxidation of tazarotenic acid to its sulfoxide metabolite was less than 0.6. However, CYP2C19-mediated hydroxylation of 5-methoxytio was used to phenotype CYP2C activity. This probe substrate is specific to CYP2C19 but not to other CYP2C isoforms, namely CYP2C8. Overall, these correlation studies indicated that further experimental work was required to characterize the specific P450 enzymes responsible for the metabolism of tazarotenic acid. Hence, the studies using cDNA-expressed P450 and FMO isoforms and specific inhibitor studies were conducted to determine which metabolic enzymes were responsible for the sulfoxidation of tazarotenic acid.

The specific human microsomal enzymes responsible for the metabolism of tazarotenic acid were identified through incubation with cDNA expressed isoforms. These studies demonstrated that the bio-transformation of tazarotenic acid to the sulfoxide metabolite, in human liver microsomes, can be mediated by CYP2C8 and FMO. These findings were supported by inhibition of tazarotenic acid metabolism by specific inhibitors for either CYP2C8 or FMO. Furthermore, CYP2C8 mediation of tazarotenic acid metabolism to the sulfoxide metabolite was supported by the findings of an independent study (Tang-Liu et al., 1999). This study evaluated the effect of tazarotenic acid as an inhibitor of human P450 enzymes. The results of the study concluded that tazarotenic acid most potently inhibited CYP2C8-mediated paclitaxel 6α-hydroxylation as compared with other assayed enzymatic activities. Paclitaxel (Taxol) is a drug commonly administered to cancer patients. CYP2C8-mediated metabolism of tazarotenic acid, an acetylenic retinoid, is further supported by...
a recent study that examined the metabolism of retinoic acid isomers. This study identified CYP2C8 to be active in the metabolism of both 9-cis-retinoic acid and 13-cis-retinoic acid (Marill et al., 2002).

The chemical inhibition studies with CYP2C8 inhibitors determined $K_i$ values of compounds that may be coadministered with tazarotene. The in vivo predicted fractional inhibition ($i$) value is useful in establishing the potential for interaction between coadministered drugs. The degree of potential competitive inhibition will depend on the substrate concentration, the inhibitor concentration, and the $K_m$ and $K_i$ values (Lin and Lu, 1998). This $i$ value is based on the ratio of the anticipated human plasma concentration of a drug to the $K_i$ of that drug, on the oxidation of tazarotenic acid to its sulfoxide metabolite. Generally, if this ratio is less than 0.01, the likelihood of a significant interaction between the two drugs is low. As listed in Table 1, the predicted fractional inhibition by paclitaxel (Taxol) on tazarotenic acid metabolism to the sulfoxide metabolite is 0.00743 to 0.123 depending on the dose of Taxol administered to the patient. The predicted fractional inhibition by 9-cis-retinoic acid (Panretin) on the sulfoxidation of tazarotenic acid is between 0.00483 and 0.0242. The $i$ value for the potential 13-cis-retinoic acid (Accutane)-tazarotenic acid interaction is between 0.0178 and 0.0474. The potential fractional inhibition on tazarotenic acid metabolism by the dietary flavonoid, quercetin is between 0.00108 and 0.0207. However, the present study demonstrates that the metabolism of tazarotenic acid to the sulfoxide metabolite is mediated by two different enzymes, namely, CYP2C8 and FMO. Potentially, if one metabolic pathway is inhibited the alternative pathway can mediate the metabolism of tazarotenic acid. The present study demonstrated that paclitaxel inhibited the formation of the sulfoxide metabolite of tazarotenic acid in a concentration-dependent manner. At the maximal inhibitor concentration of 1000 $\mu$M, 60% control activity was still observed. This residual activity may represent the contribution of other enzymes capable of mediating the sulfoxidation of tazarotenic acid such as FMOs. Furthermore, methimazole also inhibited the sulfoxidation of tazarotenic acid in a concentration-dependent manner. At the maximal inhibitor concentration of 1000 $\mu$M, 40% control activity was still observed. In this experiment, the residual activity may be the contribution of enzymes other than FMOs capable of mediating the sulfoxidation of tazarotenic acid such as CYP2C8.

Concomitant administration of two or more drugs can result in undesirable drug-drug interactions. Competitive inhibition of the metabolism of one drug by another may result in undesirable elevations in plasma drug concentrations, which is of clinical importance for both therapeutic and toxicological reasons (Lin and Lu, 1997). Through identifying the enzymes responsible for the metabolism of a drug, drug-drug interactions can be predicted and managed, usually by appropriate dosage adjustment (Lin and Lu, 1997). The results of this study demonstrate that both CYP2C8 and FMO enzymes are responsible for the metabolism of tazarotenic acid in humans.

References


Oxidoreductase (PDR) (1999) entry for Taxol TM Injection (Bristol-Myers Squibb Oncology/Lymphology division) and entry for Accutane (Roche Laboratories) PDR web site; www.pdr.net.


