Identification of a Tryptanthrin Metabolite in Rat Liver Microsomes by Liquid Chromatography/Electrospray Ionization-Tandem Mass Spectrometry

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Tryptanthrin originally isolated from Isatis tinctoria L. has been characterized to have anti-inflammatory activities through the dual inhibition of cyclooxygenase-2 and 5-lipoxygenase mediated prostaglandin and leukotriene syntheses. To characterize Phase I metabolite(s), tryptanthrin was incubated with rat liver microsomes in the presence of NADPH-generating system. One metabolite was identified by liquid chromatography/electrospray ionization-tandem mass spectrometry. M1 could be identified as a metabolite mono-hydroxylated on the aromatic ring of indole moiety from the MS² spectra of protonated tryptanthrin and M1. The structure of metabolite was confirmed as 8-hydroxytryptanthrin with a chemically synthesized authentic standard. The formation of M1 was NADPH-dependent and was inhibited by SKF-525A, a general CYP inhibitor, indicating the cytochrome P450 (CYP)-mediated reaction. In addition, it was proposed that M1 might be formed by CYP 1A in rat liver microsomes from the experiments with enriched rat liver microsomes.

Key words tryptanthrin; rat liver microsome; LC-ESI/MS; metabolite; cytochrome P450 (CYP)

Tryptanthrin (indolo-[2,1-b]-quinazoline-6,12-dione, Fig. 1) is an alkaloid originally isolated from Isatis tinctoria L. (woad, Brassicaceae). I. tinctoria, a medicinal plant in temperate climate zones, has been used since antiquity as a source for indigo dye production. The medicinal properties of woad were esteemed in different cultures. Throughout the source for indigo dye production. The medicinal properties of woad were esteemed in different cultures. Throughout the history, I. tinctoria was employed in Europe for the treatment of wounds, ulcers, snake bites, hemorrhoids and various inflammatory ailments. Tryptanthrin is also an active ingredient of traditional Japanese herbal remedies for fungal infections. Roots and leaves of I. tinctoria Fort. have been used in China, and they are official drugs in the Chinese Pharmacopoeia.

In vivo studies in acute and chronic models of inflammation, investigation of skin penetration, and a clinical pilot study with topical woad preparations and pure substances demonstrated anti-inflammatory properties of lipophilic Isatis extracts. Studies extended the spectrum of antimicrobial activity of tryptanthrin to include bacteria, particularly Mycobacterium tuberculosis. Tryptanthrin is an agonist of the rat AhR. Therefore, it could induce CYP 1A1, which was confirmed by immunoblotting and CYP 1A1 activity assay.

For the synthesis of derivatives of tryptanthrin and its related compounds, only few methods have been reported for the introduction of some substituents at C6. A simple synthetic procedure has been reported by our group for tryptanthrin from readily available starting materials, which would be a useful method to introduce a substituent at C6.

Understanding the metabolic profile of certain compounds would be a critical step in the process of drug development nowadays. In this regard, no studies have been reported to determine tryptanthrin metabolism to our knowledge. Prior to in vitro approaches, it was necessary to determine possible metabolite(s) of tryptanthrin in vitro. Therefore, the objectives of our present studies were to identify phase I metabolite(s) of tryptanthrin and to characterize the production of tryptanthrin metabolite(s) in rat liver microsomes.

Recently, the tandem mass spectrometry has been widely used for the determination of various compounds because of its inherent accuracy, excellent sensitivity and selectivity. Considering these points, a reversed-phase liquid chromatography tandem mass spectrometry (LC-MS) technique with a small amount of sample following metabolism seemed to be the best choice for the investigation of tryptanthrin metabolite(s).

MATERIALS AND METHODS

Materials Tryptanthrin (purity, >99.8%) was obtained by the chemical synthesis described previously. Glucose 6-phosphate, NADPH, glucose 6-phosphate dehydrogenase, dexamethasone, phenobarbital, ammonium formate and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile, acetone and methanol were HPLC-grades from Merck Ltd. (Poole, U.K.). 3-Methylcholanthrene and formic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Ammonium hydroxide was obtained from Fisher Scientific (Somerset, NJ, U.S.A.). Other reagents were of analytical grade and used as received.

Animal Treatment Specific pathogen-free male Sprague Dawley rats (270—300 g) were obtained from Orient Co. (Seoul, Korea). The animals received at 5 weeks of age were acclimated for at least 1 week. Upon arrival, animals were

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randomized and housed two per cage. The animal quarters were strictly maintained at 23 ± 3 °C and 50 ± 10% relative humidity. A 12 h light and dark cycle was used with an intensity of 150—300 lux. For cytochrome P450 (CYP) enrichment, rats were pretreated with either 3-methylcholanthrene (40 mg/kg, i.p., 3 d) in corn oil, dexamethasone (50 mg/kg, i.p., 3 d) in corn oil, phenobarbital (80 mg/kg, i.p., 3 d) in saline, or acetone (5 ml/kg, p.o., 1 d). Twenty-four hours after the last dose in case of 3-methylcholanthrene, dexamethasone and phenobarbital, and 2 d after the dose in case of acetone, the animals were sacrificed.

**Preparation of Liver Microsomes** The livers wereperfused with saline to remove excess blood and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 9000 × g for 10 min at 4 °C and the resulting supernatants were centrifuged again at 105000 × g for 60 min at 4 °C. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. Aliquots of liver microsomes were stored at −80 °C until use. The content of microsomal protein was determined according to the method of Lowry et al. using bovine serum albumin as a standard.

**Biotransformation of Tryptanthrin** Metabolism of tryptanthrin (100 μM, final concentration) was determined with 1 mg/ml of microsomal protein in 0.1 M potassium phosphate buffer, pH 7.4. The reactions were initiated by the addition of a NADPH-generating system containing 0.14 mg/ml of microsomal protein in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% phenobarbital (80 mg/kg, i.p., 3 d). The reactions were incubated at 37 °C for 20 min in a final incubation volume of 500 μl. The reactions were then quenched by the addition of 1 ml ethyl acetate.

**Synthesis of Metabolite of Tryptanthrin** 8-Hydroxytryptanthrin (1.33 g, 4.31 mmol) in freshly distilled CH₂Cl₂ (300 ml) was added AlCl₃ (3.05 g, 22.43 mmol) slowly. The resulting mixture was stirred at 0 °C until reaction was complete. The mixture was then concentrated at 300 °C until a residue was obtained.

When the color of the reaction mixture turned to purple, isatoic anhydride (1.16 g, 7.01 mmol) in dry DMF (15 ml) was slowly added. The resulting mixture was stirred overnight and heated at 50 °C for 30 min. The yellow precipitate formed was collected and washed with CH₂OH to give 1.33 g of a. Concentration of the filtrate afforded additional 0.14 g of 3 (overall yield 77%): mp 215 °C (sublimed). 1H-NMR (CDCl₃, 250 MHz) δ 8.49 (d, 1H, J = 8.8 Hz, H10), 8.40 (dd, 1H, J = 7.8, 1.1 Hz, H1), 8.00 (d, 1H, J = 8.1 Hz, H4), 7.81 (td, 1H, J = 8.1, 1.2 Hz, H2), 7.64 (td, 1H, J = 8.1, 1.1 Hz, H3), 7.35 (d, 1H, J = 2.5 Hz, H7), 7.28 (dd, 1H, J = 8.8, 2.5 Hz, H9), 3.87 (s, 3H). 13C-NMR (CDCl₃, 62.5 MHz) δ 182.68, 158.71, 157.71, 146.59, 144.73, 134.91, 130.69, 130.21, 127.39, 125.04, 123.88, 122.93, 119.14, 113.94, 108.35, 55.97. Anal. Calcd for C₁₅H₁₃NO₅: C, 69.26; H, 3.59; N, 10.05. Found C, 69.26; H, 3.62; N, 10.07. Compound C, 69.26; H, 3.59; N, 10.05.

8-Hydroxytryptanthrin (4): Into a mixture of 8-methoxytryptanthrin (1.2 g, 4.31 mmol) in freshly distilled CH₂Cl₂ (300 ml) was added AlCl₃ (3.05 g, 22.43 mmol) slowly. The resulting mixture was refluxed for 18 h and cooled to room temperature. The reaction mixture was carefully added water (ca. 200 ml) to afford red precipitate (945 mg). The filtrate was extracted with CH₂Cl₂, from which an additional 3.35 mg of 4 was collected (overall yield 86%): mp > 275 °C (sublimed). 1H-NMR (CDCl₃, 250 MHz) δ 8.26 (d, 2H, J = 8.0 Hz, H1, H10), 7.90 (m, 2H, H3, H4), 7.70 (ddd, 1H, J = 8.1, 7.8, 1.2 Hz, H2), 7.20 (dd, 1H, J = 8.5, 2.5 Hz, H7), 7.13 (d, 1H, J = 2.5 Hz, H7). Anal. Calcd for C₁₅H₁₃NO₅: C, 68.18; H, 3.05; N, 10.60. Found C, 68.26; H, 3.08; N, 10.59.

**LC-MS Spectrometry** The HPLC consisted of a surveyor system with the LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) equipped with an electrospray ionization (ESI) source. The column used for the separation was an Intersil® ODS-3, 5 μm (2.1 × 150 mm, GL Science). The HPLC mobile phases consisted of 100% A (water) and 100% acetonitrile (B). A gradient program was used for the HPLC separation at a flow rate of 250 μl/min. The initial composition was programmed 10% A and 90% B during 15 min. Nitrogen was used as the sheath gas at the flow rate of 1.05 l/min. ESI spray voltage was set at 5.30 kV. Capillary temperature was set at 300 °C and capillary voltage was set at 1.5 V. Tube lens offset was set at 58.80 V. The mass spectrometer was operated in the positive ion mode in m/z range of 100—300. Helium was used as the collision gas for the tandem mass spectrometric experiments, followed by the isolation of ions over a selected mass window of 1 Da.

**HPLC** HPLC analyses of tryptanthrin and its metabolite(s) in microsome-incubated samples were performed with a PU/610 pump and a UV/620 detector (GL Sciences Inc., Tokyo, Japan). The samples (20 μl) were injected into the chromatographic system and separated on an Intersil® ODS-3 column, 5 μm (4.6 × 150 mm, GL Sciences Inc., Tokyo, Japan) with a Phenomenex® SecurityGuard™ cartridge C18 (3.0 × 4.0 mm, Torrance, CA, U.S.A.). Separation was conducted with a linear gradient system from 20:80 to 80:20 for 30 min (90% acetonitrile with 10% 20 mM ammonium formate: 20 mM ammonium formate buffer) at pH 4.0. The analyses were performed at room temperature at a flow rate of 1.0 ml/min and an UV detection at 250 nm.
Statistics  The mean value ± standard error (S.E.) was determined for each treatment group of a given experiment. Dunnet’s t-test was used to compare statistical significance of data. The significant values at either \( p<0.05 \) (*) or \( p<0.01 \) (**) were represented as asterisks.

RESULTS AND DISCUSSIONS

Identification of Tryptanthrin Metabolite(s)  Tryptanthrin was biotransformed to one metabolite by rat liver microsomes in the presence of NADPH-generating system (data

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Fig. 2. Extracted Ion Chromatograms of Protonated Tryptanthrin and M1
Tryptanthrin was incubated in rat liver microsomes (2 mg/ml) in the presence of NADPH-generating system for 2 h at 37 °C (A), 10 μM synthesized authentic 8-hydroxytryptanthrin (B) and incubated sample spiked with synthesized 8-hydroxytryptanthrin (C).

Fig. 3. Representative CID Spectra and Proposed Structures of Fragment Ions from Protonated Tryptanthrin (A), M1 (B) and Synthesized 8-Hydroxytryptanthrin (C)
not shown). No metabolites were generated in the absence of NADPH, indicating a likely involvement of CYP enzymes in the metabolism of tryptanthrin (data not shown).

As shown in Figs. 2 and 3, the incubation of tryptanthrin with rat liver microsomes in the presence of NADPH-generating system generated a mono-hydroxylated metabolite. Their protonated molecules of tryptanthrin and M1 were observed at \( m/z 249.2 \) and \( m/z 265.2 \), respectively (Fig. 3). These results indicated that M1 would be a mono-hydroxylated form of tryptanthrin. Subsequently, the structure of protonated molecule of metabolite was identified by the full-scan MS detection.

The MS\(^2\) spectrum of protonated tryptanthrin \( (m/z \ 249.2) \) was depicted in Fig. 3A. The MS\(^2\) spectrum of protonated tryptanthrin showed product ions at \( m/z \ 221.2, \ 146.1, \ 130.1 \) and 121.0. The ion at \( m/z \ 221.2 \) corresponded to the loss of CO (28 Da) from the protonated tryptanthrin. The characteristic ion at \( m/z \ 146 \) was assigned to the formation of an odd-electron cation from the homolytic cleavage between the benzaldehyde and indol-2-ylidene-ammonium moieties.\(^{10} \) Fragmentation on quinazoline moiety might produce ions at \( m/z \ 130.1 \) as an even-electron ion, and \( m/z \ 121.1 \) as an odd-electron ion from the heterolytic cleavage between the indole and quinazoline moieties, respectively (Fig. 3A).

The MS\(^2\) spectrum of protonated M1 \( (m/z \ 265.2) \) was shown in Fig. 3B. M1 was observed at the retention time of 11.8 min (Fig. 2). The product ion at \( m/z \ 265.2 \) could be suggested as a hydroxylation on the aromatic ring of indole moiety from MS\(^2\) spectrum of protonated M1. The ion at \( m/z \ 237.1 \) was rationalized by CO elimination. The ions at \( m/z \ 146.1 \) and 162.8 were found to be characteristic ions for the presence of a hydroxyl group at one of the positions of the aromatic ring of indole moiety. The characteristic ion at \( m/z \ 121.0 \) was the same as observed in the MS\(^2\) spectrum of protonated tryptanthrin. Although MS\(^2\) spectra of protonated tryptanthrin and M1 indicated that the metabolite could be mono-hydroxylated on the indole ring, the exact structure of M1 could not be assigned.

Based on the mechanism and the electronic aspects in the CYP-mediated oxidation process of aromatic ring, 8-hydroxytryptanthrin was expected as the possible metabolite of tryptanthrin. To the best of our knowledge, this compound was not reported yet in the literature. We, thus, attempted to prepare 8-hydroxytryptanthrin using a method employed previously for the preparation of tryptanthrin as shown in Chart 1.\(^{7,13} \) Following the retention time and MS\(^2\) spectrum of synthesized M1, the synthesized 8-hydroxytryptanthrin was probed as M1 formed by rat liver microsomes (Figs. 2C, 3C).

Although numerous studies have been performed for biological activities of tryptanthrin and its derivatives, no studies on 8-hydroxytryptanthrin were attempted to date. In the literatures, 8-amino derivative of tryptanthrin showed better anticancer activity than tryptanthrin and 8-halogenated tryptanthrin had better anti-trypanosomal activities than tryptanthrin.\(^{15,16} \) These results indicate that the 8-substituted derivatives may have good biological activities. An attempt to compare the anti-inflammatory activity of tryptanthrin with metabolite M1 is currently under investigation. Because the metabolite M1 is a product formed by CYP enzymes and can be possibly formed in vivo, its biological activity would be significant to understand pharmacological action of tryptanthrin.

**Characterization of Tryptanthrin Metabolism in Rat Liver Microsomes** The formation of M1 was increased according to the duration of incubation time from 0 to 240 min, protein concentration of 0.5 to 5 mg/ml and substrate concentration of 50 to 200 \( \mu \)M (data not shown). In addition, the formation of M1 was significantly inhibited when SKF-525A was added into the reaction mixture (Fig. 4), indicating once again that the metabolism of tryptanthrin in rat liver microsomes was CYP-dependent.

To investigate the effects of different CYP isoforms on tryptanthrin metabolism, M1 formed by the incubation of tryptanthrin in either control, 3-methylcholanthrene-, phenobarbital-, dexamethasone- and acetone-induced rat liver microsomes were determined (Fig. 4). The production of metabolite M1 was increased in 3-methylcholanthrene- and dexamethasone-induced microsomes, by 3.73- and 1.49-fold, respectively, when compared with the production of M1 in un-induced microsomes. Take together, the present results indicate that CYP 1A might catalyze the production of M1 from tryptanthrin in rat liver microsomes.

In the present study, the production of metabolite M1 was reduced in the acetone-induced microsomes (Fig. 4). We
don’t have an explanation on this at the present time, but the further metabolism of M1 might be possible in these microsomes. In fact, in the rat liver microsomes, two metabolites were produced: one as metabolite M1 characterized in the present study and the other as a dihydroxy metabolite of which structure is still uncharacterized (data not shown). Although further studies are needed to determine the reason for reduced production of metabolite M1, the result suggested a further metabolism of metabolite M1 to uncharacterized metabolite M2 in acetone-induced microsomes. The hypothesis is currently under investigation in vitro and in vivo.

In the present study, tryptanthrin was found to be metabolized by CYP-dependent manners. It was also found that tryptanthrin might be metabolized to a mono-hydroxylated form. The structure of metabolite could be proposed, based on the results from the LC/ESI-MS and the synthesized authentic 8-hydroxytryptanthrin (Chart 1). The proposed metabolic pathway for tryptanthrin is summarized in Fig. 5. Knowledge of the proposed structures of the metabolite will be helpful in further studies on in vivo metabolism of tryptanthrin.

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REFERENCES