Mouse Pharmacokinetics and in Vitro Metabolism of (±)-Cremastranone

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Received July 21, 2018; accepted November 12, 2018

The objective of this study was to characterize pharmacokinetics and metabolism of (±)-cremastranone (CMT) in mouse. Plasma concentrations of CMT following a single oral dose (10 mg/kg) were all below quantitation limit throughout 24-h time course, indicating poor oral bioavailability. Its plasma levels declined rapidly, with a half-life ($t_{1/2}$) of 1.5 ± 0.3 min following a single intravenous dose (5 mg/kg). They were below the quantitation limit after 15 min post-dosing. CMT showed a high plasma clearance ($CL_{p}$) of 7.73 ± 3.09 L/h/kg. Consistently, CMT was metabolized rapidly, with a $t_{1/2} < 1$ min when it was incubated with liver or intestine S9 fractions of mouse and human in the presence of cofactors for CYP450, uridine 5’-diphosphoglucuronosyltransferase (UGT), and sulfotransferase (ST). Further studies showed that CMT was metabolized by CYP450, UGT, and ST in vitro in liver S9 fractions of mouse and human, with UGT being the major enzyme responsible for its rapid metabolism. CMT was metabolized by UGT and ST in intestine S9 fractions of mouse and human. Mono-demethylated (M1), mono-glucuronide (M2), and mono-sulfate (M3 and M4) metabolites were tentatively identified in vitro. In conclusion, the pharmacokinetics of CMT is suboptimal as a systemic agent, especially as an oral therapy, due to its extensive metabolism. This report provides possible structural modifications to design CMT derivatives with better pharmacokinetic properties.

Key words cremastranone; homoisoflavanone; pharmacokinetics; metabolism

INTRODUCTION

Cremastranone [5,7-dihydroxy-3-(3-hydroxy-4-methoxy-benzyl)-6-methoxyxchroman-4-one] is a homoisoflavanone natural product isolated from various plants, including Muscari armeniacum, Scilla natalensis, Chionodoxa luciliae, Mervilla plumbea, and Cremastra appendiculata (D. Don) 1–5. Cremastranone has been shown to possess some important biological activities such as anti-inflammatory and anti-angiogenic activities both in vitro and in vivo. 6) Its anti-angiogenic properties have been thoroughly demonstrated in various in vitro and in vivo models, including human retinal endothelial cells (HRECs), human umbilical vein endothelial cells (HUVECs), laser-induced choroidal neovascularization, and oxygen-induced retinopathy mouse models, suggesting that cremastranone may be useful as a therapeutic agent for treatment of proliferative ocular vascular diseases such as wet age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity. 7–9) Recently, (±)-cremastranone (CMT) has been synthesized and its anti-angiogenic activities have been demonstrated. 6) A series of homoisoflavanoids have also been synthesized using cremastranone as a scaffold and their anti-angiogenic activities have been tested both in vitro and in vivo with an aim to produce a drug candidate with more potent and selective anti-proliferative activity. 10,11) Furthermore, a forward chemical genetics approach has identified ferrochelatase as a molecular target of cremastranone. 12)

Although the therapeutic potential of cremastranone has well been demonstrated, especially for the treatment of ocular proliferative diseases, its pharmacokinetic and metabolic properties have not been reported previously to our knowledge. Cremastranone and its synthetic derivatives showing efficacy in animal models so far were all administered by intravitreal injection. It is inevitable to evaluate its pharmacokinetic and metabolic characteristics to test its potential as a systemic therapy agent. Thus, the objective of this study was to investigate the pharmacokinetics and metabolism of CMT using mouse animal model. We also conducted in vitro metabolism studies using mouse and human liver tissues to determine whether there might be species difference.

MATERIALS AND METHODS

Materials CMT was synthesized as described previously. 6) Glipizide, uridine diphosphoglucuronic acid (UDPGA), β-nicotinamide adenine dinucleotide phosphate reduced (NADPH), 3’-phosphoadenosine-5’-phosphosulfate (PAPS), dimethylacetamide (DMA), and hydroxypropyl-β-cyclodextrin (HP/βCD) were purchased from Sigma-Aldrich Korea (Kyunggi, Republic of Korea). Pooled male CD-1 mouse liver S9 (MSL9) fraction, pooled human liver S9 (HLS9) fraction, and uridine 5’-diphosphate (UDP)-glucuronosyltransferase (UGT) Reaction Mix Solution B (250 mM Tris·HCl, 40 mM MgCl2, and 0.125 mg/mL α-lamethin) were purchased from BD Korea (Seoul, Republic of Korea). Pooled male CD-1 mouse intestine S9 (MIS9) fraction and pooled human intestine S9 (HIS9) fraction were purchased from XenoTech, LLC (Lenexa, KS, U.S.A.). HPLC-grade water and acetonitrile were purchased from JT Baker (Phillipsburg, NJ, U.S.A.).

Pharmacokinetic Studies Animal studies were approved by the Institutional Animal Care and Use Committee of Korea University. Eight-week-old male ICR mice (30–35 g; DBL Co., Ltd., Chungbuk, Republic of Korea) were acclimated to the testing facility in a temperature- and humidity-controlled environment for approximately a week prior to the study. CMT was dissolved in DMA/20% aqueous HP/βCD (10/90 vol%)
at a concentration of 1 mg/mL for animal dosing. In each experiment, a group of three animals were dosed orally via oral gavage (p.o.) at a dose of 10 mg/kg with dose volume of 10 mL/kg or intravenously (i.v.) via tail vein injection at a dose of 5 mg/kg with a dose volume of 5 mL/kg. This p.o. dose was chosen for a human equivalent dose of ca. 50 mg based on average human body weight of 70 kg. The i.v. dose was given at 1/2 of the p.o. dose assuming ca. 50% oral bioavailability. About 40 µL of blood samples were collected into BD Microvette® 100 LH plasma separator tubes (BD Korea, Seoul, Republic of Korea) at 0, 5, 10, 15, 30 min, 1, 2, 4, 6, 8, and 24 h for i.v. and 0, 10, 20, 30 min, 1, 2, 4, 6, 8, and 24 h for p.o. through saphenous vein. Blood samples were centrifuged at 6000 × g for 5 min to separate plasma and stored in a freezer until analysis.

Calibration standards and quality control (QC) samples were prepared by spiking 15 µL of blank plasma with 5 µL of analyte working solution prepared in 50% aqueous acetonitrile. For sample preparation, 15 µL aliquot of plasma sample was mixed with 3 volumes of ice-cold acetonitrile containing glipizide (500 ng/mL) as analytical internal standard. The mixture was briefly vortexed, sonicated, and centrifuged at 3000 × g for 20 min. The supernatant was then mixed with the same volume of HPLC grade water before analysis. Sample analyses were conducted using Agilent 6460 Triple Quadrupole LC-MS/MS system equipped with a dual AJS ESI ion source and Agilent 1290 Infinity HPLC system (Agilent Technologies Korea, Seoul, Republic of Korea). Chromatographic separation was performed on a Phenomenex Kinetex XDB-C18 column (2.1 × 50 mm, 2.6 μm) with a Phenomenex Security Guard C18 guard column (4 × 20 mm) maintained at 40°C. The mobile phase consisted of water (A) and acetonitrile (B): 0–2 min 5% B, 2–3 min to 95% B, and 3–5 min 95% B. Total run time including an equilibration time of 3 min was 8 min. The flow rate was 0.4 mL/min. The injection volume was 5 µL. Detection of analyte ions was performed in multiple reaction monitoring (MRM) mode to monitor the following transitions: m/z 345 > 330 and 444 > 319 in negative ion mode for CMT and glipizide, respectively. Ion source parameters of the mass spectrometer were set as follows: capillary voltage, −3 kV; drying gas, 12 L/min; nebulizer gas, 35 L/min; sheath gas, 11 L/min; sheath gas heater, 400°C; and drying gas temperature, 340°C. The fragmentor voltage was set at −150 and −100 V for CMT and glipizide, respectively. The collision energy (CE) was set at −30 and −15 eV for CMT and glipizide, respectively. The developed analytical method was specific as no interfering peaks were observed in the chromatograms of the blank mouse plasma. The lower limit of quantitation (LLOQ) was 15.6 ng/mL with a signal-to-noise ratio based on root mean square noise > 10. The calibration curves were linear in the range of 15.6–4000 ng/mL (r² > 0.997). The precision and accuracy of the assay were assessed using LLOQ samples and QC samples prepared in triplicate at 50, 500, and 3000 ng/mL. Precision was evaluated by examining the coefficient of variation (%CV) of the QC samples. Accuracy was assessed by the relative error (%RE) of the observed concentration from the nominal concentration. The %RE and %CV of the LLOQ samples were in the ranges of −19.0–14.8 and 4.5–17.0%, respectively. Those of the QC samples were in the ranges of 7.7–13.9 and 2.1–7.7%, respectively. Extraction recovery, matrix effect, and process efficiency were in the ranges of 99.5–105.7, 87.9–98.2, and 89.9–103.8%, respectively.

**Metabolism Studies** To investigate the metabolic stability, CMT was incubated with MLS9, MIS9, HLS9, and HIS9 fractions in the absence or presence of a mixture of cofactors (NADPH, UDPGA, and PAPS). The final CMT concentration was 1 µM as has been the case in common in vitro metabolic stability studies. Four microliters of 40 µM CMT in 40% aqueous acetonitrile was mixed with a solution containing 32 µL of UGT Reaction Mix Solution B, 40 µL of 4 mg protein/mL MLS9, MIS9, HLS9, or HIS9 fraction, and 68 µL of de-ionized water. The mixture was then warmed at 37°C for 5 min. The reaction was initiated by adding 16 µL of 10 mM mixture of NADPH, UDPGA, and PAPS dissolved in de-ionized water. The final incubation mixture contained 50 mM Tris–HCl (pH 7.5), 1 mg protein/mL MLS9, MIS9, HLS9, or HIS9 fraction, 1 µM CMT, 25 µg/mL alamethicin, 8 mM MgCl₂, and 1 mM cofactors in a volume of 160 µL. The reaction was terminated after 30 min of incubation at 37°C by adding 160 µL of ice-cold acetonitrile containing glipizide (500 ng/mL). The resulting mixture was centrifuged at 3000 × g for 20 min. Aliquots of the supernatant were subjected to LC-MS/MS analysis described below.

To identify metabolic enzyme(s) involved, CMT was incubated in a similar manner with MLS9, MIS9, HLS9, or HIS9 fractions in the absence or presence of an individual cofactor (NADPH, UDPGA, or PAPS). Potassium phosphate buffer (0.1 M, pH 7.4) was used as the medium with NADPH and PAPS whereas UGT Reaction Mix Solution B was used with UDPGA.

The LC-MS/MS system used for sample analyses consisted of Agilent 6530 Q-TOF LC-MS/MS system equipped with dual AJS ESI ion source and Agilent 1290 Infinity HPLC system. Chromatographic separation was conducted in the same manner as pharmacokinetic sample analysis described above with the exception that the mobile phase gradient was as follows: 0–2 min 5% B, 2–3 min to 95% B, and 3–5 min 95% B. Total run time including an equilibration time of 3 min was 8 min. The mass spectrometer was operated in negative auto MS/MS scan mode with a full scan mass range of m/z 50–800, fragmentor voltage of −150 V, and CE of −30 eV, selecting the two most intense precursor ions for collision induced dissociation. Ion source parameters of the mass spectrometer were set as follows: capillary voltage, −3.5 kV; drying gas, 12 L/min; nebulizer gas, 35 L/min; sheath gas, 11 L/min; sheath gas heater, 400°C; and drying gas temperature, 340°C. Putative metabolites were identified using Agilent Mass Hunter Metabolite ID software (ver.B.04.00) followed by manual interpretation of the spectral data. Chromatograms and mass spectra were extracted using Agilent Mass Hunter Qualitative Analysis software (ver. B.05.00).

**Data Analysis** Pharmacokinetic parameters were calculated by compartmental analysis of plasma concentration–time curves with linear 1-compartment model using PKSolver. Plasma half-life (t_half) was calculated by the equation, \( t_{1/2} = 0.693 / k \), where k was the first-order elimination rate constant. Plasma clearance (CL_p) was calculated by the equation, \( CL_p = Dose \times k / C_0 \), where C_0 was the estimated plasma concentration at time 0. Volume of distribution (V_d) was calculated by the equation, \( V_d = Dose / C_0 \).

In vitro half-life (t_half), intrinsic clearance (CL_int), hepatic clearance (CL_LH), and hepatic extraction ratio (E_int) were calculated from in vitro studies as described previously. The following scaling factors were
used in the calculation of $CL_{\text{int}}$ and $CL_{H}$. S9 content = 143 (mouse) and 121 (human) mg Liver S9 protein/g liver, liver weight = 87.5 (mouse) and 26 (human) g liver/kg body weight, and hepatic blood flow = 90 (mouse) and 20 (human) mL/min/kg body weight.  

RESULTS

Pharmacokinetics of CMT Pharmacokinetic characteristics of CMT was investigated in mouse following a single i.v. (5 mg/kg) and p.o. (10 mg/kg) dose. Mean plasma concentration–time profiles of CMT obtained in this study are shown in Fig. 1. Plasma concentrations following p.o. dose were all below the quantitation limit (15.6 ng/mL) throughout the time course. Plasma levels of CMT declined rapidly, with $t_{1/2}$ of 1.5 ± 0.3 min following i.v. dose. They were below the quantitation limit after 15 min post-dosing (Fig. 1). CMT showed a high $CL_{H}$ of 7.73 ± 3.09 L/hr/kg, exceeding the hepatic blood flow (ca. 5.4 L/h/kg). Its $V_{d}$ was 0.30 ± 0.17.

Metabolic Stability of CMT Metabolic stability of CMT was studied in vitro using MLS9, MIS9, HLS9, and HIS9 fractions fortified with a mixture of NADPH, UDPGA, and PAPS as cofactors for CYP, UGT, and sulfotransferase (ST), respectively (Figs. 2, 3). CMT was metabolized rapidly in all tested tissues, with <10% remaining after 5 min of incubation (Fig. 2, top). Its $t_{1/2}$ values were all <1 min (Table 1). As a result, CMT showed a high predicted $CL_{H}$ nearly identical to the hepatic blood flow in both mouse and human. Correspondingly, its $E_{H}$ values were close to unity (Table 1). The observed dis-
crepancy in $CL_{int}$ and $CL_{H}$ between mouse and human (Table 1) was due to differences in the scaling factors used in the calculations (see data analysis in Materials and Methods).

CMT was also incubated in the same manner with a cofactor added individually to identify the metabolic enzyme(s) involved (Figs. 2, 3). As shown in the bottom of Fig. 2, CMT was metabolized in the presence of NADPH, PAPS, or UDPGA in both MLS9 and HLS9 fractions, suggesting that

![Fig. 3. Metabolic Stability of CMT in MIS9 (A) and HIS9 (B) Fractions](image-url)

CMT (1 μM) was incubated in triplicates with intestinal S9 fractions (1 mg protein/mL) for 10 (top) or 30 (bottom) min at 37°C. Cofactors (NADPH, UDPGA, and PAPS) were added at 1 mM either as a mixture (top) or individually (bottom). Buspirone and antipyrine incubated in the presence of NADPH were used as positive and negative controls, respectively (bottom). Data are expressed as % remaining of CMT vs. 0 min (mean ± S.D., n = 3). Open and closed symbols represent without and with a cofactor, respectively. ND: not detected.

Table 1. Prediction of in Vivo $CL_{H}$ from in Vitro Metabolic Stability for CMT

| Species   | Tissue | $t_{1/2}$ (min) | $CL_{int}$ (mL/min/kg) | $CL_{H}$ (mL/min/kg) | $E_{H}$ | CL class |
|-----------|--------|----------------|------------------------|----------------------|---------|----------|
| Mouse     | Liver  | 0.91           | 9499.5                 | 89.2                 | 0.99    | High     |
|           | Intestine | 0.66         | NA                     | NA                   | NA      | NA       |
| Human     | Liver  | 0.64           | 3429.1                 | 19.9                 | 0.99    | High     |
|           | Intestine | 0.25         | NA                     | NA                   | NA      | NA       |

*In vitro* metabolic stability was determined using MLS9, MIS9, HLS9, and HIS9 fractions as described for the tops of Figs. 2 and 3. $CL_{H}$ was estimated using the 'well-stirred' model of the liver (see Materials and Methods). CL class is defined as follows: $E_{H} < 0.3$, low; $0.3 \leq E_{H} \leq 0.7$, moderate; and $E_{H} > 0.7$, high. NA: not applicable.

Table 2. Putative Metabolites of CMT Identified in Vitro in MLS9 and HLS9 Fractions

| No. | Biotransformation | Formula | $[M - H]^{-}\ (m/z)$ | $\Delta m^{0}$ (ppm) | $t_{R}^{0}$ (min) | Product ions (m/z) |
|-----|-------------------|---------|----------------------|-----------------------|------------------|-------------------|
| M0  | Parent            | C18H18O7 | 345.0994             | 0                     | 5.55             | 330, 312, 193, 165, 137, 109 |
| M1  | Demethylation    | C17H16O7 | 331.0816             | −2.2                  | 6.19             | 313, 298, 194, 138 |
| M2  | Glucuronidation   | C24H26O13 | 521.1335            | 7.1                   | 5.15             | 345, 330, 312, 193, 175, 113, 85, 59 |
| M3  | Sulfation         | C18H18O10S | 425.0542           | −1.5                  | 5.01             | 345, 330, 312 |
| M4  | Sulfation         | C18H18O10S | 425.0547           | −0.3                  | 5.25             | 345, 330, 312, 193, 165 |

CMT (1 μM) was incubated with MLS9 or HLS9 fraction (1 mg protein/mL) for 30 min at 37°C in the presence of 1 mM NADPH, UDPGA, or PAPS as a cofactor. Putative metabolites were identified by Q-TOF LC-MS/MS system run in negative auto MS/MS mode. a) Observed mass. b) Deviation of the observed mass from the theoretical mass. c) Retention time on chromatograms.
it was metabolized by CYP450, ST, and UGT in the liver. Especially, CMT was undetectable in the presence of UDPGA at the end of 30-min incubations with both MLS9 and HLS9 fractions (Fig. 2, bottom). This indicates that CMT might be metabolized mainly by UGT in the liver. CMT was metabolized almost completely in both MIS9 and HIS9 fractions in the presence of PAPS or UDPGA (Fig. 3, bottom). On the contrary, it was not metabolized in either of the intestine S9 fractions when NADPH was present as the cofactor (Fig. 3, bottom). These results suggest that CMT is likely metabolized mainly by UGT and ST in the intestine.

**Identification of CMT Metabolites** One oxidative (M1) and three conjugative metabolites (M2–M4) were identified after incubating CMT with MLS9 and HLS9 fractions containing NADPH, UDPGA, or PAPS as a cofactor (Table 2). No metabolite was detected in the absence of these cofactors, indicating that these detected metabolites were produced by enzyme-dependent reactions (data not shown).

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**Fig. 4. Product Ion MS Spectra of CMT and Its Putative Metabolites Identified in Vitro in MLS9 and HLS9 Fractions**

A: CMT; B: mono-demethylated CMT; C: mono-glucuronide conjugate; and D and E: mono-sulfate conjugate; Glu: glucuronide.
The [M − H]− ion of CMT was detected at m/z 345.0994 (Fig. 4A). Upon fragmentation, loss of a methyl group (−15u) gave the product ion of m/z 330, followed by a neutral loss of H2O (−18u), yielding product ion of m/z 312 (Fig. 3A). The C–C bond cleavage between B and C rings and a concomitant loss of the methyl group from A ring generated product ion of m/z 193 (Fig. 4A). Subsequent losses of COs from m/z 193 ion yielded product ions of m/z 165, 138, and 110 (Fig. 4A).

M1 was detected in both MLS9 and HLS9 fractions after incubating CMT in the presence of NADPH. The [M − H]− ion of M1 was detected at m/z 331.0816, corresponding to a mono-demethylated metabolite (i.e., −14u from the parent) of CMT (Fig. 4B). M1 exhibited a similar fragmentation pattern as the parent, with product ions detected at m/z 313 (−H2O), 298 (313−CH3), 194 (bond cleavage between B and C rings and −CH3), and 138 (194−2CO) (Fig. 4B). M2 was detected in both MLS9 and HLS9 fractions after incubating CMT with UDPGA. The [M − H]− ion of M2 was detected at m/z 521.1335, corresponding to a mono-glucuronidated metabolite (i.e., +176u from the parent) (Fig. 4C). Fragmentation of M2 generated [M − H]− ion of the parent (m/z 345) together with its product ions (m/z 330, 312, 193) (Fig. 4C). Product ions characterized of glucuronic acid were also detected at m/z 175, 113, 85, and 59 (Fig. 4C). M3 and M4 were detected in both MLS9 and HLS9 fractions after incubating CMT with PAPS. [M − H]− ions of M3 and M4 were detected at m/z 425.0542 and 425.0547, respectively, corresponding to a mono-sulfated metabolite (i.e., +80u from the parent) (Figs. 4D, E). Both M3 and M4 yielded [M − H]− ion of the parent (m/z 345) together with its product ions (m/z 330, 312) (Figs. 4D, E). M4 also had additional product ions of the parent at m/z 193 and 165 (Fig. 4E).

DISCUSSION

The pharmacokinetics of oleracne C, a homoisoflavanone isolated from the plant Portulaca oleracea L., in rats has recently been published as the first report for homoisoflavone pharmacokinetics. This compound exhibited a low oral bioavailability (8.32%) and a short plasma t1/2 (1.4h) in rats. Similar to oleracne C, pharmacokinetics of CMT is characterized by high CL and poor oral bioavailability in mouse based on the current study. Consistent with these in vivo results, CMT showed a very short t1/2 and a corresponding high predicted CL in vitro in MLS9 fractions fortified with cofactors for major drug-metabolizing enzymes (DMEs) such as CYP450, UGT, and ST. These results suggest that the high CL of this compound in mouse is likely due at least in part to extensive hepatic metabolism. CMT also showed a rapid metabolism in MIS9 fraction fortified with the same cofactors. In addition, CMT has shown a high permeability in parallel artificial membrane permeability assay (PAMPA) (in-house data). It also has a calculated logP optimal for lipid membrane permeation (i.e., 1.99 by ACD/Labs LogP and 2.08 by CLogP of PerkinElmer, Inc. ChemBioDraw 14.0) without violating Lipinski’s rule-of-five, suggesting that its low oral bioavailability is likely due to its extensive first-pass metabolism in both the intestine and the liver. CMT was also metabolized very rapidly in both HLS9 and HIS9 fractions. CMT was shown to be metabolized by the same DMEs in both species in the current study. Taken together, CMT is likely to exhibit pharmacokinetic characteristics in human similar to those in mouse.

CMT was shown to be metabolized by O-demethylation, glucuronidation, and sulfation in both mouse and human. It appears that only one of its two methoxy groups is metabolized to form demethylated metabolite M1 as only one chromatographic peak with a mass corresponding to demethylated CMT is detected. The obtained mass spectral data, however, were insufficient to assign the methoxy group that was metabolized. Three phenolic alcohols on B and C rings can be metabolized by glucuronidation and sulfation. In this study, one glucuronide (M2) and two sulfate (M3 and M4) metabolites were identified. Again, phenolic groups metabolized by either glucuronidation or sulfation could not be assigned by the mass spectral data obtained. Proposed metabolic pathway of CMT in both mouse and human is shown in Fig. 5.

In conclusion, the pharmacokinetics of CMT is suboptimal.
for a systemic agent, especially as an oral therapeutic agent. The main reason for its poor pharmacokinetic properties appears to be extensive metabolism mainly by glucuronidation and sulfation. This report provides options for structural modifications that could potentially lead to a CMT derivative with better pharmacokinetic properties (e.g. modifications that could potentially lead to a CMT derivative and sulfation. This report provides options for structural...}

**Acknowledgments** This work was supported by a Grant (NRF-2014R1A1A1007304) of the Basic Research Lab Program and a Grant (NRF-2017M3A9C8027781) of the Bio & Medical Technology Development Program of the National Research Foundation funded by the Korea government. It was also supported by a Korea University Grant.

**Conflict of Interest** The authors declare no conflict of interest.

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