A NEW METABOLITE OF IRINOTECAN IN WHICH FORMATION IS MEDIATED BY HUMAN HEPATIC CYTOCHROME P-450 3A4

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ABSTRACT:

Irinotecan (CPT-11) is an anticancer prodrug. It is converted by carboxylesterase to yield an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), which acts as a topoisomerase I inhibitor. Several oxidative metabolites of CPT-11 have been identified in humans, including 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxyacamptotelin (APC) and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxyacamptothecin (NPC), generated by cytochrome P-450 3A4 (CYP3A4). Other minor metabolites in which metabolic pathways and biologic activities have not been identified also exist. To further investigate the metabolism of CPT-11 in human liver, we analyzed metabolites of CPT-11 in human hepatic microsomes using a high-performance liquid chromatography/mass spectrometry (HPLC/MS) system and detected a new metabolite that was the major one produced in the microsomal system.

Irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxyacamptothecin (CPT-11) is a water-soluble derivative of camptothecin, a plant alkaloid isolated from the Chinese tree Camptotheca acuminata. CPT-11 displays antitumor activity in a variety of solid tumors, and it is currently used for the treatment of patients with colon cancer (Rougier et al., 1991), lung cancer (Negoro et al., 1991), and other types of cancer (Saijo, 1996). The antitumor activity of CPT-11 is attributed to an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) (Kawato et al., 1991), which is produced after enzymatic cleavage by carboxylesterase (CES) in the liver (Slatter et al., 1997; Humerickhouse et al., 2000), small intestine (Khanna et al., 2000), and plasma (Kehr et al., 2000). SN-38 is 100-fold more cytotoxic than CPT-11 (Kawato et al., 1991). The mechanism of action of SN-38 is thought to be its interaction with the cleavable complex of DNA and a nuclear protein topoisomerase I, resulting in a blockade of DNA replication (Hsiang and Liu, 1988; Hertzberg et al., 1989). SN-38 is further metabolized by conjugation via uridine diphosphate glucuronosyltransferase 1A1 in the liver to yield SN-38 glucuronide (SN-38G) (Iyer et al., 1998), which is excreted in the urine and bile (Lokiec et al., 1996). SN-38, CPT-11, and SN-38G are known to be excreted in the bile with the aid of transporters, such as the canalicular multispecific organic anion transporter (Chu et al., 1998) and P-glycoprotein (MRD1) (Sugiyama et al., 1998), and to undergo enterohepatic recirculation. SN-38 is thought to be regenerated by hydrolysis of SN-38G by β-glucuronidase in the intestinal microflora (Takasuna et al., 1995, 1996).

Major adverse effects of CPT-11 treatment are severe gastrointestinal toxicities, such as acute and delayed-onset diarrhea (de Forni et al., 1994). It is postulated that the involvement of anticholinergic activity resulting from the inhibition of acetylcholinesterase by CPT-11 is the mechanism of acute phasic diarrhea (Hecht, 1998). The cytotoxic action of the SN-38 excreted in the bile (Gupta et al., 1994, 1997) and its regeneration from SN-38G by β-glucuronidase in the intestinal microflora are suggested to be responsible for the delayed-onset diarrhea (Takasuna et al., 1995, 1996).

CPT-11 is metabolized by cytochrome P-450 (CYP) 3A. Major oxidation products catalyzed by CYP3A4 are 7-ethyl-10-[4-N-(5-...
aminopentanoic acid)-1-piperidino)carbonyloxycamptothecin (APC) (Rivory et al., 1996; Haaz et al., 1998b) and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin (NPC) (Dodds et al., 1998; Haaz et al., 1998a). The latter might be produced by at least two successive oxidation steps. APC is not a substrate for CES in the human, whereas NPC is converted to SN-38 by human CES in the liver (Dodds et al., 1998; Haaz et al., 1998b) or plasma (Kehrer et al., 2000). Other hydroxylated (Lokiec et al., 1996) and de-ethylated (Santos et al., 2000) metabolites in which biologic actions have not yet been identified, have been detected in human liver microsomes (Fig. 1). It is likely that other as yet unknown metabolites of CPT-11 are present in humans.

As a result of our efforts to understand more about the metabolic pathways of CPT-11, we have detected, using a developed high-performance liquid chromatography/mass spectrometry (HPLC/MS) system, a new metabolite that is the major metabolite produced in human liver microsomes. The molecular weight of this compound is less than that of parent compound (CPT-11) by 2 atomic mass units, and HPLC-tandem mass spectrometry (HPLC/MS/MS) analysis indicates that this metabolite is an oxidation product formed by the loss of two hydrogen atoms from the terminal piperidine ring. We conducted studies using specific CYP inhibitors and recombinant CYP isoforms and correlation analyses to identify enzyme(s) responsible for the formation of the new metabolite.

Materials and Methods

**Chemicals.** CPT-11 (lot 115122), SN-38 (lot 970507R), APC (lot 970730), NPC (lot 000014), and SN-38G (lot 970507R) were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan). (S)-(+)-camptothecin (CPT) was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Pooled and individual human hepatic microsomes (H161, HG3, HG6, HG23, HG30, HG42, HG43, HG56, HG66, HG70, HG89, HG93, and HG112), insect microsomes expressing human CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9*1, CYP2C18, CYP2C19, CYP2D6*1, CYP2E1, CYP3A4, CYP3A5, CYP3A7, and CYP4A11), CYP reductase and cytochrome b5 as a control, and antibodies for human CYPs (CYP1A1/1A2, CYP2B6, CYP2C, CYP2D6, CYP2E1, CYP3A4, and CYP4E1) were obtained from GENTEST (Woburn, MA). SKF525A, quinidine, ketoconazole, and sulfaphenazole were purchased from Ultrafine Ltd. (Manchester, England). 8-NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, uridine 5′-diphosphoglucuronic acid, α-naphthoflavone, coumarin, and chlorzoxazone were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol, and ammonium acetate were reagent grade and obtained from Wako Pure Chemical Industries (Osaka, Japan).

**Incubation of CPT-11 with Human Hepatic Microsomes.** A typical reaction mixture in a total volume of 200 μl contained 1 mg of protein/ml of pooled or individual human hepatic microsomes, 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM MgCl2, 5 mM uridine diphosphoglucuronic acid, and 10 μM CPT-11 in 100 mM potassium phosphate buffer, pH 7.4. Reactions were incubated at 37°C for 10 min. Increases in amounts of the metabolites formed were linear up to 10 min of incubation. For kinetic analyses, CPT-11 was incubated at concentrations ranging from 2.5 to 750 μM. In the experiment testing the effects of inhibitors of CYPs, the reaction mixture included either SKF525A (40 and 200 μM), α-naphthoflavone (0.2 and 1 μM), coumarin (2 and 10 μM), quinidine (1 and 5 μM), chlorzoxazone (1 and 5 μM), sulfaphenazole (1 and 5 μM), or ketoconazole (0.4 and 2 μM). For investigations of the effects of inhibitory antibodies, human hepatic microsomes were preincubated with 20 and 40 μl (10 mg of protein/ml) of each polyclonal antibody (anti-CYP1A1/1A2, anti-CYP2B6, or anti-CYP3A4) at room temperature for 30 min or with 2 and 4 μl (10 mg of protein/ml) of each monoclonal antibody (anti-CYP2A6, anti-CYP2B6, or anti-CYP2E1) on ice for 15 min. For incubations with insect

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**Fig. 1.** Structures of CPT-11 metabolites and responsible enzymes established previously.

SN-38 (active metabolite), SN-38G, APC, NPC, and M2 were detected both in vivo and in vitro systems, and M1 was detected in vitro system.
identification of a new metabolite of CPT-11 generated in human hepatic microsomes. Since the maximum level of CPT-11 in the plasma of patients treated with a therapeutic dose is 5 to 10 μM (Sparreboom et al., 1998; Kehrer et al., 2000), we chose 10 μM as the concentration for CPT-11 in this study. We detected several peaks on an HPLC chromatogram obtained from metabolism of CPT-11 by pooled human hepatic microsomes in vitro (Fig. 2, A and B). Metabolite peaks were not detected when NADPH* was absent from the reaction mixture, and peak intensities were dependent on the amount of microsomal proteins in the system (data not shown). We selected major peaks of metabolites for HPLC/MS analysis by selected ion monitoring (Fig. 2 C). Major reported metabolites were detected in the present study: peak a, M1 (a de-ethylated product; mol. wt., 558); peak b, NPC (an oxidative metabolite; mol. wt., 518); peak c, M2 (a hydroxylated product of the piperidine ring; mol. wt., 602); peak d, APC (an oxidative metabolite; mol. wt., 618); and peak f, SN-38 (a hydrolyzed product; mol. wt., 392). Along with the known metabolites, the most intensive peak (e*) in the chromatogram was found to be a new metabolite, M3 (unknown) in which the mol. wt. is 584, 2 atomic mass units smaller than the parent compound, CPT-11 (mol. wt., 586). In our system, SN-38G was not detectable, presumably due to the lower concentration of substrate for uridine diphosphate glucuronosyltransferase (SN-38). The production of SN-38 was less than 20 nM, even after incubation of the highest concentration of CPT-11 (750 μM) in this study.

For prediction of the detailed structure of M3 (unknown), HPLC/MS/MS analysis was conducted (Fig. 3). The fragment of m/z 502 can be attributed to the CPT frame with an inner piperidine ring, and m/z 458 can be attributed to its decarboxylated form, both of which were observed in the parent compound (CPT-11) as well. Fragments derived from di-piperidine in the parent compound, corresponding to m/z 167 and m/z 195 (Fig. 3, upper panel), disappeared in the profile for M3 (unknown) (Fig. 3, lower panel). This finding indicates that M3 (unknown) is an oxidative metabolite, with loss of two hydrogen atoms expressed as human CYPs, the content of each human CYP was adjusted to 100 pmol/mg of protein/ml. The reaction was terminated by the addition of 200 μl of methanol/5% perchloric acid (1:1) containing 10 μl of internal standard (CPT; final concentration, 0.3 μM) and mixed for 3 min, followed by centrifugation at 14,000g for 3 min. The supernatant (300 μl) was mixed with 60 μl of 5 M ammonium acetate buffer, pH 4.5, and transferred to an analytical vial for HPLC/MS after filtration with a filter cartridge (0.45 μm; Millipore Corporation, Bedford, MA).

HPLC/MS. For the analysis of CPT-11 metabolites, we used an HPLC/MS system consisting of an HP 1100 model (Hewlett Packard, Les Ulis, France). The validation of the system will be described elsewhere (manuscript in preparation). Chromatographic separation was performed using an analytical column of CAPCELL PAK CN UG120, S-5 μm (4.6-mm i.d. × 150 mm) (Shiseido Co. Ltd., Tokyo, Japan) protected by a NewGuard C18 column (PerkinElmer Co., Norwalk, CT) at a temperature of 40°C. The mobile phase for separation consisted of a mixture of acetonitrile/methanol/0.05 M ammonium acetate (14:14:72), pH 4.5, and was delivered at a flow rate of 0.5 ml/min. The column outlet was split into two pathways—one directed to a fluorescence detector and the other connected to a mass spectrometer at a ratio of 3:1 for tube diameter (i.e., approximately 9:1 for volume). For fluorescence detection, the excitation wavelength was set at 368 nm, and the emission wavelengths were 432 nm for CPT-11, SN-38G, APC, and NPC and 535 nm for SN-38. MS analysis by the atmospheric pressure ionization-electrospray mode was used for identification of several metabolites of CPT-11. The selected ions monitored by the protonated molecules (MH+) were as follows: 569 for SN-38G, 559 for M1, 519 for NPC, 603 for M2, 619 for APC, 585 for M3 (unknown), 587 for CPT-11, 349 for CPT, and 393 for SN-38. The nitrogen was used as nebulization and curtain gas and was delivered at a flow rate of 7 l/min at a temperature of 250°C. The nebulizer pressure was 45 psig, the capillary voltage was 4000 V, and the fragmentation voltages were set at 400 V for SN-38G and 120 V for all other ions.

For the structure prediction of the new metabolite, daughter fragments of CPT-11 (MH+ 587) and M3 (unknown) (MH+ 585) were analyzed by an HPLC/MS/MS system. The system consists of an HP1100 instrument coupled to a Micromass Quattro II MS/MS for detection (Micromass UK Ltd., Manchester, UK). The columns and mobile phase were the same as the HPLC/MS detection method described above. The operating parameters of the MS/MS detector were set as follows: capillary voltage, 3.0 kV; cone voltage, 80 V; ion energy, 1.0 V for MS1 and 2.0 V for MS2; collision voltage, 33.0 V; source temperature, 130°C; desolvation temperature, 350°C; cone gas flow, 98 l/h; desolvation gas flow, 505 l/h; and resolution, 11.5 for low mass, and 15.0 for high mass.

Data Analysis. In each experiment, samples were assayed in duplicate. For the kinetic analysis and inhibitor experiments, three independent experiments were performed, and data were expressed as mean ± S.D. Determination of kinetic constants from Eidhof-Steeet plots and the correlation analysis of Pearson were performed using the program Prism 3.0 (GraphPad, San Diego, CA). For the correlation analysis, we adopted the data on CYP3A4 content derived from di-piperidine in the parent compound, corresponding to m/z 167 and m/z 195 (Fig. 3, upper panel), disappeared in the profile for M3 (unknown) (Fig. 3, lower panel). This finding indicates that M3 (unknown) is an oxidative metabolite, with loss of two hydrogen atoms.
Fig. 3. Spectra of HPLC/MS/MS and proposed fragmentation of CPT-11 (upper panel) and M3 (unknown) (lower panel).
atoms from the outer piperidine ring of the parent compound, although the position of the oxidation is unclear at present.

**Kinetic Analyses of CPT-11 Metabolites Generated in Human Hepatic Microsomes.** We conducted kinetic analyses of the formation of M3 (unknown) and other metabolites in pooled human hepatic microsomes to determine whether the formation of this metabolite is mediated by a single enzyme. Figure 4 shows the dose-dependent formation of CPT-11 metabolites. The Eadie-Hofstee plot of M3 (unknown) was linear, as were those of M1, M2, NPC, and APC, indicating involvement of a single enzyme. In the case of SN-38, the Eadie-Hofstee plot exhibited two linear regression curves, indicating contributions of low-affinity and high-affinity components for this metabolite, as previously reported (Slatter et al., 1997; Humerickhouse et al., 2000).

**Effects of CYP Inhibitors on the Formation of the New Metabolite.** To determine the enzyme responsible for the formation of M3 (unknown) in hepatic microsomes, we examined the effects of inhibitors of heme protein, inhibitors of major CYPs, and antibodies for human CYP isoforms. Significant inhibition of M3 (unknown) formation was observed upon coincubation with SKF525A (a heme protein inhibitor), with 90% inhibition at 200 μM, and with ketoconazole (a CYP3A inhibitor), with 99% inhibition at 2 μM (Table 1). Dose-related inhibition was also observed with treatment with an anti-CYP3A4 antibody, revealing 70% inhibition at a higher dose of antibody (Table 1). No significant inhibition was detected with α-naphthoflavone (a CYP1A2 inhibitor), coumarin (a CYP2A6 inhibitor), quinidine (a CYP2D6 inhibitor), chlorzoxazone (a CYP2E1 inhibitor), sulfaphenazole (a CYP2C9 inhibitor), or with antibodies

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Fig. 4. Dose-dependent formation of CPT-11 metabolites in human hepatic microsomes.

CPT-11 at concentrations ranging from 2.5 to 750 μM was incubated with pooled human hepatic microsomes (1 mg of protein/ml) at 37°C for 10 min. Each point is the mean ± S.D. of data from triplicate experiments. Km values were calculated from the Eadie-Hofstee plots (insertions). Since the authentic standards for M1, M2, and M3 (unknown) were not available, determination of the amounts of those three metabolites was not conducted.
for CYP1A1/1A2, CYP2A6, CYP2B6, CYP2C, and CYP2E1. α-Naphthoflavone is known to act as a CYP1A1/1A2 inhibitor at lower concentrations (0.01–1 μM) and also a CYP3A4 activator at higher concentrations (10–100 μM) in human liver microsomes (McManus et al., 1990). In our experimental doses (0.2 and 1 μM), no significant enhancement was detected for the formation of M3 (unknown).

**Formation of CPT-11 Metabolites in Insect Microsomes Expressing Human CYP3A4.** To identify the human CYP enzyme that generates M3 (unknown), we incubated CPT-11 with insect microsomes expressing each human CYP isoform (100 pmol/mg of protein/ml) at 37°C for 10 min. Signal intensity of each metabolite was quantified by liquid chromatography–mass spectrometry. Table 1 shows that the formation of M3 (unknown) was significantly correlated with CYP3A4 content in individual human hepatic microsomes. Our results, revealing a significant correlation of M3 formation, were consistent with previous reports by other investigators (Lokiec et al., 1996; Rivory et al., 1996; Dodds et al., 1998; Haaz et al., 1998a,b; Santos et al., 2000). We found that CYP3A4 catalyzed the formation of M1, a de-ethylated product, more efficiently than CYP3A5, which was shown to catalyze exclusively the formation of M3 (unknown), indicating that CYP3A4 is responsible for the generation of M3 (unknown) in human hepatic microsomes. This finding was supported by the fact that M3 (unknown) formation was significantly correlated with CYP3A4 content in individual human hepatic microsomes. Our observation that the production of NPC, APC, and M2 was also significantly correlated with CYP3A4 content in individual human hepatic microsomes, were also consistent with our results using the recombinant enzymes.

**Correlation between the Formation of CPT-11 Metabolites and CYP3A4 Contents in Individual Human Hepatic Microsomes.** To support the above finding that CYP3A4 is the enzyme responsible for the generation of M3 (unknown), we incubated CPT-11 with microsomes expressing either CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9*, CYP2C18, CYP2C19, CYP2D6*, CYP2E1, CYP3A7, or CYP4A11 (data not shown).

**Discussion**

In the present study, we detected a new oxidative metabolite, M3 (unknown), of CPT-11 in an in vitro human liver microsomal system. CYP3A4 inhibitors specifically inhibited M3 formation, and CYP3A4-expressing microsomes produced M3 (unknown), indicating that CYP3A4 is responsible for the generation of M3 (unknown) in human hepatic microsomes. This finding was supported by the fact that M3 (unknown) formation was significantly correlated with CYP3A4 content in individual human hepatic microsomes. Our observation that the production of NPC, APC, and M2 was also mediated by CYP3A4 is consistent with previous reports by other investigators (Lokiec et al., 1996; Rivory et al., 1996; Dodds et al., 1998; Haaz et al., 1998a,b; Santos et al., 2000). We found that CYP3A4 catalyzed the formation of M1, a de-ethylated product, more efficiently than CYP3A5, which was shown to catalyze exclusively the reaction by Santos et al. (2000) (Table 2). Considering the generally lower level of CYP3A5 expression compared with the dominant level of CYP3A4 expression in the human liver and the lower affinity of CYP3A5 for CPT-11 (Santos et al., 2000), the enzyme is most likely to be responsible for M1 formation in the human liver is CYP3A4. Our results, revealing a significant correlation of M1 formation with CYP3A4 contents in individual hepatic microsomes, were also consistent with our results using the recombinant enzymes.

Kinetic analyses revealed that SN-38 formation is mediated by lower and higher affinity enzymes, presumably corresponding to CES-1 and CES-2 (Humerickhouse et al., 2000), respectively. The CES activity in the individual hepatic microsomes, evaluated by
The production of SN-38 using 10 \( \mu \text{M} \) CPT-11 as a substrate, ranged from 1.2 to 2.9 pmol/min/mg of protein in this study. Under the low concentration (10 \( \mu \text{M} \)) of CPT-11 in the current study, CES-2 may be contributed to the SN-38 production. Since NPC, but not APC, was demonstrated to be converted by human CES in the liver (Dodds et al., 1998), a conversion of NPC to form SN-38 in our study might be taken into account. However, this possibility is unlikely in the current study because 1) the \( K_m \) value of NPC for CES determined in the human hepatic microsomes was reported to be 66.2 to 86.5 \( \mu \text{M} \) (Dodds et al., 1998), which is much higher than NPC level produced in our system (100 nM at most); 2) conversion of NPC to SN-38 is reported to be 4-fold lower than of CPT-11 as a substrate for human hepatic carboxylesterase (Dodds et al., 1998); 3) the concentration of CPT-11 is much higher (1000-fold) than NPC produced in our experimental system; and 4) formation of SN-38 was undetectable after incubation of 100 nM NPC (the maximum level as shown in Fig. 4) in the current experimental condition (data not shown). Therefore, CPT-11 may be a much better substrate than NPC for SN-38 production in the current study. A possible conversion of APC to NPC also seems unlikely since no experimental evidence has been reported about such conversion to our knowledge, and we also observed no formation of NPC after incubation of 15 nM APC (the maximum level as shown in Fig. 4) in our experimental condition (data not shown).

The HPLC/MS/MS spectra experiment indicated that M3 (unknown) was formed by removal of two protons from the outer piperidine ring, suggesting CYP3A4 may play a role for removing two protons from a heterocyclic alkyl group of a molecule. There are reports showing a role of CYP3A4 in the oxidation of haloperidol 1,2,3,6-tetrahydropyridine to yield haloperidol pyridinium by two successive removal of two protons (Igarashi et al., 1995; Fang et al.,

**Fig. 5.** Correlations between CYP3A4 contents and formations of CPT-11 metabolites in individual human hepatic microsomes.

CPT-11 (10 \( \mu \text{M} \)) was incubated with individual human hepatic microsomes (1 mg of protein/ml) at 37°C for 10 min. Each point represents the mean of formation of each metabolite from duplicate experiments. Since the authentic standards for M1, M2, and M3 (unknown) were not available, determination of the amounts of those three metabolites was not conducted.
The same manner of oxidation processes by CYP3A4 was also demonstrated in the case of bromperidol, an analog of haloperidol (Sato et al., 2000). Therefore, it is likely that M3 (unknown) formation was mediated by such a function of CYP3A4. However, details in molecular mechanisms of this formation were still unclear in this study.

Slatter et al. (2000) investigated the disposition of radiolabeled CPT-11 in humans and demonstrated that CPT-11 (the parent compound) and three major metabolites (SN-38G, APC, and SN-38) accounted for 93% of the administered dose. They also showed other minor metabolites, including NPC, hydroxylated products, and photodegradation products, whereas neither the de-ethyalted product (M1) nor M3 (unknown) was detected in their work. As in other previous pharmacokinetics studies (Sparreboom et al., 1998; Kehrer et al., 2000), they detected APC as one of the major metabolites, whereas NPC was a minor one. This is in contrast to the observation in microsomal studies by us and others (Dodds et al., 1998), showing much higher production of NPC than APC. The reason why NPC is lower in humans may be explained by the fact that NPC, but not APC, is converted to SN-38 by CES in the liver (Slatter et al., 1997; Humerezikhouse et al., 2000) and plasma (Kehrer et al., 2000). Recent research also suggests that a contribution of plasma esterase for keeping a lower level of NPC and for sustaining a steady-state level of SN-38 in the plasma during the enterohepatic circulation of CPT-11 and its metabolites (Kehrer et al., 2000).

Levels of M3 (unknown) and M1 and M2 were not able to be determined due to no availability of the authentic standards in this study. However, considering the close values of response factors between APC and NPC in both fluorescence detector and mass-selected detector (data not shown) and the structural similarities between APC, NPC, and M3 (unknown), the response factor of M3 (unknown) might not be much different from those of APC and NPC. Therefore, M3 (unknown) might be substantially produced as a major metabolite at least in the hepatic microsomal system used since the relative intensity of M3 (unknown) was the highest among all the metabolites monitored in the chromatograms of both in fluorescence detector and mass-selected detector. The reason why M3 (unknown) was not recognized previously in patients might be due to different analytical conditions and/or unstable properties of M3 (unknown) (i.e., spontaneous decomposition or further conversions by enzymes including CYP3A4, CES, or other microsomal enzymes). In our preliminary studies, we observed that the maximum level of M3 (unknown) was obtained after 2 h-incubation of CPT-11 in the human liver microsomal system used and that further incubation up to 20 h caused a dramatic decrease in the amount of M3 (unknown) (6% of the maximum level), whereas it caused slight increases in other CYP3A4-mediated products and a 5-fold increase in SN-38. Considering the similarity between the structures of M3 (unknown) and CPT-11, having one double bond in the outer piperidine ring of CPT-11, the main metabolite produced by CYP3A4 in human hepatic microsomes, other human microsomal enzymes, still resulted in significant decrease in the amount of M3 (unknown) after subsequent incubation. However, the extent of decrease was much less in the insect CYP3A4-expressed microsomes, suggesting a possible involvement of human microsomal enzyme(s) other than CYP3A4 in conversion of M3 (unknown). Further investigation is planned using an authentic standard of M3 (unknown) to elucidate its fate and a responsible enzyme for the conversion.

The fate of M3 (unknown) in patients is also of interest, and it is possible that this metabolite, if produced in the human liver, may undergo enterohepatic circulation similar to CPT-11 and its metabolites by the responsible transporters, such as canalicular multispecific organic anion transporter (Chu et al., 1998) and P-glycoprotein (MDR1) (Sugiyama et al., 1998). Further study is scheduled for more precise characterization of M3 (unknown) in vitro systems and for clinical investigation of its presence in patients and individual differences in its production, possibly depending on the CYP3A4 expression level.

In conclusion, we identified a new metabolite of CPT-11, which is the main metabolite produced by CYP3A4 in human hepatic microsomes. Our study also showed the possible involvement of CYP3A4 and CYP3A5 in the formation of a de-ethylated product from CPT-11. Further study is scheduled for detection and characterization of this compound in clinical samples.

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References


Gupta E, Lastingi TM, Mick R, Ramirez J, Vokes EE, and Ratain MJ (1994) Metabolic fate of CPT-11, M3 (unknown) might be a substrate for CES to yield SN-38, a minor metabolite at least in the hepatic microsomal system used since the maximum level), whereas it caused slight increases in other CYP3A4-mediated products and a 5-fold increase in SN-38. They also showed other minor metabolites, including NPC, hydroxylated products, and photodegradation products, whereas neither the de-ethylated product (M1) nor M3 (unknown) was detected in their work. As in other previous pharmacokinetics studies (Sparreboom et al., 1998; Kehrer et al., 2000), they detected APC as one of the major metabolites, whereas NPC was a minor one. This is in contrast to the observation in microsomal studies by us and others (Dodds et al., 1998), showing much higher production of NPC than APC. The reason why NPC is lower in humans may be explained by the fact that NPC, but not APC, is converted to SN-38 by CES in the liver (Slatter et al., 1997; Humerezikhouse et al., 2000) and plasma (Kehrer et al., 2000). Recent research also suggests that a contribution of plasma esterase for keeping a lower level of NPC and for sustaining a steady-state level of SN-38 in the plasma during the enterohepatic circulation of CPT-11 and its metabolites (Kehrer et al., 2000).

Levels of M3 (unknown) and M1 and M2 were not able to be determined due to no availability of the authentic standards in this study. However, considering the close values of response factors between APC and NPC in both fluorescence detector and mass-selected detector (data not shown) and the structural similarities between APC, NPC, and M3 (unknown), the response factor of M3 (unknown) might not be much different from those of APC and NPC. Therefore, M3 (unknown) might be substantially produced as a major metabolite at least in the hepatic microsomal system used since the relative intensity of M3 (unknown) was the highest among all the metabolites monitored in the chromatograms of both in fluorescence detector and mass-selected detector. The reason why M3 (unknown) was not recognized previously in patients might be due to different analytical conditions and/or unstable properties of M3 (unknown) (i.e., spontaneous decomposition or further conversions by enzymes including CYP3A4, CES, or other microsomal enzymes). In our preliminary studies, we observed that the maximum level of M3 (unknown) was obtained after 2 h-incubation of CPT-11 in the human liver microsomal system used and that further incubation up to 20 h caused a dramatic decrease in the amount of M3 (unknown) (6% of the maximum level), whereas it caused slight increases in other CYP3A4-mediated products and a 5-fold increase in SN-38. Considering the similarity between the structures of M3 (unknown) and CPT-11, having one double bond in the outer piperidine ring of CPT-11, the main metabolite produced by CYP3A4 in human hepatic microsomes, other human microsomal enzymes, still resulted in significant decrease in the amount of M3 (unknown) after subsequent incubation. However, the extent of decrease was much less in the insect CYP3A4-expressed microsomes, suggesting a possible involvement of human microsomal enzyme(s) other than CYP3A4 in conversion of M3 (unknown). Further investigation is planned using an authentic standard of M3 (unknown) to elucidate its fate and a responsible enzyme for the conversion.

The fate of M3 (unknown) in patients is also of interest, and it is possible that this metabolite, if produced in the human liver, may undergo enterohepatic circulation similar to CPT-11 and its metabolites by the responsible transporters, such as canalicular multispecific organic anion transporter (Chu et al., 1998) and P-glycoprotein (MDR1) (Sugiyama et al., 1998). Further study is scheduled for more precise characterization of M3 (unknown) in vitro systems and for clinical investigation of its presence in patients and individual differences in its production, possibly depending on the CYP3A4 expression level.

In conclusion, we identified a new metabolite of CPT-11, which is the main metabolite produced by CYP3A4 in human hepatic microsomes. Our study also showed the possible involvement of CYP3A4 and CYP3A5 in the formation of a de-ethylated product from CPT-11. Further study is scheduled for detection and characterization of this compound in clinical samples.


