Metabolism of Irinotecan (CPT-11) by CYP3A4 and CYP3A5 in Humans

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ABSTRACT

Irinotecan (CPT-11) is a hemisynthetic, water-soluble derivative of CPT, with a heterocyclic ring structure isolated from the Chinese tree Camptotheca acuminata (Fig. 1). CPT-11 has been shown to display strong antitumor activity against a wide spectrum of human solid tumor xenografts in mice, including pediatric tumors (1–4). CPT-11 is also active in the treatment of numerous malignancies such as colon and lung cancers (5, 6).

The biological activity of CPT-11 results from its capacity to bind to the transient cleavable complex formed between DNA and topoisomerase I, a nuclear protein involved in DNA replication, preventing dissociation of the DNA-topoisomerase I complex and thereby inhibiting enzyme activity (7–10).

In vivo, produg CPT-11 is converted by carboxylesterase into its active metabolite SN-38 (Fig. 1), the cytotoxicity of which is far greater than that of unchanged CPT-11 (11). SN-38 is further conjugated by UDP-glucuronosyltransferase 1A1 (12) primarily in the liver to yield SN-38-G excreted in urine and bile. Hydrolysis of SN-38-G by the intestinal microflora can occur and allows possible recycling of SN-38 in humans (13).

Several oxidative CPT-11 metabolites have been identified in the human plasma: the major derivative is APC [RPR 121056A (Fig. 1)], resulting from a double oxidation of the terminal piperidine ring of CPT-11. M3 had a molecular mass of 602, but its urine concentration in patients was too low to establish its chemical structure by liquid chromatography/mass spectrometry.

In vitro incubations with cells expressing CYP2C8, CYP2C9, CYPIA1, CYPIA2, or CYPIA3 did not produce any detectable metabolites. Only CYPIA4 produced both APC and NPC, resulting from the oxidation of the piperylindipiperidin side chain of CPT-11 along with metabolite M2. The metabolism of CPT-11 by CYPIA5 was markedly different because neither APC nor NPC nor M2 was produced, whereas only one new metabolite, M4 (molecular weight, 558), was generated by de-ethylation of the CPT moiety. No previous study has reported the presence of the M4 metabolite. Production of APC, NPC, M2, and M4 was prevented by ketoconazole, a specific CYPIA inhibitor. The parameters of CPT-11 biotransformation into M2 and M4 were examined using cell lines expressing, respectively, with CYPIA4 and CYPIA5, indicating that CPT-11 is preferentially metabolized by CYPIA4. In conclusion, CYPIA3 plays a major role in the metabolism of CPT-11, with some differences of the metabolic profile exhibited by 3A4 and 3A5.

INTRODUCTION

Irinotecan (CPT-11) is a hemisynthetic, water-soluble derivative of CPT, with a heterocyclic ring structure isolated from the Chinese tree Camptotheca acuminata (Fig. 1). CPT-11 has been shown to display strong antitumor activity against a wide spectrum of human solid tumor xenografts in mice, including pediatric tumors (1–4). CPT-11 is also active in the treatment of numerous malignancies such as colon and lung cancers (5, 6).

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Several oxidative CPT-11 metabolites have been identified in the human plasma: the major derivative is APC [RPR 121056A (Fig. 1)], resulting from a double oxidation of the terminal piperidine ring and generated by CYPIA4 (14). Recently, Dodds et al. (15) identified in the plasma of patients receiving CPT-11 and in human liver microsomal incubations a new metabolite, NPC, produced by cleavage of the distal piperidine ring of irinotecan by cytochrome CYPIA4. Several other oxidation metabolites have been identified in the bile and urine of a female patient (53-yr-old) treated with CPT-11 (16). Only

Received 9/22/99; revised 1/18/00; accepted 1/27/00.

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1 Supported by the Ligue Nationale Contre le Cancer and Rhône-Poulenc Rorer S.A. This study was presented in part at the 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, Pennsylvania, 1999.
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APC and CPT-11 have been evaluated for their biological activity and demonstrated a weaker inhibitors capacity of cell growth in culture than SN-38 (17). The cytotoxic capacity of other metabolites remains unknown.

We have initiated a Phase I study of CPT-11 in children with solid tumors and treated once every 3 weeks. Preliminary pharmacokinetic data showed a wide interpatient variability in the total body clearance of CPT-11 (7–21 liters/h/m²). Only 13% of the administered dose was excreted unchanged in urine, indicating that CPT-11 is extensively biotransformed. Furthermore, up to this time, unidentified metabolites were found in the urine of these patients.5

The objectives of the present study were to separate and identify the metabolites of CPT-11 recovered in human urine using HPLC coupled to spectrofluorometric and electrospray MS detection and to ascertain the identity of enzymes involved in biotransformation reactions after in vitro incubation of CPT-11 with recombinant human CYP450 expressed in a mammalian cell system.

**MATERIALS AND METHODS**

**Patients.** The metabolism of CPT-11 was studied in two children and two adults, respectively, in Phase I and Phase II studies of CPT-11. The drug was given by i.v. infusion over 90 min or 120 min every 3 weeks: a 16-yr-old male with a carcinoma of the cavum (patient A) received 300 mg/m²; a 4-yr-old female (patient B) with a brain tumor was treated with 600 mg/m²; a 67-yr-old male and a 73-yr-old female with a glioblastoma (patients C and D) received 350 mg/m². These patients received concomitant medication with valproic acid, carbamazepine, phenobarbital, or corticoid.

After the first dose, urine samples were collected as 12-h fractions over a 0–72-h period, and all samples were stored at −20°C until analysis.

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5 Unpublished data.
CYP-expressing Cell Lines. Full-length cDNAs coding for human CYP 1A1, 1A2, 2C8, 2C9, 3A4, 3A5, and 3A7 were inserted in the correct orientation in either the EcoRI or the KpnI site of the expression vector pMT2. Ad 293 cells were transfected with 50 μg of CYP-pMT2 plasmid and 5 μg of vector conferring the resistance to geneticin. Clones growing in the presence of G418 and expressing a high level of CYP protein determined by immunoblotting were selected, as described previously (18, 19). In case of CYP3A4, 3A5, and 3A7, stable cell lines were further transfected with the full-length cDNA encoding human NADPH-CYP reductase inserted into pMT2. The addition of multiple copies of the reductase gene markedly increased the production and activity of the reductase protein and consequently stimulated monoxygenase activities supported by CYP3A genes, but had no action on the catalytic activity of other CYP proteins. For transient transfections with CYP3A5, Ad 293 cells were transfected for 48 h with 50 μg of DNA. CPT-11 dissolved in ethanol as a 10-mM stock solution was added to 5 ml of culture medium for 24 h (except when otherwise mentioned) in 75-cm² flasks containing either mock-transfected cells (Ad 293 transfected with pMTII alone) or CYP-transfected cell lines. When needed, ketoconazole (5 μM) was added to the culture medium before adding CPT-11. After incubation, culture medium was removed and processed as urines.

Enzyme kinetic studies were performed with either CYP3A4 or 3A5 stable transfected cell lines. Cell lines were incubated with CPT-11 concentrations ranging from 10–150 μM (stock solution at 10 mM in methanol) for 24 h. The Michaelis-Menten Kₘ parameter was estimated by double reciprocal plots with a computerized program (GraphPad Prism 2.01; Graph Software, San Diego, CA).

It has been checked that evaluation of Kₘ performed on whole cells were in agreement with values calculated from in vitro experiments with microsomes prepared from stable cell lines.

Human Liver Microsomes. Microsomes were prepared from adult human liver, as described previously (20). The CYP3A4 and CYP3A5 content of microsomal preparations was immunochemically determined by Western blotting with a polyclonal antibody raised against human CYP3A4 (Daichi, Tokyo, Japan) or against a hexapeptide including the COOH-terminal of 3A5 (Affiniti, Exeter, United Kingdom) and reacting exclusively with 3A5. After initial screening, two microsomal preparations were selected, one containing a high level of CYP3A5 (“3A5 positive”) and a second totally devoid of CYP3A5 (“3A5 negative”). Incubations were carried out for 30 min with 50 μM CPT-11 in 5 mM MgCl₂, 10% glycerol 1-ml incubation mixture containing 50 mM sodium phosphate buffer (pH 7.4), a NADPH-generating system consisting in 1 mM glucose-6-phosphate and 0.1 mM NADP and glucose-6-phosphate dehydrogenase. The reaction was stopped by the addition of acetonitrile/methanol (50:50, v/v).

HPLC. Pure CPT-11 and metabolites SN-38, SN-38-G, APC (RPR121056A), NPC (RPR132695A), RPR112524, and RPR112526 were provided by Rhône-Poulenc Rorer (Vitry-sur-Seine, France). CPT-11 and its metabolites were separated by a HPLC and analyzed by LC/MS according to the procedure described by Rivory et al. (17), modified as: 50 μl of thawed urine, culture medium, or microsome incubation were vortexed with 0.1 ml of a mixture of acetonitrile and methanol 50/50 (v/v) to precipitate proteins. After centrifugation the supernatant was spiked with 1N HCl to form lactone for further analysis. Separation of metabolites was achieved on a Nucleosil C18 column (10 μm, 250 × 4.6 mm i.d.) with a gradient of acetonitrile (17%–34%) in sodium phosphate buffer (0.1 M, pH 4.0) and heptanesulfonic acid (3 mM) at a flow rate of 1.0 ml/min. The spectrofluorometric detector was set at an excitation wavelength of 355 nm and an emission wavelength of 515 nm.

To ascertain the presence of sulfato or glucuroconjugates, 50-μl samples of urine were incubated at 37°C for 2 h with sulfatase (30 units, Helix promatia, type H-1) or β-glucuronidase (1000 units, Escherichia coli, type IX) before HPLC separation. Control incubations were carried out without β-glucuronidase or sulfatase.

Identification of CPT-11 Metabolites. For LC/MS analysis, solid-phase extraction on Bond Elut C18 columns allowed to remove salts in excess. Chromatography was carried out using a HP1100 model HPLC system (Hewlett-Packard, Les Ulis, France) fitted with a microbore C18 Nucleosil column (5 μm, 1 mm i.d. × 250 mm) supplied by Interchim (Montluçon, France). Mobile phases (flow-rate of 50 μl/min) were a mixture of acetonitrile and deionized water adjusted to pH 4 either a 17:83 (v/v; solvent A) or 34:66 (v/v) ratio (solvent B). The following conditions were used for LC/MS: after injection of 10 μl of extract in solvent A, a linear gradient of 100% A to 100% B was achieved within 20 min, and after a 10-min step in 100% B was returned in 5 min to the initial 100% A, with a final equilibration for 10 min.

The column outlet was connected, without splitflow, to a PLATFORIM II quadrupole mass spectrometer (VG Biotech, Micromass, Manchester, United Kingdom) supplied with the electrospray atmospheric ionization interface. The capillary tip and cone voltage were set at 3000 V and 100 V, respectively. The nitrogen drying gas was set at 300 liters/h, and the nebulization gas was set at 15 liters/h. LC/MS chromatograms were obtained by collecting multiple ions scans over the mass ranging from 100–700 a.m.u. in 6 s. Positive ion spectra were processed with Masslynx software by averaging scans for each compound and subtracting background noise.

RESULTS

Urinary Excretion of CPT-11 Metabolites in Children and Adults. In a first attempt, metabolites of CPT-11 present in urine from patient A were identified by comparing their retention times with those of reference compounds.

Several peaks could be observed unequivocally in urine collected between 12 and 24 h after infusion (Fig. 2). Compounds 1, 3, 6, 7, and 8 (eluting, respectively, at 11.9, 18.8, 21.8, 25.8, and 30.3 min) were identified as SN-38-G, NPC, APC, CPT-11, and SN-38, respectively. The peak ascribed to SN-38-G disappeared totally in urine aliquots treated by β-glucuronidase, leading to a significant increase of the SN-38 peak area, whereas sulfatase had no effect. Other metabolites were not affected by glucuronidase or sulfatase treatment, suggesting that they were not conjugated. Three peaks corresponding to
compounds 2 (M1, 18 min), 4 (M2, 20 min), and 5 (M3, 20.9 min) were unknown.

The presence of these metabolites was carefully examined in urine collected from another child (patient B) and two adults (patients C and D; chromatograms not shown). All CPT-11 derivatives (APC, NPC, SN-38, SN-38-G, M1, M2, and M3) were consistently observed in these samples. The concentration of M2 was equivalent to or even higher than that of NPC, as already seen in pediatric patient A. M1 and M3 were present at a much lower concentration than M2.

**Identification of Unknown Metabolites.** The electrospray-positive ion mass spectra of CPT-11 metabolites are summarized in Table 1.

Positive MS analysis of the compound M1 (Fig. 3A) indicated a monohydroxylation on the CPT moiety of CPT-11 (MW, 602) with fragment ions at m/z 347 and 518, whereas the lateral chain was unaffected (fragments at 167 and 195). Positive MS analysis of the M2 compound (Fig. 3B) displayed a molecular mass of 602 and fragment ions at m/z 331, 375, 502, and 559, indicating an unchanged CPT moiety and a lateral chain at 183, suggesting a monohydroxylation on the terminal piperidine ring of CPT-11. The M3 compound had a molecular mass of 602, but its concentration was too low to allow a reliable fragmentation.

**Identification of CYPs Involved in the Formation of CPT-11 Metabolites in Vitro.** The identification of CYPs involved in the biotransformation of CPT-11 was performed with stable cell lines expressing individual human CYP.

Native Ad 293 cells did not produce any detectable metabolites of CPT-11 (chromatograms not shown), except metabolite M1 (17.9 min; Fig. 4A), as did cells expressing CYP2C8, CYP2C9, CYP1A1, CYP1A2, and CYP3A7 (data not shown). Since native Ad 293 cells one devoid of known CYP proteins, this would indicate that the formation of metabolite M1 is unlikely supported by CYP proteins. APC (peak 3), NPC (peak 6), and metabolite M2 were formed in an appreciable amount by cells expressing CYP3A4 (Fig. 4B), whereas CYP3A5 produced a new metabolite, M4 (19.5 min),
found exclusively in the incubation medium of CYP3A4-expressing cells (Fig. 4C).

Metabolite M3 (MW, 602), detected in urine samples, was not formed or in a too low concentration to be accurately detected during in vitro incubations.

The fragmentation mass spectrum of metabolite M4 (m/z 559), isolated from incubations of CPT-11 with CYP3A5, exhibited fragment ions at m/z 167, 195 (unchanged lateral chain), 275, 303, 430, and 515, suggesting a de-ethylation on the CPT moiety (Fig. 5). This structural information has also been confirmed by its MS/MS daughter mass spectrum. Both spectra confirmed the CYP3A5-dependent formation of M4, evidenced in vitro incubation conducted with microsomes devoid of CYP3A5 and microsomes containing only CYP3A5, whereas the formation of M1 was unaffected.

To estimate the relative affinity of CPT-11 to CYP3A4 and 3A5, cells were incubated with increasing concentrations of CPT-11. The following parameters were calculated from double reciprocal plots based on a linear dose-response relationship within the range of concentrations tested: \( K_{M/AC} = 67 \mu M \), \( K_{M/NPC} = 111 \mu M \), and \( K_{M/M2} = 29 \mu M \) with CYP3A4 and \( K_{M/M4} > 200 \mu M \) with CYP3A5.

To confirm the CYP3A5-dependent formation of M4, CPT-11 was further incubated with human liver microsomes containing only CYP3A4 or microsomes containing both CYP3A4 and CYP3A5: we observed the formation of M1, NPC, M2, and APC with microsomes containing only CYP3A4. Microsomes positive for CYP3A5 produced, in addition, a significant amount of metabolite M4 that was totally absent from the incubation conducted with microsomes devoid of CYP3A5 (data not shown).

Metabolite M4 generated by CYP3A5 was not detected in urines from patients examined in this study.

**DISCUSSION**

A recent study, performed by Lokiec et al. (16), on a 53-yr-old female patient treated with CPT-11 for liver metastatic colon carcinoma, showed eight urinary metabolites: six compounds were already known, namely SN-38-G, SN-38, APC, NPC, RPR112524 (5 hydroxy-CPT-11), and RPR112526 (decarboxylation of the acid form of the lactone ring of CPT-11). In addition, HPLC profiles showed the presence of unknown metabolites, M1 and M2 (16).

In urines of two children and two adults treated by CPT-11 the same metabolites were recovered: SN-38, SN-38-G, APC, NPC, and three unknown metabolites (M1, M2, and M3) were detected by HPLC coupled to fluorescence detection. We identified metabolites M1 and M2: these two compounds had a molecular mass of 602, resulting, respectively, from a monohydroxylation either on the CPT ring or the terminal piperidine. According to its HPLC retention time and its fragmentation mass spectrum, the structure of M1 is different from that of compound RPR112526, which displays the same MW (MW, 558) but resulted from decarboxylation on the CPT ring (17) with fragment ions at m/z 167, 195, 318, and 474.

To confirm the involvement CYP3A4 and 3A5, ketoconazole was incubated in vitro with CPT-11 and CYP3A3-expressing cells. The production of APC, NPC, M2, and M4 was almost completely prevented by the addition of 5 \( \mu M \) ketoconazole (chromatograms not shown), whereas the formation of M1 was unaffected.
Fig. 3  Identification of M1 (A) and M2 (B) metabolites in a urine sample from patient 107. LC/MS spectrum of M1 and M2, and description of their fragmentation pattern.
Fig. 4  HPLC chromatogram using spectrofluorometric detection of cells transfected with pMT2 alone (A), cells expressing 3A4 (B), and cells expressing 3A5 (C) incubated with CPT-11 (50 μM). NPC (3), APC (6), CPT-11 (7), M1, M2, and the unknown metabolite (M4).
exact position of the hydroxyl group on piperidine or CPT rings by nuclear magnetic resonance. In addition, a new metabolite, M4, was generated in in vitro experiments but thus far was not characterized in urine. M4 has a molecular mass of 558, consistent with a de-ethylation on the CPT moiety. The de-ethylation position is currently being investigated by exact mass LC-MS and by nuclear magnetic resonance in our laboratory.

To determine which CYP was involved in the formation of M1, M2, M3, and M4, we used cell lines transfected with human CYPs. M1 was present in urines of patients, in mock-transfected and in transfected cells, and its formation was not inhibited in vitro by ketoconazole. This strongly suggests that M1 is not a CYP3A-dependent product of CPT-11. Among CYP, only CYP3A4 and CYP3A5 were capable of metabolizing CPT-11. CYP1A1, 1A2, 2C8, 2C9, and 3A7 had no oxidative activity. As already demonstrated, CYP3A4 promotes the synthesis of NPC (15) and APC (14) but also of M2, a monohydroxy derivative of CPT-11 on the terminal piperidine. CYP3A5 did not generate M2 and M3, but the new CPT-11 metabolite termed M4. The formation of APC, NPC, M2, M3, and M4 was totally prevented by ketoconazole, a well-known potent inhibitor of CYP3A proteins, demonstrating the involvement of CYP3A4 and 3A5 in these reactions.

Kinetic studies performed on human cell lines transfected with CYP3A4 or CYP3A5 indicated that CPT-11 is preferentially metabolized by CYP3A4. Km values were higher with CYP3A5 than with CYP3A4, suggesting that the affinity of CPT-11 for CYP3A5 is lower than for CYP3A4. Similarly, among CYP3A4 reactions, the formation of APC and M2 displayed the highest affinity (Km/PC and Km/M2 ≪ Km/NPC). Several studies have demonstrated that CYP3A5 is polymorphically expressed, because only 20–25% of human livers have been found to contain detectable levels of CYP3A5 (21). Furthermore, the catalytic activity of CYP3A5 was generally weaker than those of CYP3A4. For example, docetaxel was oxidized by CYP3A5 with a Km 10-fold higher than that of CYP3A4 (22). Similar results were observed for the relative affinity of CPT-11 to CYP3A4 and 3A5. Taken together, the lower affinity for CYP3A5 and the polymorphic expression of CYP3A5 could partly explain why the M4 metabolite was not detected in the urine of the four patients studied here.

Patients with ongoing chemotherapy often received concomitant medications (corticoids, antiarrheics, antiemetics). The metabolism of CPT-11 may be influenced by coadministration of drugs that can modify the activity and/or toxicity of CPT-11 in patients. If such drugs are metabolized by the same enzymes (CYP3A4 and UDPGT), they competitively inhibit and partly prevent the oxidation and conjugation of CPT-11. Thus, Haaz et al. (14) demonstrated that the formation of APC by liver microsomes was inhibited by loperamid and racecadotril (two antiarrheics) and ondansetron (antiemetic). However, drug concentrations to inhibit the formation of APC exceed those observed in clinical use. On the other hand, comedication can increase the level and activity of drug-metabolizing enzymes by inducing their synthesis. Indeed, Gupta et al. (23) have shown
that valproic acid inhibits hepatic conjugation of SN-38 by UDP-glucuronosyltransferase 1A1 in rats. This led to an increase in plasma SN-38 concentration, whereas phenobarbital had a reverse effect and caused induction of SN-38 conjugation (23). Recently, Friedman et al. (24) demonstrated an increase in CPT-11 clearance and reduced SN-38 and SN-38-G concentrations in adults with a brain tumor, receiving CPT-11 together with anticonvulsants (carbamazepine, phenytoin, and phenobarbital), known as potent inducers of CYP.

In the present study, the patients received concomitant medication that could be inducers or inhibitors of CPT-11 metabolism. Therefore, we currently try to identify the qualitative and quantitative modifications in the metabolism of CPT-11 ascribable to concomitant medication used in clinical practice.

In conclusion, CYP3A4 and 3A5 play a major role in the metabolism of CPT-11, with some differences in their metabolic profile.

ACKNOWLEDGMENTS

We are grateful to all of the nurses and physicians at the Gustave-Roussy Institute (Villejuif, France) who devoted care to the patients included in this study. We acknowledge the generous assistance of Eric Roselier and Christian Claude (Mass Spectrometry Laboratory, Micromass, Lyon, France) for the LC/MS/MS analyses. We also thank Lorna Saint-Ange for assistance in editing the manuscript.

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