Identification and Properties of a Major Plasma Metabolite of Irinotecan (CPT-11) Isolated from the Plasma of Patients

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ABSTRACT

Irinotecan [7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11)] is a promising water-soluble analogue of camptothecin [S. Sawada et al., Chem. & Pharm. Bull. (Tokyo), 39: 1446-1454, 1991]. We have previously reported the presence of a major polar metabolite, in addition to 7-ethyl-10-hydroxycamptothecin (SN-38) β-glucuronide, in plasma samples taken from patients undergoing treatment with CPT-11 (L. P. Rivory and J. Robert, Cancer Chemother. Pharmacol. 36: 176-179, 1995; L. P. Rivory and J. Robert, J. Chromatogr., 661: 133-141, 1994). Plasma samples (0.5 ml) containing comparatively large amounts of this metabolite were extracted by solid-phase columns and subjected to high-performance liquid chromatography and mass spectrometry in parallel to fluorometric detection. The metabolite yielded [M + l] ions with a m/z of 619, representing the addition of 32 atomic mass units to CPT-11. Purified fractions were subjected to proton nuclear magnetic resonance, and the structure determined, 7-ethyl-10-[4-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC), was further validated following synthesis. Like CPT-11, APC was found to be only a weak inhibitor of the cell growth of KB cells in culture (IC₅₀, 2.1 versus 5.5 µg/ml for CPT-11 and 0.01 fig/ml for SN-38, the active metabolite of CPT-11) and was a potent inhibitor of the synthesis. Like CPT-11, APC was found to be only a weak inhibitor of the hydrolysis of CPT-11. In this study, we have identified the other principal circulating metabolite observed previously by using a HPLC and MS system with an electrospray interface coupled to fluorometric detection followed by NMR spectrometry of purified fractions. The properties of this metabolite in terms of the inhibition of cell growth, induction of topo I-mediated DNA cleavage, conversion to SN-38, and inhibition of AChE were then examined following its chemical synthesis.

MATERIALS AND METHODS

Chemicals and Reagents. Pure CPT-11, SN-38, and CPT were supplied by Rhône Poulenc Rorer SA (Vitry sur Seine, France). Solvents and reagents were supplied by the highest grade available commercially. ATCh, 5,5'-dithiobis-[2-nitrobenzoic acid] (Ellman's reagent), and AChE (EC 3.1.1.7) from the electric eel (Torpedo californicus) were obtained from Sigma Chemical Co. (St. Louis, MO). Neostigmine methylsulfate was from Astra Pharmaceuticals Pty., Ltd. (North Ryde, New South Wales, Australia).

Sample Handling and Extraction. Blood samples were collected from patients taking part in Phase I and II studies of CPT-11 (300–600 mg/m²) in Bordeaux and Toulouse, France. Plasma was separated rapidly and kept frozen at −70°C until analysis. Samples were chosen in which relatively high concentrations of the unknown metabolite were present when analyzed by HPLC, as described previously for the presence of total SN-38 β-glucuronide (4, 5). To each 500 µl thawed plasma was added 1 ml of a 50:50 (v/v) mixture of acetonitrile and methanol to precipitate proteins. The tubes were centrifuged in an Eppendorf microfuge (12,000 rpm at 4°C), and the supernatant was transferred to an extraction tube and dried down under a stream of nitrogen. Solid-phase extraction columns (Sep-Pak C-18; Waters, Brisbane, Australia) were conditioned with 10 ml methanol, followed by 10 ml of 10 M acetic acid. The dried-down residue of the sample was then resuspended in 0.5 ml of 1 M acetic acid and loaded onto the extraction column, which was then washed with 5 ml of 10 M acetic acid. The column eluate (5 ml of methanol) was dried down under a stream of nitrogen. The residue was stored at 4°C and reconstituted just prior to analysis by the addition of 10 µl each of methanol and 1 M acetic acid, followed by 50 µl mobile phase buffer (see below). The sample was vortex mixed and transferred to a small glass vial and centrifuged for 30 s at 10,000 rpm. An aliquot of the supernatant (20 µl) was injected onto the column. The use of acidic conditions during the Sep-Pak extraction of plasma and for reconstitution of residues ensured that camptothecins and metabolites were present in their lactone forms.

HPLC. HPLC separation was carried out using a micorbore C18 column (250 × 1 mm, 5 µm; Alltech, Brisbane, Australia). The mobile phase solvent was a mixture of methanol and 10 mM ammonium acetate buffer adjusted to pH 5.5 with glacial acetic acid delivered at 0.05 µl/min by an ABI 140B (Perkin-Elmer, Brisbane, Australia) syringe pump as follows. After equilibrating the column with 45% methanol, the sample was injected and the content of methanol was increased linearly to 65% over a period of 40 min and to 80% over the following 20 min. A 40-min column wash step (100% methanol)
followed. The bulk of the column outflow was directed to a Waters 470 fluorimeter (5-μl flow cell) with the excitation and emission wavelengths set at 355 and 515 nm, respectively (5), and the data were collected with MAX-IMA software (Waters).

The remaining column outflow (approximately one-seventh) was directed into a PE SCIEX API III triple quadrupole mass spectrometer (Perkin Elmer, Australia), where it was vaporized at atmospheric pressure, and ions were introduced via an electrospray interface. The OR and interface voltages were set at 100 and 4200 V, respectively, when collecting Q1 data in the positive ion detection mode. Multiple ion scans were collected for m/z values ranging from 30 to 1000. The hulk of the outflow of the column was directed to a Waters 470 fluorescence detector (5-μl flow cell) with the excitation and emission wavelengths set at 360 and 450 nm, respectively. The final reaction mixture contained 0.1 M Tris·HCl (pH 7.4), 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 2 × 10⁴ dpm end-labeled DNA, and a range of drug concentrations. The reaction was initiated by the addition of topo I (20 units in a 20-μl final volume) and allowed to proceed for 10 min at 37°C. Reactions were stopped by adding SDS at a final concentration of 0.25% (w/v) and proteinase K at 250 μg/ml, followed by incubation for 30 min at 50°C. Samples were denatured by the addition of 10 μl denaturing buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, and 0.1% (w/v) bromocresol green and were loaded onto 1% agarose gels in Tris-borate-EDTA buffer containing 0.1% SDS. The samples were electrophoresed at 2 V/cm overnight. The resulting gels were dried between two sheets of Whatman 3MM paper and autoradiographed with MP hyperfilms (Amersham) for 1 day. The X-ray films were scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed using Image Quant software (Molecular Dynamics).

**Biotransformation of Metabolite to SN-38.** The enzyme-catalyzed conversion of the metabolite to SN-38 was estimated with both human liver microsomes and purified human liver carboxylesterase. The human liver microsomes used in this study were provided by C. Riché and D. Ratanasavan (Laboratory of Pharmacology, Faculty of Medicine, University of Brest). They were prepared according to standard procedures (12) from human livers obtained following approval by the relevant institutional ethical committees. A pool of microsomes from five patients was used. Microsomes were solubilized by the addition of 0.5% (w/v) Triton X-100 and incubated for 10 min on ice. The final reaction mixture consisted of 0.9 mg microsomal protein in 0.2 ml Tris buffer (pH 7.2 at 37°C). This mixture was incubated for 5 min in a thermostatted agitating water bath (37°C), and the reaction was initiated by the addition of CPT-11. In preliminary experiments, we have observed different rates of hydrolysis for the two forms of CPT-11, and the drug was added, therefore, as either the lactone or ring-opened carboxylate. Samples (50 μl) were taken every 10 min over a 1-h incubation and assayed for total SN-38 concentrations by HPLC, as described previously (5). Two independent experiments were performed. We verified these observations using purified human liver carboxylesterase as described recently (13). Briefly, incubations of the metabolite and CPT-11 (50 μM) with purified human liver carboxylesterase (EC 3.1.1.11) were carried out in PBS (pH 7.4) at 37°C using 2.0 μM enzyme. The substrates were always equilibrated in the reaction buffer at room temperature prior to the initiation of the reaction and were present, therefore, as a mixture of the lactone and ring-opened carboxylate forms. Samples were withdrawn during the steady-state phase of the reaction and analyzed for total concentrations of SN-38 by HPLC. These concentrations were corrected for the presence of SN-38 as a contaminant of the synthesized metabolite (~0.4%) in both types of study. The possible inhibitory effects of the metabolite on the conversion of CPT-11 to SN-38 were investigated in both systems by the coinoculation of the metabolite and CPT-11 at equal concentrations (microsomes, 10 and 25 μM; purified enzyme, 50 μM). These experiments were performed in triplicate.

**Inhibition of AChE.** CPT-11 and other CPT analogues featuring nitrogenous substitutions at C10 have been shown to be inhibitors of AChE in vitro (14). Therefore, the inhibition of AChE by CPT-11 and the metabolite was studied using the prototypic enzyme from the electric eel. Incubations of AChE were carried out with neostigmine methylsulfate for comparative purposes. A modification of the method of Ellman et al. (15) was used. Briefly, the formation of thionitrobenzene was followed spectrophotometrically (λ = 412 nm) at 30°C in 0.1 M phosphate buffer (pH 7.3; 0.3 mM 5,5'-dithiobis[2-nitrobenzoic acid]) with a UVikon 810 spectrophotometer (Kontron, Schlesien, Switzerland) following the addition of ATChI at a final concentration corresponding to the Km of AChE under the same conditions (2.83 mM) and 0.05 units of AChE. A preincubation of the drug with AChE for 3 min prior to the addition of ATChI was required to achieve a maximal effect for neostigmine. In the case of the metabolite and CPT-11, however, there was no increased effect with preincubation; therefore, none was used.

**RESULTS**

The HPLC and MS conditions were optimized with the pure standards of CPT, SN-38, and CPT-11 as their lactones. Lower OR voltages

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{Fig. 1. Structures of the compounds discussed in the text.}

3 SN-38
4 APC (RPR-121056)

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IRINOTECAN METABOLITE

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Fig. 2. Fluorescence (A) and total ion count (B) traces obtained with the HPLC and MS analysis of an extract of 0.5 ml plasma. This sample was taken 30 min after infusion from a 47-year-old patient receiving 600 mg/m² CPT-11 in a first cycle for a colonic tumor. Peaks in A: 1. SN-38 ß-glucuronide; 2. APC; 3. SN-38; 4. CPT-11.

(40 V) were found to yield lower signal clarity, and the camptothecins were detected partially as dimers and acetate adducts under such Q1 conditions. Furthermore, as can be seen in Fig. 2, A and B, the high OR used (100 V) resulted in fragmentation characteristic of the loss of CO₂ (−44), although this was more pronounced for SN-38 and CPT than for CPT-11. The neutral loss of 44 atomic mass units was found to be a useful property, which in addition to the fluorescence trace, was used to detect several metabolites in addition to the readily identifiable SN-38 ß-glucuronide, SN-38, and CPT-11 (Fig. 2A, peaks 1, 3, and 4, respectively) in extracts of patient plasma. CPT-11 also yielded fragments consistent with loss of the distal piperidine (m/z, 502) and the loss of CO₂ from this species. The principal unknown metabolite was well resolved from the other peaks and yielded spectra with a major peak at m/z 619 and, as for the standards, significant fragmentation (Fig. 3C).

The presence of the 393 and 227 fragments indicate that the addition of 32 mass units to the principal unknown metabolite ([M+1] = 619), relative to CPT-11, occurs on the bipiperidino moiety. Specifically, the signal at m/z 502 indicates that the distal piperidine is the site of metabolism (Fig. 3C). Spectra obtained from collision-associated dissociation experiments using argon did not provide further structural information regarding this compound (data not shown), which was suspected to be either a bihydroxylated or ring-opened carboxylate metabolite. The purified metabolite yielded NMR spectra with the aromatic part very similar to that of CPT-11 (δ = 8.24, d, J = 8 Hz; δ = 8.04, s; δ = 7.67, d, J = 8 Hz), and the two methylenes at δ = 5.4 and 5.6 (AB system) and δ = 5.38 (A₂ system) indicated that no modification occurred on the camptothecin moieties. The two α protons of the proximal piperidine residue were also clearly identified at δ = 4.55 and 4.37, as well as two of the β protons at δ = 2.24. On the other hand, three signals at δ = 1.9 (m, 4H), δ = 2.3 (t, 2H), and δ = 3.1 (t, 2H) characteristic of a n-butyl chain suggested that scission of the distal piperidine had occurred at the level of a N-C bond of this ring and that the metabolite was APC (Fig. 1). Another four metabolites were detected, which eluted in proximity to SN-38 (Fig. 2A). Three of these yielded [M+1] ions with m/z of 603, indicating monohydroxylation of CPT-11 and the fourth ([M+1] = 519) had a pattern of fragmentation suggesting a N-dealkylated metabolite of APC. These metabolites require further characterization to be certain of their identity.

Fig. 3. Mass spectra collected from the peaks of SN-38 (A), CPT-11 (B), and APC (C). Insets, proposed fragmentation.
The cytotoxic potential of APC was assessed by a cell growth inhibition assay using the KB human epidermoid cell line. For comparative purposes, CPT-11 and SN-38 were evaluated in parallel. Under the experimental conditions used (4 days of continuous exposure), APC was found to be a weak inhibitor of cell proliferation. The IC_{50} found (mean ± SD; n = 4) was 2.1 ± 0.09 μg/ml, whereas those of CPT-11 and SN-38 were 5.5 ± 0.4 and 0.01 ± 0.002 μg/ml, respectively.

In vivo, topo I enables normal replication and transcription to be carried out by relaxing the supercoiling of DNA (2). Topo I cleaves and resells one strand of the DNA duplex through a transesterification reaction that leads to the formation of transient covalent complexes between nicked DNA and topo I. These intermediate complexes are often referred to as “cleavable,” because they are transformed into permanent single-stranded breaks following the addition of protein denaturants. Because the camptothecins greatly stabilize these intermediate complexes by inhibiting the religation step of topo I, the evaluation of topo I-mediated DNA cleavage offers a sensitive and selective system for determining the potency of camptothecin analogues. To test the topo I-mediated activity of APC, we used such a DNA cleavage assay, and the cleavage products were separated by alkaline agarose gel electrophoresis. An autoradiograph of a gel from a typical experiment is shown in Fig. 4. Purified calf thymus topo I was incubated with linearized pBR 322 DNA in the presence of absence of the test compounds. Reactions were carried out for 10 min at 37°C, then stopped with SDS-proteinase K treatment, and single-stranded DNA fragments were analyzed on a 1% alkaline agarose gel. Lane 1, DNA alone; Lanes 2 and 15, DNA and topo I without drugs; Lanes 3–6, as in Lane 2 with CPT-II at 100, 10, and 0.1 μg/ml, respectively; Lanes 7–10, as in Lane 2 with APC at 10, 1, 0.1, and 0.01 μg/ml, respectively; Lanes 11–14, as in Lane 2 with SN-38 at 100, 10, and 0.1 μg/ml, respectively.

Fig. 4. Effect of CPT-11, APC, and SN-38 on the topo I-mediated DNA cleavage reaction. Purified calf thymus topo I was incubated with linearized pBR 322 DNA in the presence or absence of the test compounds. Reactions were carried out for 10 min at 37°C, then stopped with SDS-proteinase K treatment, and single-stranded DNA fragments were analyzed on a 1% alkaline agarose gel. Lane 1, DNA alone; Lanes 2 and 15, DNA and topo I without drugs; Lanes 3–6, as in Lane 2 with CPT-11 at 100, 10, and 0.1 μg/ml, respectively; Lanes 7–10, as in Lane 2 with APC at 10, 1, 0.1, and 0.01 μg/ml, respectively; Lanes 11–14, as in Lane 2 with SN-38 at 100, 10, and 0.1 μg/ml, respectively.

The cleavage resulted in the reduction of the intensity of the band corresponding to the DNA substrate and the appearance of an increasing proportion of fragments with lower molecular weights. The relative potency of the three compounds could be estimated by inspection of the autoradiograph and comparison of the concentrations of drugs required to cause a similar extent of cleavage, as determined from the intensity of the band corresponding to the DNA substrate as a percentage of total lane absorbance. APC at 10 μg/ml produced cleavage (%A, 19.7) equivalent to a concentration of CPT-11 of between 10 (%A, 35.3) and 100 μg/ml (%A, 18.3), suggesting that APC is more potent than CPT-11. However, this interpretation should be made carefully, given that SN-38 was found to be a minor contaminant of the synthesized APC. In any case, the cleavage observed at the highest concentration of APC was less intense than that observed with the lowest concentration of SN-38 (0.1 μg/ml), indicating that APC is at least 100-fold less active than SN-38 in the stabilization of topo I-cleavable complexes.

APC was found to be an extremely poor substrate of human liver microsomes in either its lactone or ring-opened carboxylate forms. Indeed, no significant release of SN-38 could be measured (Fig. 5). Similar observations were made with purified human liver carboxylesterase (Fig. 6), which appears to be the principal CPT-11-convert ing enzyme of human liver (13). APC did not inhibit the biotransformation of CPT-11 by microsomes (data not shown) or by human liver carboxylesterase (Fig. 6) when present in concentrations equal to those of CPT-11.

Unlike CPT-11, APC was a weak inhibitor of AChE, with an IC_{50} of approximately 45 μM. The corresponding IC_{50} values for CPT-11 and neostigmine were 0.19 and 0.053 μM, respectively (Fig. 7). SN-38 did not have any detectable activity over a comparable range of concentrations.

**DISCUSSION**

It has been noted that the kinetics of CPT-11 and its active metabolite SN-38 display large interindividual variations (6, 7), and the role of variable metabolism and disposition of CPT-11 on antitumoral...
activity and systemic toxicity is unclear. The contribution of the biotransformation to SN-38 appears to be a minor metabolic route for CPT-11, with plasma, bile, and urine levels generally one to two orders of magnitude lower for SN-38 than for CPT-11 (6–8). Furthermore, the total proportion of the dose recovered as SN-38 plus CPT-11 in the urine is on the order of 20–50% (6–8), which suggests that a hitherto unknown metabolic route exists for CPT-11 (8). This has been suspected, because we discovered a major circulating fluorescent metabolite in the plasma of patients (4, 5), but the small amounts of plasma available precluded the direct identification of this metabolite until now. The use of electrospray HPLC and MS in combination with fluorescence detection and NMR has enabled us to identify this compound with only 6–7 ml patient plasma. The conditions used here appear suitable for the studies of the metabolism of other camptothecin analogues.

Recently, we reported a preliminary study of the pharmacokinetics of APC in patients in whom we observed large interindividual variation in the concentrations of this metabolite (16). In all patients, however, the plasma APC concentrations were greater than for SN-38 except for late time points (12–24 h after infusion). On the other hand, they were generally lower than for CPT-11, although in some patients, the reverse was true 2–3 h after the end of the infusion. These particular plasma samples were the ones selected for the present study. The plasma kinetics of APC and their relationship to the other known metabolites of CPT-11 are to be published in detail elsewhere.

APC is most likely a product of cytochrome P450-mediated metabolism (17) and appears to be metabolized further to a N-dealkylated metabolite. Other metabolites were observed, which suggests that several metabolic pathways, which are possibly interrelated (e.g., monohydroxylation as an intermediate step toward piperidine ring opening), are active in the elimination of CPT-11. Unfortunately, the concentrations of these other metabolites were considerably lower than APC, preventing their characterization using the same methodology.

It is noteworthy that APC was not significantly converted to SN-38 by human liver microsomes or human liver carboxylesterases. Although CPT-11 is itself only moderately transformed in comparison to avid substrates such as p-nitrophenylacetate (13, 18), it appears that APC will not act directly as a prodrug of SN-38 in vivo. Studies carried out with human liver carboxylesterase have shown that the rate of enzymatic hydrolysis of a range of acylsalicylic acids increases dramatically with the length of the acyl chain (19). Likewise, the conversion of SN-38 prodrugs by the rat serum CPT-11-converting enzyme is greatly influenced by the structure of the CIO side chain (18). Specifically, the rate of hydrolysis of 4-(N-dialkylamino)-1-piperidino esters of CPT-11 is dependent on the hydrophobicity of the alky substitution, with the 4-(N-dibutylamino)-1-piperidino analogue being cleaved at almost twice the rate of CPT-11 (18). Thus, a probable reason for the negligible rate of APC conversion to SN-38 is the considerable hydrophilicity of the side chain of APC compared with CPT-11.

APC was found to be a poor inhibitor of cell growth in culture, with an IC50 comparable to that of CPT-11. Like CPT-11, APC was only a weak inducer of topo I-mediated DNA cleavage in vitro. This is likely to be due to the presence of the bulky substitution. In both systems, APC was found to be approximately 100-fold less active than the active metabolite SN-38. However, APC differed significantly from CPT-11 as far as inhibition of acetylcholinesterase was concerned. Therefore, it is unlikely that APC plays a direct role in the activity and toxicity profile of CPT-11. Nevertheless, because the exact mechanism for the tardive diarrhea associated with CPT-11 remains to be determined, an involvement of APC in this toxicity cannot be ruled out. In any case, the presence of APC in the plasma of patients is evidence of an important route of metabolism, in addition to the conversion of CPT-11 to SN-38 by carboxylesterase and the conjugation of the latter to glucuronic acid. The route of metabolism involving the production of APC may be an important source of variability in the disposition of CPT-11 in patients.

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REFERENCES


Fig. 6. Conversion of 50 μM CPT-11 (●) and 50 μM APC (□) to SN-38 by purified human liver carboxylesterase as a function of time (mean, n = 3). Incubations were also carried out with both compounds simultaneously at 50 μM (▲) to determine whether APC modifies the biotransformation of CPT-11. Bars, SD.

Fig. 7. Inhibition of the hydrolysis of ACh catalyzed by AChE as a function of the concentration of neostigmine (●), CPT-11 (●), and APC (△). SN-38 did not have any significant activity over this range of concentrations (mean, n = 3). Bars, SD.

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