Metabolism of Irinotecan (CPT-11) by Human Hepatic Microsomes: Participation of Cytochrome P-450 3A and Drug Interactions

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ABSTRACT

Irinotecan (CPT-11) is a water-soluble analogue of camptothecin showing activity in colon cancer. Recently, we identified a major metabolite of CPT-11 in patients' plasma, 7-ethyl-10-[4-(1-piperidino-1-piperidino)carbonyloxycamptothecin (APC), which is produced by the oxidation of the distal piperidine ring (P. Rivory et al., Cancer Res., 56: 3689–3694, 1996). As with all active camptothecin derivatives, CPT-11 is subject to spontaneous interconversion between a lactone and a carbonylate form in aqueous media. The kinetics of biotransformation of the two forms of CPT-11 into APC was studied using pooled human liver microsomes. The formation of APC was characterized by the following parameters: \(k_m = 18.4 \pm 1.4 \) and \(39.7 \pm 11.6 \mu M\); and \(V_{max} = 26.0 \pm 0.6 \) and \(13.4 \pm 1.7 \) pmol/min/mg protein for the lactone and carbonylate forms of CPT-11, respectively. This reaction was found to be catalyzed primarily by cytochrome P-450 (CYP) 3A because of three key results: (a) the CYP 3A-selective inhibitors ketoconazole (1 \mu M) and troleandomycin (100 \mu M) inhibited APC formation by 98 and 100%, respectively, mostly in a competitive way; (b) using microsomes from transfected lymphoblastoid cells expressing specific CYPs, we found that only those from CYP 3A4 cDNA-transfected cells transformed CPT-11 into APC; and (c) using 15 individual preparations of human liver microsomes, we observed highly significant correlations between the activity of CPT-11 metabolism into APC and both immunoreactivity with anti-CYP 3A antibodies and testosterone 6\beta hydroxylation, an activity specifically mediated by CYP 3A. The effect on this metabolism of 11 drugs used at 100 \mu M was studied with CPT-11 lactone at 25 \mu M. Amikacin, Bactrim, ciprofloxacin, rocephine, 5-flourouracil, metoclopramide, morphine, and paracetamol had no effect, but ondansetron, loperamide, and raccadotril inhibited this pathway by 25, 50, and 50%, respectively. These concentrations exceed those expected in vivo. APC formation in patients may thus be influenced by coadministered ketoconazole therapy and may decline after administration of CPT-11 because of the lactonolysis of the latter.

INTRODUCTION

Camptothecin is a pentacyclic alkaloid first isolated from extracts of the Chinese tree *Camptotheca acuminata*, which was shown to display antitumor activity in the National Cancer Institute drug screening program in the 1960s (1). CPT-11 is a semisynthetic water-soluble derivative of camptothecin showing activity in colon cancer. Recently, we identified a major metabolite of CPT-11 in patients' plasma, 7-ethyl-10-[4-(1-piperidino-1-piperidino)carbonyloxycamptothecin (APC), which is produced by the oxidation of the distal piperidine ring (P. Rivory et al., Cancer Res., 56: 3689–3694, 1996). As with all active camptothecin derivatives, CPT-11 is subject to spontaneous interconversion between a lactone and a carbonylate form in aqueous media. The kinetics of biotransformation of the two forms of CPT-11 into APC was studied using pooled human liver microsomes. The formation of APC was characterized by the following parameters: \(k_m = 18.4 \pm 1.4 \) and \(39.7 \pm 11.6 \mu M\); and \(V_{max} = 26.0 \pm 0.6 \) and \(13.4 \pm 1.7 \) pmol/min/mg protein for the lactone and carbonylate forms of CPT-11, respectively. This reaction was found to be catalyzed primarily by cytochrome P-450 (CYP) 3A because of three key results: (a) the CYP 3A-selective inhibitors ketoconazole (1 \mu M) and troleandomycin (100 \mu M) inhibited APC formation by 98 and 100%, respectively, mostly in a competitive way; (b) using microsomes from transfected lymphoblastoid cells expressing specific CYPs, we found that only those from CYP 3A4 cDNA-transfected cells transformed CPT-11 into APC; and (c) using 15 individual preparations of human liver microsomes, we observed highly significant correlations between the activity of CPT-11 metabolism into APC and both immunoreactivity with anti-CYP 3A antibodies and testosterone 6\beta hydroxylation, an activity specifically mediated by CYP 3A. The effect on this metabolism of 11 drugs used at 100 \mu M was studied with CPT-11 lactone at 25 \mu M. Amikacin, Bactrim, ciprofloxacin, rocephine, 5-flourouracil, metoclopramide, morphine, and paracetamol had no effect, but ondansetron, loperamide, and raccadotril inhibited this pathway by 25, 50, and 50%, respectively. These concentrations exceed those expected in vivo. APC formation in patients may thus be influenced by coadministered ketoconazole therapy and may decline after administration of CPT-11 because of the lactonolysis of the latter.

MATERIALS AND METHODS

Chemicals and Reagents. Pure CPT-11 and APC (RPR 21056) were supplied by Rhône-Poulenc Rorer (Vitry-sur-Seine, France). Solvents and reagents were of the highest grade available commercially.

Human Liver Microsomes. Human liver microsomes were prepared according to standard subcellular fractionation procedures (12) from human livers obtained after approval by the relevant institutional ethical committees. A pool of preparations containing ~15 mg/ml microsomal proteins was used for the kinetic studies, CYP identification, and drug interactions. In addition, we used two other sources of human microsomes for CYP isoform identification: (a) microsomes originating from human lymphoblastoid cell lines transfected with specific CYP cDNAs (CYP 1A2, CYP 2C9, CYP 2D6, and CYP 3A4), which were obtained from Gentest Corp. (Woburn, MA); and (b) microsomes originating from the livers of 15 individuals and already characterized for the activity of various cytochromes, which were purchased from the International Institute for the Advancement of Medicine (Exton, PA). All microsomes were stored at ~80°C and were never refrozen after use.

Optimization of the Incubation Conditions. Preliminary experiments were conducted to determine optimal incubation conditions. They were selected so that CPT-11 biotransformation was linear with respect to incubation time (20 min) and microsomal protein concentration (1 mg/ml final concentration). Incubations were done in 0.1 M phosphate buffer (pH 7.4), to which NADPH (1 mM) and CPT-11 were added as a substrate at the appropriate concentrations in a final volume of 500 \mu l. Polypropylene tubes containing the mixture were gently vortex-mixed and incubated in a shaking water bath at 37°C for 5 min. The enzymatic reaction was started by adding the microsomes that were kept on ice until use. At least three independent experiments were always carried out.

High-Performance Liquid Chromatography. Quantitative evaluation of APC was performed using a high-performance liquid chromatography technique with fluorescence detection adapted from the technique of Rivory and...
Thermo Separation Products. Les Ulis. France). Detection was carried out with data were automatically recorded, and concentrations were calculated by excitation and emission wavelengths set at 355 and 515 nm, respectively. Peak parameters (apparent \( K_m \) and \( V_{max} \)) were then determined by nonlinear regression of the APC concentrations versus time. The Michaelis-Menten

0.01 M citric acid (pH 3) or as carboxylate (diluted in 0.1 M sodium carbonate reference to a standard calibration curve obtained before each series of injection, using PC1000 software (Thermo Separation Products).

Enzyme Kinetics. These studies were performed using the original pool of human liver microsomes. CPT-11 was used either as lactone (stock diluted in 0.01 M citric acid (pH 3)) or as carboxylate [diluted in 0.1 M sodium carbonate (pH 10)], at concentrations ranging between 2 and 100 \( \mu \)M. Samples were taken at 0, 5, 10, 15, and 20 min. Reaction velocities were evaluated by linear regression of the APC concentrations versus time. The Michaelis-Menten parameters (apparent \( K_m \) and \( V_{max} \)) were then determined by nonlinear regression (SigmaPlot; Jandel Scientific, Erkrath, Germany).

CYP Identification. Three independent methods were used for the identification of the CYP isoform involved in the biotransformation of CPT-11 into APC:

(a) The first approach relied on the inhibition of APC formation by known selective inhibitors of CYPs (Ref. 14; Table 1), with the concentration of CPT-11 lactone set at 25 \( \mu \)M. Control incubations were carried out in the presence of the appropriate solvent (Table 1). The concentration of inhibitor required to inhibit APC formation by 50% (IC\(_{50}\)) and its effect on the kinetics of APC formation (\( K_m \) and \( V_{max} \)) were determined when inhibition was higher than 50% with the recommended concentration of inhibitor.

(b) The second approach used the microsomes obtained from human lymphoblastoid cells transfected with the cDNA of specific CYPs; these microsomes were incubated as described above, with 100 \( \mu \)M CPT-11 lactone. However, with the microsomes of the CYP 2C9-expressing cells, the phosphate buffer was replaced by 0.1 M Tris buffer (pH 7.5), as recommended by the supplier.

(c) The third approach relied on the correlation that may exist between the level of a given CYP, evaluated by enzymatic or immunological reactions, and the biotransformation of CPT-11 into APC. CYP activities on specific substrates were indicated by the supplier of the 15 individual microsomal preparations, and these values were used without further characterization. In addition, CYP 3A was quantified in these microsomes by Western blotting. For this purpose, 25 \( \mu \)g of protein from the 15 individual microsomal preparations were separated for 15 h by electrophoresis on a 10% polyacrylamide-SDS gel according to procedures described by Laemmli (15). Proteins were then electrothermally transferred onto nitrocellulose filters and blotted overnight in 10 mM Tris buffer (pH 7.5) containing 3% immunoglobulin-free BSA. Blots were incubated with antihuman CYP 3A antibodies (Valbiotech, Paris, France) and an alkaline phosphatase-labeled polyclonal antibody (DAKO, Trappes, France). Finally, the blots were revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma Chimie, Saint-Quentin-Fallavier, France). The relative amount of CYP 3A in human microsomes was estimated by densitometric scanning of the blot using the Densyslab software (Bioprobe, Montreuil, France). The levels of immunoreactive CYP 3A of 15 human microsomal preparations were then compared to the velocities of APC formation in these preparations.

Drug Interactions. The effect of 11 drugs that could be coadministered with CPT-11 in the clinical setting was studied on this biotransformation: the antibiotics amikacin, trimethoprim/sulfamethoxazole (Bactrim), ciprofloxacin, and rocephin; the antineoplastic 5-fluorouracil; the antiemetics metoclopramide and ondansetron; the antidiarrheics loperamide and racedotril (ace- torphan); and the analgesics morphine and 4-acetamidophenol (paracetamol). These studies were performed under the conditions described above at fixed concentrations of CPT-11 lactone (25 \( \mu \)m) and of each drug (100 \( \mu \)M). When the inhibition of APC formation was significant under these conditions, the IC\(_{50}\) of the inhibitor was evaluated. This was followed by a kinetic study in which five different concentrations of CPT-11 were incubated in the presence of the previously determined IC\(_{50}\) of the inhibitor.

RESULTS

In Vitro Kinetics of APC Formation by Human Liver Microsomes. After incubation of CPT-11 with human liver microsomes in the presence of NADPH, several peaks were detected (Fig. 2) that did not appear when NADPH was omitted from the reaction mixture (data not shown). In a typical chromatographic profile, CPT-11 eluted at 13.0 min and camptothecin (internal standard) eluted

Table 1. Inhibition of APC formation by various inhibitors of CYP isoforms

<table>
<thead>
<tr>
<th>CYP isoform</th>
<th>Inhibitor (concentration)</th>
<th>Solvent</th>
<th>APC formation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyaclent</td>
<td>Cimetidine (100 ( \mu )M)</td>
<td>Ethanol</td>
<td>71.0 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>a-Naphthoflavone (1 ( \mu )M)</td>
<td>Ethanol</td>
<td>86.9 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>Coumarin (100 ( \mu )M)</td>
<td>Ethanol</td>
<td>88.3 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>Quinidine (5 ( \mu )M)</td>
<td>Buffer</td>
<td>95.6 ± 12.5</td>
</tr>
<tr>
<td></td>
<td>Chloroxazone (100 ( \mu )M)</td>
<td>Ethanol</td>
<td>81.6 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole (1 ( \mu )M)</td>
<td>Ethanol</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Troloxandemycin (100 ( \mu )M)</td>
<td>Methanol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3C/9</td>
<td>Sulphaphenazole (1 ( \mu )M)</td>
<td>Ethanol</td>
<td>97.2 ± 6.9</td>
</tr>
</tbody>
</table>

of APC formation (\( K_m \) and \( V_{max} \)) were determined when inhibition was higher than 50% with the recommended concentration of inhibitor.
The second metabolite peak corresponded to ARC, which had been characterized by mass spectrometry and nuclear magnetic resonance at 16.8 min, whereas three metabolites eluted at 8.0, 9.0, and 10.2 min.

Incubations with 1 mg/ml protein yielded rates of formation of APC that were linear over 20 min (data not shown). For CPT-11 lactone, apparent \( K_m \) and \( V_{\max} \) values were 18.4 ± 1.4 \( \mu M \) and 26.0 ± 0.6 pmol/min/mg protein, respectively. Corresponding values for the carboxylate form were 39.7 ± 11.6 \( \mu M \) and 13.4 ± 1.7 pmol/min/mg protein, respectively (Fig. 3).

Identification of the CYP Involved in APC Formation. Several approaches have been used to unequivocally identify the CYP responsible for the biotransformation of CPT-11. First, we examined inhibitors with known selectivity for CYPs with CPT-11 lactone set at 25 \( \mu M \). The general CYP inhibitor cimetidine (100 \( \mu M \)) inhibited APC formation by 29\% (Table 1). Table 1 also shows that the CYP 3A inhibitors troleandomycin (100 \( \mu M \)) and ketoconazole (1 \( \mu M \)) inhibited APC formation by 100 and 98\%, respectively. The remaining compounds had no appreciable effect. The inhibition of APC formation by troleandomycin and ketoconazole was further evaluated by determining the concentrations of inhibitor able to inhibit 50\% of APC formation. These values, as well as the apparent \( K_m \) and \( V_{\max} \) values determined in the presence of the \( IC_{50} \) of these inhibitors, are indicated in Table 2. It seemed that the inhibition exerted by these two compounds on APC formation was essentially of mixed type.

In a second step, we used human cDNA-transfected human lymphoblastoid cells expressing a single isoform of CYP. The only microsomes able to metabolize CPT-11 into APC originated from the CYP 3A4-transfected cells (data not shown). Incubation of CPT-11 with microsomes originating from control cells (no CYP) or from cells transfected with the cDNA of CYP 1A2, CYP 2D6, or CYP 2C9 did not produce any detectable formation of APC.

We compared the rates of transformation of CPT-11 into APC by 15 individual human liver microsomal preparations to the CYP activities determined with specific substrates by the supplier of the microsomes (7), and chemically synthesized (16).

The IC\(_{50}\) of each inhibitor was first estimated with 15 \( \mu M \) CPT-11 lactone, and then the effect of this concentration of inhibitor on the kinetic parameters of CPT-11 transformation into APC with a range of concentrations of CPT-11 was studied on APC formation by the pool of human liver microsomes. The results are presented in Fig. 5 as a percentage of the control (velocity measured in the presence of drug/velocity measured in the absence of drug \( \times 100 \)). They show that loperamide and racecadotril inhibited APC formation by about 50\% \( (P < 0.001) \), whereas ondansetron inhibited the reaction by 25\% \( (P < 0.05) \). In contrast, the remaining drugs did not significantly inhibit the metabolism of CPT-11 into APC. The concentrations of loperamide, racecadotril, and ondansetron able to inhibit 50\% of APC formation are indicated in Table 2, together with the apparent \( K_m \) and \( V_{\max} \) values of CPT-11 transformation into APC.

![Fig. 2. A typical chromatogram showing the separation of two unknown metabolites (1 and 3), APC (2), CPT-11 (4), SN-38 (5), and camptothecin (internal standard; 6).](image)

![Fig. 3. Kinetics of CPT-11 biotransformation into APC when the substrate was supplied as lactone (●) or carboxylate (●). The solid lines represent fits of the data with the Michaelis-Menten equation by nonlinear regression.](image)

### Table 2 Kinetic data for inhibition of APC formation by various drugs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( IC_{50} ) of inhibitor (( \mu M ))</th>
<th>( K_m ) of CPT-11 (( \mu M ))</th>
<th>( V_{\max} ) of APC formation (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.14</td>
<td>19.2 ± 3.9</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>0.40</td>
<td>53.2 ± 4.3</td>
<td>14.9 ± 0.6</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>56.1</td>
<td>25.0 ± 1.3</td>
<td>14.1 ± 0.3</td>
</tr>
<tr>
<td>Loperamide</td>
<td>23.0</td>
<td>25.3 ± 5.4</td>
<td>10.3 ± 0.8</td>
</tr>
<tr>
<td>Racecadotril</td>
<td>46.1</td>
<td>15.6 ± 4.9</td>
<td>5.6 ± 0.5</td>
</tr>
</tbody>
</table>
between the lactone and carboxylate forms are at the level of the ring E opening (see Fig. 1). However, there is an important difference in water solubility and lipophilicity between the two forms; a better penetration of the lactone form into the microsomal membranes where the CYPs are located could explain its preferential handling by the enzyme. However, by using a detergent able to solubilize the membranes and equalize the accessibility of both forms, we obtained the same kinetic differences between the two forms (data not shown).

Our results demonstrate that CYP 3A is involved in CPT-11 metabolism: (a) of the CYP inhibitors tested, only those selectively inhibiting the CYP 3A subfamily, troleandomycin and ketoconazole, or the general inhibitor cimetidine inhibited the biotransformation of CPT-11 into APC; (b) only the microsomes of lymphoblastoid cells transfected with CYP 3A4 cDNA were able to produce measurable amounts of APC, albeit at lower rates than human liver microsomes; and (c) correlations were established between APC formation and both the enzyme activity characteristic for CYP 3A (testosterone 6β hydroxylation) and the CYP 3A immunoreactive protein. Unfortunately, the various CYP 3A isoforms are closely related, and the antibody we used cannot distinguish between CYP 3A4, the major CYP 3A isoform of human liver, and CYP 3A5 or CYP 3A7. Similarly, enzyme inhibition studies do not allow us to formally identify the CYP 3A isoform responsible for the formation of APC. In contrast, because cDNA transfection is specific, our results show unambiguously that CYP 3A4 is involved in APC formation, although this does not exclude participation by other CYP 3A isoforms. Based on the various approaches used, the intervention of other CYP families can be excluded.

An important question that cannot be addressed directly in human patients concerns the possible influence of comediations on CPT-11 biotransformation. Drugs metabolized by the same CYP isoform as CPT-11 may inhibit its biotransformation and, therefore, its disposition. This is the case for cyclosporine, steroids, erythromycin, macrolide antibiotics, and anticancer agents such as the Vinca alkaloids, tamoxifen, and Taxol (18–22) that could, in theory, substantially reduce the formation of APC and favor other metabolic pathways of CPT-11. In addition, repeated administration of drugs inducing CYP 3A (20) could modify the metabolism and elimination of CPT-11. When testing a panel of 11 drugs that might be used concomitantly with CPT-11 in the clinical setting, we observed a significant inhibition of APC formation with loperamide, racecadotril (two anti-diarrheics used for the treatment of CPT-11-induced diarrhea) and ondansetron, an antiemetic. It has been established that

**DISCUSSION**

CPT-11 is metabolized by human liver microsomes into several metabolites. We have already studied the characteristics of the formation of the active metabolite of CPT-11, SN-38, by human liver microsomes (17). However, other metabolites are quantitatively more important than SN-38, both in the plasma of patients treated with CPT-11 and after incubation of CPT-11 with liver microsomes. We have identified two metabolites as the β-glucuronide of SN-38 (5) and APC (7). Although APC is not a substrate for the carboxylesterases (7), its formation may play an important role in CPT-11 disposition in patients (11), and it was necessary to explore the pathway giving rise to this metabolite.

The kinetic study we performed on a pool of human microsomes revealed that the two forms of CPT-11 were not metabolized to the same extent to APC. The apparent $K_m$ was 2-fold higher and the $V_{max}$ was 2-fold lower when CPT-11 carboxylate was used as a substrate of the reaction as compared with CPT-11 lactone. We had also observed a similar preferential transformation of CPT-11 lactone to SN-38 by the carboxylesterases of human liver microsomes (17), but this was not the case for the glucuronidation of SN-38 using the same enzyme source (6). It is difficult to assign a cause to the preferential transformation of CPT-11 lactone to APC, particularly given that the site of the reaction is the distal piperidine ring, whereas the difference in water solubility and lipophilicity between the two forms; a better penetration of the lactone form into the microsomal membranes where the CYPs are located could explain its preferential handling by the enzyme. However, by using a detergent able to solubilize the membranes and equalize the accessibility of both forms, we obtained the same kinetic differences between the two forms (data not shown).

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loperamide (23) and ondansetron (24) are substrates of CYP 3A, whereas we could find no such information for racemadotril. Our kinetic studies are in favor of mixed mechanisms of inhibition of APC formation by loperamide and ondansetron, as with the well-known CYP 3A substrates troleandomycin and ketoconazole. In view of the relatively high concentrations required for the inhibition of APC formation by most drugs, however, it is doubtful that significant interactions may occur in the clinical setting.

Comparing the structures of CPT-11 and APC leads to the conclusion that the biotransformation of the former to the latter cannot occur in only one step. It is possible that the reaction proceeds via an α-hydroxylated metabolite that, because of its inherent instability, would be expected to decompose to APC. Alternatively, if other enzyme-catalyzed reactions are implicated in APC formation, these are likely, based on our findings, to also be mediated by CYP 3A. As indicated in Fig. 2, other fluorescent products are generated during incubations of CPT-11 with human liver microsomes, and work is continuing in our laboratory to identify their role in the disposition of CPT-11. During the process of identification of the CYP involved in APC formation, we were able to show that the formation of peak 1 was also dependent on CYP 3A.

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