ABSTRACT:

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecine) is a water-soluble derivative of camptothecine (Sawada et al., 1991) that is currently being used in the treatment of advanced colorectal adenocarcinoma (Wiseman and Markham, 1996). Irinotecan is converted to SN-38 (fig. 1), a very potent inhibitor of topoisomerase I, through the action of carboxyles-terases (Haaz et al., 1996). Irinotecan is converted to SN-38 (fig. 1), a very potent inhibitor of topoisomerase I, through the action of carboxylesterases (Haaz et al., 1996; Slatter et al., 1997). A number of other important plasma metabolites have been identified in patients, and these include a β-glucuronide of SN-38 (Rivory and Robert, 1995) and APC (RPR 121056) (Rivory et al., 1996). Recently, we identified an additional metabolite, NPC, as resulting from the incubation of irinotecan with human liver microsomes. The formal identification of this metabolite using mass spectrometry and the characterization of its pharmacological properties are the subjects of a separate report (Dodds et al., 1998).

APC is the product of a ring-opening oxidation of the distal piperidine ring of CPT-11 that is mediated by the activity of CYP3A (Haaz et al., 1998), whereas NPC has only one piperidine ring, bearing a primary amine on the γ-carbon relative to the ring nitrogen (fig. 1). Despite its lack of cytotoxicity and its weak induction of topoisomerase I-mediated cleavable complex formation, NPC appears to be a functionally important metabolite, because it can be transformed into SN-38 (although to a lesser extent than irinotecan itself) (Dodds et al., 1998). In earlier studies on the metabolism of CPT-11, it was postulated that NPC could arise from the dealkylation of APC (Rivory et al., 1996, 1997; Lokic et al., 1996). However, there are no available data on the enzymes responsible for the formation of NPC or the role of APC as a precursor. In this study, we wanted to characterize the metabolic pathways leading to the formation of NPC from irinotecan and to evaluate the potential drug interactions that could occur in the clinical setting.

This study was supported by grants from the Ligue Nationale Francaise contre le Cancer (Comité Départemental de Charente-Maritime), the Rhône-Poulenc Rorer Company, and the Pôle Médicament Aquitaine.

1 Abbreviations used are: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecine; SN-38, 7-ethyl-10-hydroxycamptothecine; APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecine; NPC, 7-ethyl-10-[4-amino-1-piperidino]carbonyloxycamptothecine; CYP, cytochrome P450.

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CPT-11 lactone set at 25 m and being used at a fixed concentration of 100 m for the study of NPC formation from APC, with the latter replacing CPT-11. Independent experiments were always performed. The same conditions were used in the presence of the appropriate solvent (table 1). The concentration of inhibitor required to inhibit NPC formation by 50% (IC$_{50}$) and its effect on the kinetics of NPC formation ($K_M$ and $V_{max}$) were determined when inhibition was >50% with the initial concentration of inhibitor.

The second approach used microsomes obtained from human lymphoblastoid cells transfected with the cDNAs for specific CYPs; these microsomes were incubated as described above, with 100 m CPT-11 lactone. However, for the microsomes from CYP2C9-transfected cells, the phosphate buffer was replaced with 0.1 M Tris buffer, pH 7.5, as recommended by the supplier. The third approach relied on the correlation that existed between the levels of specific CYPs, as evaluated by enzymatic or immunological reactions, and the biotransformation of CPT-11 into NPC. CYP activities for specific substrates were indicated by the supplier of the 15 individual microsomal preparations, and these values were used without further characterization. In addition, the CYP3A content in these microsomes was quantified by Western blotting. For this purpose, 25 m of CPT-11 in the presence of selective inhibitors of CYP isoforms (Halpert et al., 1994). The rates of formation of NPC (estimated in three independent experiments) are expressed as percentages of those observed in corresponding control incubations containing equal amounts of drug solvent and are means ± SD.

**TABLE 1**

<table>
<thead>
<tr>
<th>CYP</th>
<th>Inhibitor (Concentration)</th>
<th>Solvent</th>
<th>NPC Formation % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonselective</td>
<td>Cimetidine (100 m)</td>
<td>Ethanol</td>
<td>58.8 ± 9.6</td>
</tr>
<tr>
<td>1A2</td>
<td>α-Naphthoflavone (1 m)</td>
<td>Ethanol</td>
<td>81.3</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin (100 m)</td>
<td>Ethanol</td>
<td>92.8 ± 8.2</td>
</tr>
<tr>
<td>2C8/9</td>
<td>Sulfaphenazole (1 m)</td>
<td>Ethanol</td>
<td>92.8 ± 0.7</td>
</tr>
<tr>
<td>2D6</td>
<td>Quinidine (5 m)</td>
<td>Buffer</td>
<td>75.4 ± 9.3</td>
</tr>
<tr>
<td>2E1</td>
<td>Chlorozoxazone (100 m)</td>
<td>Ethanol</td>
<td>77.2 ± 12.2</td>
</tr>
<tr>
<td>3A</td>
<td>Ketocoumazole (1 m)</td>
<td>Ethanol</td>
<td>1.4 ± 1.1</td>
</tr>
<tr>
<td>3A</td>
<td>Troleandomycin (100 m)</td>
<td>Methanol</td>
<td>ND*</td>
</tr>
</tbody>
</table>

Inhibition of NPC formation using pooled microsomes was estimated with 25 m CPT-11 in the presence of selective inhibitors of CYP isoforms (Halpert et al., 1994). The rates of formation of NPC (estimated in three independent experiments) are expressed as percentages of those observed in corresponding control incubations containing equal amounts of drug solvent and are means ± SD.

*ND, no formation detected.

Materials and Methods

**Chemicals and Reagents.** Pure CPT-11, SN-38, APC, and NPC (RPR 132595A) were supplied by Rhône-Poulenc Rorer (Vitry-sur-Seine, France). (2S)-Camptothecin was obtained from Sigma Chimie (Saint-Quentin-Fallavier, France). Solvents and reagents were of the highest grade commercially available.

**Human Liver Microsomes.** Human liver microsomes were prepared, according to standard subcellular fractionation procedures (Berthou et al., 1989), from human livers obtained after approval by the relevant institutional ethical committees. A pool of preparations containing approximately 15 mg/ml microsomal protein was used for the study of NPC formation and drug interactions.

In addition, we used two other sources of human microsomes for CYP isoform identification, i.e., 1) microsomes from human lymphoblastoid cell lines transfected with specific CYP cDNAs (CYP1A2, CYP2C9, CYP2D6, and CYP3A4), which were obtained from Gentest Corp. (Woburn, MA), and 2) microsomes from the livers of 15 individuals (already characterized for the activity of various CYPs), which were purchased from the International Institute for the Advancement of Medicine (Exton, PA). All microsomes were stored at −80°C and never refrozen after use.

**Incubation Conditions.** For the study of NPC formation from CPT-11, we used the same conditions as described for the study of NPC formation from CPT-11 (Haaz et al., 1998). Incubations were carried out in 0.1 M phosphate buffer, pH 7.4, with 1 mM NADPH and CPT-11 as a substrate at the appropriate concentrations, in a final volume of 500 μl. At least three independent experiments were always performed. The same conditions were used for the study of NPC formation from APC, with the latter replacing CPT-11 and being used at a fixed concentration of 100 m.

**Enzyme Kinetics.** Enzyme kinetics were studied using the original pooled human liver microsomes. CPT-11 was used either as the lactone (stock solution diluted in 0.01 M citric acid, pH 3) or as the carboxylate (diluted in 0.1 M sodium carbonate, pH 10), at concentrations ranging between 2 and 100 m. Samples were taken at 0, 5, 10, 15, and 20 min and analyzed by HPLC. Reaction velocities were evaluated by linear regression of the NPC concentrations vs. time. The Michaelis-Menten parameters (apparent $K_M$ and $V_{max}$) were then determined by nonlinear regression (SigmaPlot; Jandel Scientific, Erkrath, Germany).

**CYP Identification.** For the identification of the CYP involved in the biotransformation of CPT-11 into NPC, three independent techniques were used. The first relied on the inhibition of NPC formation by known selective inhibitors of CYP isoforms (Halpert et al., 1994), with the concentration of CPT-11 lactone set at 25 m. Control incubations were carried out in the presence of the appropriate solvent (table 1). The concentration of inhibitor required to inhibit NPC formation by 50% (IC$_{50}$) and its effect on the kinetics of NPC formation ($K_M$ and $V_{max}$) were determined when inhibition was >50% with the initial concentration of inhibitor.

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The second approach used microsomes obtained from human lymphoblastoid cells transfected with the cDNAs for specific CYPs; these microsomes were incubated as described above, with 100 m CPT-11 lactone. However, for the microsomes from CYP2C9-transfected cells, the phosphate buffer was replaced with 0.1 M Tris buffer, pH 7.5, as recommended by the supplier. The third approach relied on the correlation that existed between the levels of specific CYPs, as evaluated by enzymatic or immunological reactions, and the biotransformation of CPT-11 into NPC. CYP activities for specific substrates were indicated by the supplier of the 15 individual microsomal preparations, and these values were used without further characterization. In addition, the CYP3A content in these microsomes was quantified by Western blotting. For this purpose, 25 μg of protein from each of the 15 individual microsomal preparations was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels for 15 hr, according to the method of Laemmli (1970). Proteins were then transferred to nitrocellulose filters and blotted overnight in 10 mM Tris buffer, pH 7.5, containing 3% immunoglobulin-free bovine serum albumin. Blots were incubated with anti-human CYP3A antibody (Valbiotech, Paris, France) and an alkaline phosphatase-labeled polyclonal antibody (Dako, Trappes, France). Finally, the blots were treated with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma). The relative amount of CYP3A in the human microsomes was measured by densitometric scanning of the blots, using Densyslab software (Bioprobe, Montreuil, France). The content of immunoreactive CYP3A in the
15 human microsomal preparations was then compared with the corresponding velocity of NPC formation.

**Drug Interactions.** The effects on this biotransformation of 11 drugs that could be coadministered with CPT-11 in the clinical setting were studied. The antibiotics amikacin, trimethoprim/sulfamethoxazole (Bactrim), ciprofloxacin, and rocephin, the antineoplastic agent 5-fluorouracil, the antiemetics metoclopramide and ondansetron, the antiarhythmics loperamide and raccacetodrol (acetophan), and the analgesics morphine and 4-acetamidophenol (paracetamol) were selected. These studies were performed initially with fixed concentrations of CPT-11 lactone (25 μM) and of each drug (100 μM), under the conditions described above. When the inhibition of NPC 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} level of the inhibitor.

**HPLC.** Quantitative evaluation of NPC formation was performed using a HPLC method adapted from that of Rivory and Robert (1994), essentially as described recently (Haaz et al., 1998). Briefly, the samples taken from the incubation mixtures were added to 0.5-ml Eppendorf tubes containing 100 μl of ice-cold methanol/acetonitrile (50:50, v/v) and 50 ng of camptothecine (internal standard). The mixture was vortex-mixed and centrifuged at 8000 g for 2 min. After acidification by addition of 7 μl of 1 N HCl to the supernatant and vigorous stirring, samples (10 or 20 μl) were injected into the HPLC system, with a Radial-Pak C18 reverse-phase column (Waters). Elution was performed at a constant flow rate of 1.5 ml/min, with a programmed linear acetonitrile gradient (from 15 to 30%, v/v, over 23 min) in 0.075 M ammonium acetate buffer, pH 6.0, containing tetrabutylammonium phosphate at a final concentration of 5 mM. Eluent fluorescence was monitored with excitation and emission wavelengths set at 355 and 515 nm, respectively, and peak data were analyzed as reported previously (Haaz et al., 1998).

**Results**

**In Vitro Kinetics of NPC 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 HPLC separation, CPT-11 eluted at 13.0 min and camptothecine (internal standard) eluted at 16.8 min, whereas three compounds eluted at 8.0, 9.0, and 10.2 min. The first and second metabolite peaks corresponded to standards of NPC and APC, respectively.

With the lactone form of CPT-11, incubations with 1 mg/ml protein yielded rates of formation of NPC that initially were linear for 10–15 min and then decreased slowly for the remainder of the incubation (fig. 3). Because the formation of NPC was slower when CPT-11 carboxylate was used, it is likely that this decrease is the result of conversion of the substrate from the lactone to the carboxylate form. When the linear segment of the reaction was analyzed, the apparent K_{M} and V_{max} values obtained were 48.2 ± 6.8 μM and 74.1 ± 4.9 pmol/min/mg of protein, respectively, for the lactone form. With the carboxylate form of CPT-11, the kinetics of formation of NPC were linear for the entire 20-min incubation. However, the Michaelis-Menten parameters were not as well characterized, because the velocity of the reaction continued to increase appreciably within the range of CPT-11 concentrations tested. Nevertheless, the values for K_{M} and V_{max} obtained by nonlinear regression were 273 ± 122 μM and 78.6 ± 27.7 pmol/min/mg of protein, respectively (fig. 4). There was no detectable formation of NPC when APC (100 μM) was used as a substrate in the microsomal incubations for the same period of time.

**Identification of the CYP Involved in NPC Formation.** The nonselective CYP inhibitor cimetidine (100 μM) inhibited NPC formation by 40% (table 1) with CPT-11 lactone at 25 μM. Table 1 also shows that the CYP3A inhibitors troloandomycin (100 μM) and ketoconazole (1 μM) inhibited NPC formation by 100 and 99%, respectively. The other compounds tested had no appreciable effect. The inhibition of NPC formation by troloandomycin and ketoconazole was further evaluated by determining the concentrations of inhibitor able to inhibit NPC formation by 50%. These values, as well as the apparent K_{M} and V_{max} values determined in the presence of the IC_{50} levels of these inhibitors, are indicated in table 2. It appeared that the inhibition exerted by these two compounds on NPC formation was mixed.

Studies using human cDNA-transfected human lymphoblastoid cells expressing single CYP forms revealed that only microsomes from the CYP3A4-transfected cells were able to metabolize CPT-11 to NPC (data not shown). Incubation of CPT-11 with microsomes from control cells (no CYP) or from cells transfected with cDNA for CYP1A2, CYP2D6, or CYP2C9 did not produce any detectable formation of NPC.

We compared the rates of transformation of CPT-11 into NPC by 15 individual human liver microsomal preparations with the CYP activities, determined with specific substrates, reported by the supplier of the microsomes (fig. 5A). A significant correlation was found between NPC formation and testosterone 6β-hydroxylation (known to be mediated by CYP3A) (r = 0.904 and 0.825 for the CPT-11 lactone and carboxylate forms, respectively; p < 0.001). In contrast, nonsignificant correlations were found between NPC formation and phenacetin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), phenytoin 4-hydroxylation (CYP2C), dextromethorphan O-demethylation (CYP2D), or chlorozoxazone 6-hydroxylation (CYP2E). In addition, by using immunoblotting quantification of 15 individual preparations (fig. 5B), we found a significant correlation...
between CYP3A level and NPC formation (\( r = 0.873 \) and 0.737 for the CPT-11 lactone and carboxylate forms, respectively; \( P < 0.001 \) and 0.002, respectively).

**Drug Interactions with NPC Formation.** The effects on NPC formation of several drugs (100 \( \mu M \)) that could be used with CPT-11 in the clinical setting were studied with pooled human liver microsomes. The results are presented in fig. 6 as percentages of control values (velocity measured in the presence of drug/velocity measured in the absence of drug \( \times 100 \)). The results show that, at 100 \( \mu M \), loperamide and racecadotril inhibited NPC formation by about 95% (\( P < 0.001 \)), whereas ondansetron inhibited the reaction by 75% (\( P < 0.05 \)). In contrast, the remaining drugs did not significantly inhibit the metabolism of CPT-11 to NPC. The concentrations of loperamide, racecadotril, and ondansetron able to inhibit NPC formation by 50% with a CPT-11 concentration of 15 \( \mu M \) are indicated in table 2, together with the apparent \( K_M \) and \( V_{max} \) values for CPT-11 transformation into NPC in the presence of IC\(_{50}\) levels of these drugs. The inhibitions we observed were mostly mixed, except for racecadotril, which selectively decreased the \( V_{max} \) of NPC formation, consistent with a noncompetitive mechanism.

**Discussion**

Irinotecan is metabolized by human liver microsomes into several metabolites. We have already studied the characteristics of the formation of its active metabolite, SN-38, by human liver microsomes (Haaz et al., 1997b). However, other metabolites are quantitatively

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**TABLE 2**

<table>
<thead>
<tr>
<th>IC(_{50}) of Inhibitor</th>
<th>( K_M ) of CPT-11</th>
<th>( V_{max} ) of NPC Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu M )</td>
<td>( \mu M )</td>
<td>pmol/min/mg of protein</td>
</tr>
<tr>
<td>Control</td>
<td>33.5 ± 2.0</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.075</td>
<td>47.6 ± 2.7</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>0.256</td>
<td>103 ± 26</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>43.7</td>
<td>57.9 ± 3.3</td>
</tr>
<tr>
<td>Loperamide</td>
<td>18.1</td>
<td>79.5 ± 10.8</td>
</tr>
<tr>
<td>Racecadotril</td>
<td>46.8</td>
<td>44.7 ± 12.0</td>
</tr>
</tbody>
</table>

The IC\(_{50}\) of each inhibitor was first estimated using pooled microsomes with 25 \( \mu M \) CPT-11 lactone, in three independent experiments; we then estimated the effect of this concentration of inhibitor on the kinetic parameters for CPT-11 transformation into NPC with a range of concentrations of CPT-11, also in three independent experiments.
Fig. 6. Effects of 11 drugs (each at 100 μM) on the velocity of CPT-11 transformation into NPC by human liver microsomes.

Values are expressed as percentages of control values and are means ± SD of three independent experiments.

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 three quantitatively important metabolites as being the β-glucuronide of SN-38 (Rivory and Robert, 1995), APC (Rivory et al., 1996), and NPC (Dodd et al., 1998). NPC is a substrate for the carboxylesterases, and its formation could play an important role in irinotecan disposition in patients. Therefore, it was necessary to explore the pathway giving rise to this metabolite.

The kinetic study we performed with pooled human microsomes revealed that the two forms of irinotecan were not metabolized to NPC to the same extent, with the affinity of the carboxylate form for the enzyme being much lower than that of the corresponding lactone. Although the V_{max} estimates obtained for the two forms were similar, the kinetics of the formation of NPC from irinotecan carboxylate were not as well characterized, because of the lesser extent of this reaction. We previously observed a similar preferential transformation of irinotecan lactone to APC by CYP3A (Haaz et al., 1996). We previously observed a similar preferential transformation of irinotecan lactone to APC by CYP3A (Haaz et al., 1996), and NPC (Dodd et al., 1998). NPC is a substrate for the carboxylesterases, and its formation could play an important role in irinotecan disposition in patients. Therefore, it was necessary to explore the pathway giving rise to this metabolite.

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Our results demonstrate that CYP3A is involved in the formation of NPC, as for APC. This is supported by the demonstration that both ketoconazole and troleandomycin substantially inhibited the biotransformation of irinotecan into NPC. Also, only the microsomes of lymphoblastoid cells transfected with CYP3A4 cDNA were able to produce measurable amounts of NPC, albeit at lower rates than human liver microsomes. Finally, correlations were established between NPC formation and both the enzyme activity characteristic for CYP3A (testosterone 6β-hydroxylation) and immunoreactive CYP3A protein levels. Unfortunately, the various CYPs of the 3A group are closely related and the antibody we used cannot distinguish between CYP3A4 (the major CYP3A of human liver) and CYP3A5 or CYP3A7. Based on the various approaches used, the involvement of other CYP families can be excluded.

In two studies reporting preliminary evidence for the existence of NPC in the plasma and urine of patients treated with irinotecan (Rivory et al., 1996, 1997), it was postulated that NPC could be a dealkylated product of APC. However, it was impossible to obtain detectable amounts of NPC when APC replaced irinotecan in the incubations with human liver microsomes. We conclude that APC and NPC, which are both produced by CYP3A, are likely to be end-products of the metabolism of irinotecan. Because both require multiple oxidative steps, they may share common monooxidated metabolites. A plausible sequence of reactions would involve first the hydroxylation of an α-carbon of the distal piperidine ring. This unstable metabolite would most likely rearrange spontaneously to a ring-opened aldehyde. Indeed, several stable metabolites of m/z 603 have been observed in the plasma of 10 patients (Rivory et al., 1996). A significant peak with the same mass was also observed after the incubation of irinotecan with human liver microsomes (Dodd HM and Rivory LP, unpublished results). APC and NPC could be produced from this common intermediate, presumably by the action of an aldehyde dehydrogenase and by a second CYP-mediated hydroxyla-

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References


