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Report

Characterization of CPT-11 converting carboxylesterase activity in colon tumor and normal tissues: comparison with *p*-nitro-phenylacetate converting carboxylesterase activity

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Irinotecan (CPT-11) is a topoisomerase I inhibitor commonly used in the treatment of colorectal tumors. It is a prodrug, converted to an active metabolite, SN-38, by carboxylesterases (CEs). CEs are ubiquitary enzymes that react with numerous substrates. A specific CPT-11 converting enzyme was isolated from rat serum, with different kinetic properties than other CEs. We determined kinetic properties of specific CPT-11 CE activity (CPT-CE) in human normal liver and colon tumors. K_m were very similar (3.4 μM in liver and 3.8 μM in colon tumors), but V_{max} was higher in liver (2.7 pmol/min/mg protein) than in colon tumor (1.7 pmol/min/mg protein). CPT-CE and total CE (using *p*-nitro-phenylacetate as substrate) were weakly correlated in colon tumors. The large inter-patient variability observed in liver CPT-CE activity could play a potential role in the pharmacokinetic variability observed with irinotecan. [© 2000 Lippincott Williams & Wilkins.]

Key words: Carboxylesterase, colon carcinoma, CPT-11, irinotecan.

Introduction

Carboxylesterases (CEs) are a family of ubiquitary enzymes that react with many classic substrates such as *p*-nitro-phenylacetate (*p*-NPA) and nitrophenyl butyrate. They are present in all vertebrates, and classified by substrates for which they have high affinity and the specific compounds that inhibit their activity.[1,2] They are implicated in the metabolism of arylamine carcinogens and in steroid hormone catabolism. Moreover, they have been shown to play a key role in the activation of a number of anticancer drugs. Clinically, one of the most important of these agents is irinotecan or CPT-11. Irinotecan (7-ethyl-10[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) (CPT-11) is a topoisomerase I inhibitor commonly used in the treatment of colorectal tumors and promising results have been recently reported in metastatic disease.[3,4] CPT-11 is a prodrug, which differs structurally from other camptothecin derivatives by a bulky piperidino side chain located at the C-10 position of the camptothecin molecule.[5] This piperidino group must be cleaved enzymatically by a carboxylesterase to form SN-38, which is the active metabolite.[6] The antitumor activity of CPT-11 is, therefore, dependent on its activation by the CPT-11 converting CE (CPT-CE). The CPT-CE has been characterized in liver microsomes,[7,8] pointing out the relative inefficiency of the process in the liver. In fact, the low efficiency of CEs seems to be a general feature of these enzymes in humans and the interspecies comparison of the CEs with a panel of classical substrates demonstrated that the human enzymes were among the less efficient.[9] However, a specific CPT-11 converting CE was isolated from the rat serum.[10] These authors demonstrated that the CPT-CE exhibited different enzymatic properties compared to the other CEs and that the K_m was different between *p*-NPA and CPT-11. The conversion of CPT-11 into SN-38 has been studied in a wide variety of tissues, cell lines and purified enzyme preparations *in vitro*.[7,8,10–15] The sensitivity of pro-

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liferating tissues or cell lines to cytotoxic effects of CPT-11 may be related to their CE levels.[11,13] A decreased conversion of CPT-11 to SN-38 has been reported *in vitro* in resistant ovarian and non-small cell lung cancer cell lines.[13,16] However, little is known about the properties of CPT-CE in human and normal tissues; the contribution of specific CPT-CE among the overall CE activity determined with a general substrate as *p*-NPA is unknown.

Materials and methods

Chemicals

CPT-11 and SN-38 were provided by Rhône Poulenc Rorer laboratories (Vitry sur Seine, France). CE (EC 3.1.1.1.) isolated from porcine liver and *p*-NPA were purchased from Sigma (St Louis, MO).

Tissue specimens

Colon tumors were obtained from 52 patients undergoing surgery for colon carcinoma. The surgical procedure was for initial resection. Normal liver were obtained from seven patients who underwent hepatic lobectomy for solitary colorectal metastases. These tissue specimens were taken after obtaining an informed consent of patients.

Immediately after resection, portions of non-necrotic tumor and normal liver tissue were excised by a pathologist and frozen in liquid nitrogen. Samples were stored at −80°C until analysis of enzyme activity.

Cytosol preparation

Tumor or liver samples were homogeneized in 35 mM sodium phosphate buffer, pH 7.5. The cellular suspension was centrifuged at 20 000 *g* for 30 min at 4°C. Cytosolic protein concentration was determined with the Bradford method.[17] For normal liver, the protein concentration was corrected from hemoglobin concentration, by subtracting OD at 548.5 nm.

For the determination of K_m and V_{max} in the tumor and the liver, 10 colon tumor samples and six normal liver samples were pooled prior to preparation of the cytosol.

CPT-CE

The CPT-CE activity was then carried out by preincubating 80 μl of cytosolic proteins for 5 min at 37°C in eppendorf tubes and then different concentrations of CPT-11 (20 μl) for an additional time comprised between 5 and 90 min.[7] The reaction was stopped by addition of 100 μl of an ice-cold mixture of acetonitrile, methanol and 0.1 N HCl (3:3:3, v/v). After centrifugation at 4°C for 15 min at 400 *g*, the supernatant (150 μl) was recovered and 50 μl aliquots were analyzed for SN-38 concentration by HPLC according to Rivory and Robert.[18] Briefly, separation was performed on a Nucleosil C18 column (5 μm, 300 mm × 3.9 mm), eluted with a mobile phase consisting of 0.075 M ammonium acetate buffer (pH 6.4)–acetonitrile (60:40, v/v) to which one vial of PIC A solution was added. Detection of SN-38 was carried out with a Shimadzu fluorimeter (excitation at 355 nm and emission at 515 nm). Standards were prepared from a 100 μg/ml stock solution of SN-38 diluted serially in a mixture of acetonitrile, methanol and 0.1 N HCl (3:3:3, v/v). Standards curves were constructed for each batch of samples and were linear from 30 to 300 nM SN-38. CPT-CE activity was expressed as pmol SN-38 produced/min/mg protein.

p-NPA converting CE

The determination of the *p*-NPA converting CE in tumors was adapted from an assay based on the conversion of colorless *p*-NPA to yellow *p*-nitrophenol (*p*-NP).[10] Cytosols were diluted in 35 mM sodium phosphate buffer (pH 7.5) to obtain a protein concentration of 3 mg/ml. The diluted sample (180 μl) was transferred to a 96-well microtiter plate and the reaction was started by adding 20 μl of 10 mM *p*-NPA as a substrate. After an incubation period of 10 min at 37°C, the extinction of the produced *p*-NP was monitored at 405 nm on a Labsytems Multiscan Bichromatic plate reader. To correct the data for the spontaneous conversion of the substrate, 20 μl of 10 mM *p*-NPA was added to 180 μl phosphate buffer. The enzyme activity was expressed in units/mg protein (one unit of enzyme converts 1 μmol of *p*-NPA/min at 37°C). Standard curves using porcine liver CE (0–90 U) were performed in each experiment.

Statistical analysis

Gaussian distribution and homogeneity of variances were verified by prior statistical tests. The goodness of fit of the linear regressions was tested in two different ways: the coefficient of correlation was tested with a *t*-test and a variance analysis determined the statistical relevance for using a linear regression to fit the data. The level of significance was 0.05 in every test.

Results

Kinetic of hydrolysis of CPT-11 by tumor and liver CE

The formation of SN-38 from 5 μM CPT-11 by tumor and liver as a function of time is presented Figure 1. The kinetic in both tissues showed a biphasic transformation. An initial rapid formation of SN-38 was followed, after 30 min, by a slower linear stage. The SN-38 formation, in this linear part, was about 1.7-fold higher in liver than in colon tumor.

Effect of enzyme concentration

We determined the range of linearity of the SN-38 formation from CPT-11 with the protein concentration of either the tumor or the liver. We incubated increasing concentrations of either tumor or liver cytosols (0.5–4 mg/ml) with 5 μM CPT-11 for 1 h. The formation of SN-38 is linear in the range of protein concentrations that we investigated (Figure 2). The slope of SN-38 formation is though deeper for liver (0.195 pmol/min/mg protein) than for tumor (0.105 pmol/min/mg protein). The goodness of fit of the linear regression was statistically evaluated. The coefficients of regression were highly significant both for the liver (r^2=0.95, $p<0.001$) and the tumor (r^2=0.97, $p<0.001$).

Determination of the apparent K_m and V_{max} in liver and tumor tissues

Previous studies, based on the mechanism of action of the CEs,[7,9] estimated the CEs kinetic properties from the second part of the kinetics, the steady-state formation. We used the steady-state rate of formation as a function of CPT-11 concentration to determine the apparent K_m and V_{max}. The CPT-CE activity in colon tumor and liver were determined using a 60 min incubation and a concentration of 3 mg/ml of proteins. The double reciprocal plot, allowing the determination of K_m and V_{max}, is presented Figure 3. The data should be taken as approximate values since the enzyme used was not purified. The K_m for liver was 3.4 μM, very close to the K_m in tumor, 3.8 μM. However, the V_{max} in liver (2.7 pmol/min/mg protein) was 2-fold higher than in tumor (1.7 pmol/min/mg protein).

Comparison of *p*-NPA versus CPT-CE activity in colon tumors

The relationship between specific CPT-CE activity (using CPT-11 as a substrate) and total CE activity (using *p*-NPA as a substrate) was tested in colon tumors (Figure 4). Even if a significant correlation seemed to exist between CPT-CE activity and total CE activity (r^2=0.35, $p<0.001$), a large variability was observed.

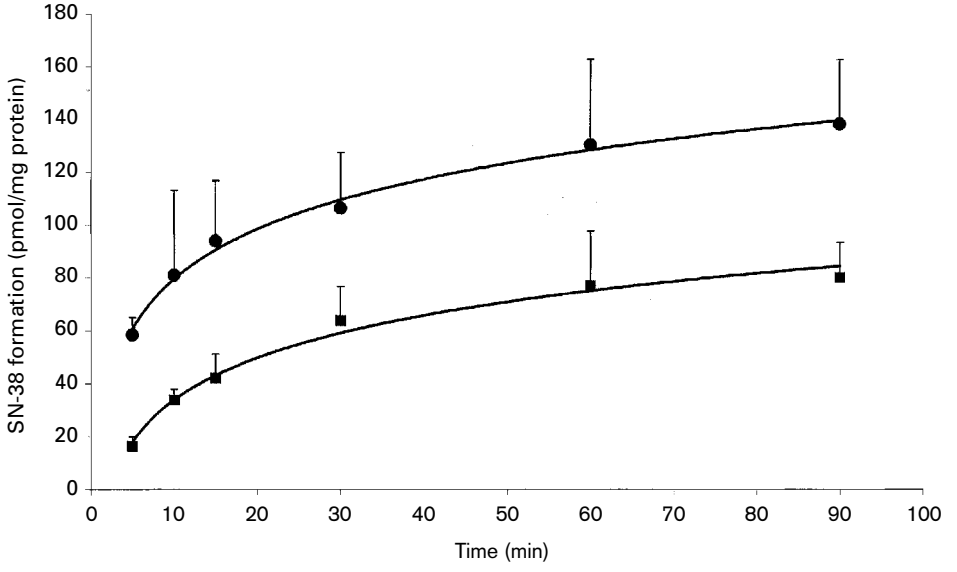

Figure 1. Formation of SN-38 (pmol/min/mg protein) as a function of time in tumor and liver cytosol in presence of CPT-11 (5 μM). The kinetic profile is biphasic, the velocity calculated on the later linear phase (after 30 min). The data are means $\pm$ SD, n=3.

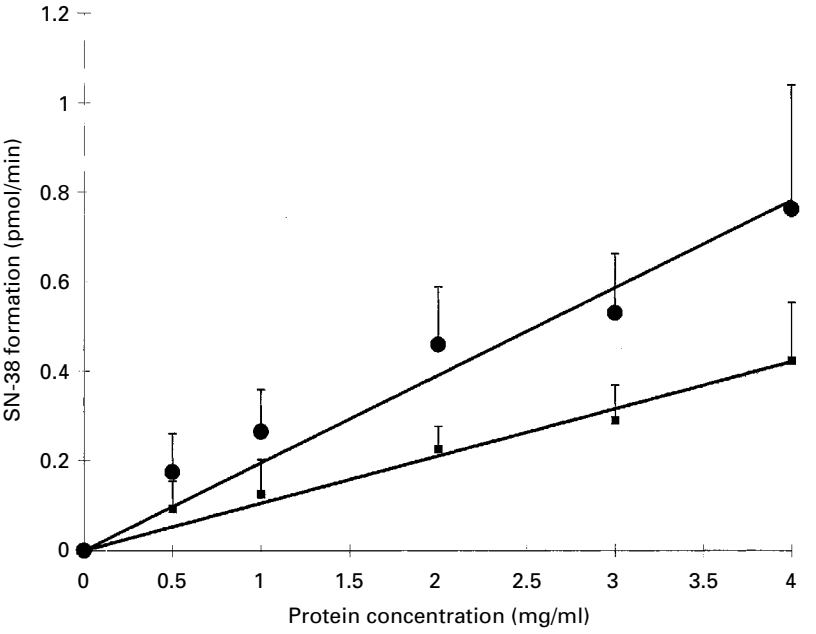

Figure 2. Rate of formation of SN-38 (pmol/min) in tumor and liver cytosol as a function of protein concentration (0.5–4 mg/ml) in the presence of 5 μM of CPT-11 for 60 min. Data are means $\pm$ SD of three independent experiments.

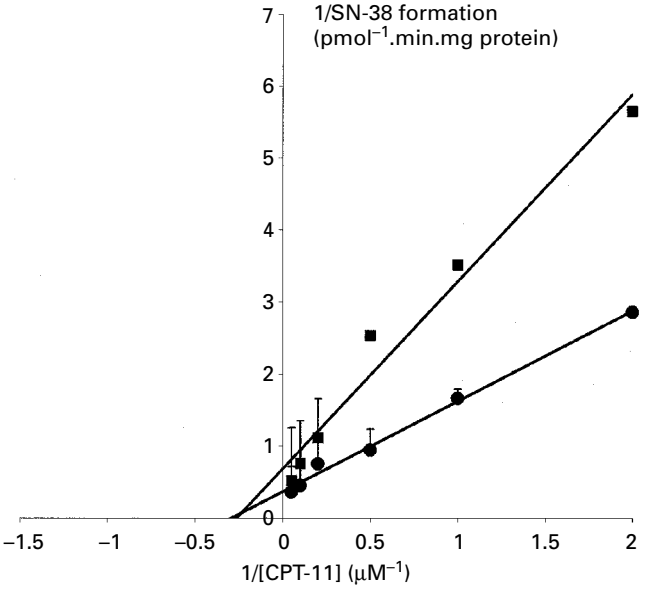

Figure 3. Representation of Lineweaver–Burk (double reciprocal plot) of tumor and liver cytosol. Apparent K_m and V_{max} are calculated from this graph. Data are mean $\pm$ SD of three independent experiments.

Discussion

Irinotecan or CPT-11 has become an important compound in the chemotherapeutic treatment of colorectal cancer.[19,20] However, the gastrointestinal toxicity and the high variability of the pharmacokinetics of both CPT-11 and its active metabolite, SN38, impair the good efficiency of the drug.

Tsuji *et al.* isolated a specific CPT-11 CE activity in rat serum[10] that exhibited different kinetic properties than other CE. So, we decided to evaluate specific CE activity using CPT-11 as a substrate (CPT-CE activity) in

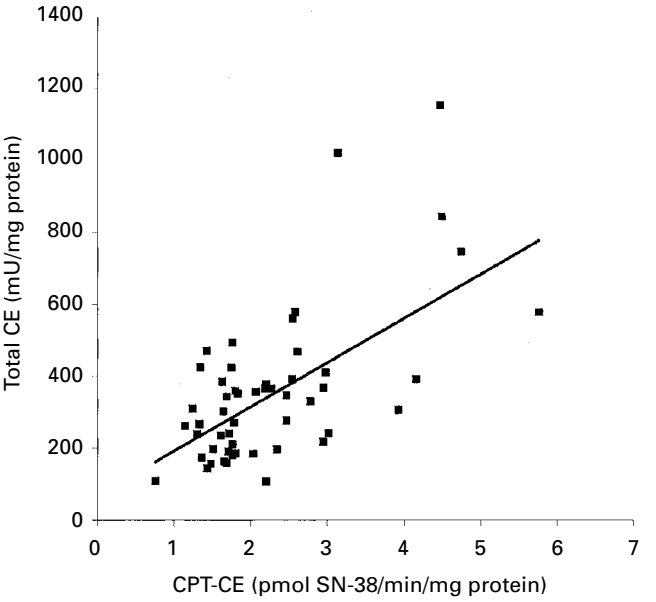

Figure 4. Correlation between total CE activity and CPT-CE activity in colon tumor cytosol. The total CE activity (mU/mg protein) was determined using *p*-NPA as substrate. CPT-CE activity (pmol SN-38/min/mg protein) was determined using CPT-11 as substrate. r^2=0.35, $p<0.001$.

tumor and normal tissues relevant for CPT-11 cytotoxicity or metabolism (liver and colon mucosa), and to compare this activity with the total CE activity determined using *p*-NPA as a substrate.

The kinetic study of CPT-CE activity demonstrated that colon tumor and normal liver CPT-CE had similar apparent K_m but a different apparent V_{max}. V_{max} in the liver, albeit low, was about 1.5-fold higher than V_{max} in tumor and consistent with the report of Slatter *et al.* in liver microsomes.[8] This low V_{max} was consistent with the fact that irinotecan bio-activation was a relatively slow and inefficient process in human, with a low metabolic ratio between irinotecan and SN-38, and an AUC of SN-38 representing only 1–3% of irinotecan AUC.[21,22] Moreover, we pointed out that V_{max} observed with colon tumor cytosols was lower than that obtained with normal liver cytosols. These data are in agreement with those published by Ahmed *et al.*[23] The kinetic study also showed that the K_m in colon tumors and in normal liver were not significantly different. The value in the liver was again in good agreement with the one reported by Slatter *et al.*,[8] corresponding to the high-affinity isoform.

Since CPT-11 requires CE for its conversion, several studies investigated the role of CE activity in CPT-11 cytotoxicity.[14,15] The CE activity as a limiting factor of irinotecan cytotoxicity was confirmed by Jansen *et al.*[14] The addition of CE in the culture medium of a panel of colon cell lines increased the irinotecan cytotoxicity by a factor of 10–100. However, while Van-Ark-Otte *et al.*[15] found a significant correlation between CE and irinotecan cytotoxicity in non-small cell lung cancer cell lines, Jansen *et al.*[14] failed to demonstrate such a correlation in human colon cancer cell lines. In these two studies, the CE activity was determined using *p*-NPA. Our study demonstrated that CPT-CE activity and total CE activity in colon tumor are poorly correlated, even if a statistical significant activity existed. So, the determination of total CE activity (like in the Jansen's study) instead of CPT-CE activity could mask a possible relationship between CE activity and CPT-11 cytotoxicity.

Because of the low efficiency of carboxylesterases to convert irinotecan into SN-38, targeting the CE gene to tumors cells might offer a new strategy for prodrugs that are activated by CE. Rat serum or rabbit liver CEs were reported to enhance the cytotoxic activity of irinotecan.[24,25] Promising results of *in vivo* transfer of the human CE cDNA to potentiate concomitant local administration of irinotecan have been reported by Kojima *et al.*[26]

Conclusion

This report demonstrates substantial contributions of both the tumor and hepatic metabolism in the bioactivation of irinotecan. The biochemical para-

meters of CPT-CE activity in colon tumor and liver showed a similar K_m, and a V_{max} in the liver only 1.5-fold higher than in the tumor. When comparing CPT-CE and total CE (using *p*-NPA as substrate) in colon cancer, a weak correlation between CPT-CE and total CE was observed suggesting that specific CPT-CE activity was more relevant for CPT-11 activity. Moreover, in liver tissue, no correlation existed between these two activities and a large interpatient variability was observed, phenomena that could play a potential in the pharmacokinetic variability observed with irinotecan.

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