Conversion of the CPT-11 Metabolite APC to SN-38 by Rabbit Liver Carboxylesterase[1]

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ABSTRACT

The anticancer drug CPT-11 (7-ethyl-[4(1-piperidino)-1-piperidino]carbonyloxycamptothecin) is a water-soluble derivative of camptothecin. We report here the conversion of APC (7-ethyl-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin), an inactive metabolite of CPT-11, to SN-38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of CPT-11, by a rabbit liver carboxylesterase. This reaction is not catalyzed by any known human enzyme. The formation of SN-38 from APC was characterized by an apparent K_m of 37.9 ± 7.1 μM and a V_{max} of 16.9 ± 0.9 pmol/units/min. SN-38 was confirmed as a reaction product by high-performance liquid chromatography and mass spectrometry. A 24-h incubation of 10 μM APC with 500 units/ml of rabbit carboxylesterase produced 4 μM SN-38. The product of this reaction inhibited the growth of U373 MG human glioblastoma cells *in vitro*. The IC_{50} for a 24-h exposure of U373 MG cells to APC in the presence of 50 units/ml of rabbit carboxylesterase was 0.27 ± 0.08 μM, whereas APC alone demonstrated no inhibition of growth at concentrations up to 1 μM. The IC_{50} of U373 MG cells transfected with the cDNA encoding the rabbit carboxylesterase (U373pIRESrabbit) and exposed to APC for 24 h was 0.8 ± 0.1 μM APC, whereas the growth of cells transfected with vector control (U373pIRES) was unaffected by up to 1 μM APC. Because APC is nontoxic to human cells, we are investigating the possibility of using APC/rabbit carboxylesterase in a prodrug/enzyme therapeutic approach.

INTRODUCTION

CPT-11[3] is a water-soluble derivative of camptothecin. Sixteen metabolites of CPT-11 have been identified in the bile and urine of patients receiving this drug (1). Among the metabolites that have been described are SN-38 and APC. The former compound is a potent inhibitor of topoisomerase I and is cytotoxic; the latter has little effect, if any, on cell proliferation (2). The chemical structures of CPT-11, SN-38, and APC (Fig. 1) contain a lactone ring that is reversibly hydrolyzed to an inactive carboxylate form in a pH-dependent manner. CPT-11 is converted to SN-38 by human liver carboxylesterase(s) *in vitro* (3, 4). The conversion of CPT-11 to its active metabolite SN-38 by human liver carboxylesterases is relatively low, and the concentration of SN-38 in the plasma is only in the range of 1–5% of the concentration of the parent drug (5). Conversion of CPT-11 by human cytochrome P450 3A to the inactive metabolite APC decreases the amount of the parent compound available to be converted to its active form (6). APC has not been shown to be converted to SN-38 by any known human enzyme activities (2).

CPT-11 has shown encouraging results in clinical use thus far (7); however, secretory diarrhea and bone marrow suppression constitute the dose-limiting toxicities of this drug. *In vitro* studies of the inactive metabolite APC demonstrated no inhibition of tumor cell growth by this compound (2). However, Lokiec *et al.* (1) hypothesized that several metabolites of CPT-11, among them APC, could be substrates for carboxylesterases and could be converted to SN-38.

Carboxylesterases are ubiquitous enzymes involved in the hydrolysis of a wide variety of endogenous and xenobiotic ester- or amide-containing compounds (8). Multiple enzymes and isoforms from various animal species have been described, and each form exhibits a unique pattern of substrate specificity (9). The liver carboxylesterases are serine hydrolases containing an NH_2-terminal domain that targets proteins to the endoplasmic reticulum, an active serine residue, two signature sequence motifs (B-1 and B-2), and four COOH-terminal residues responsible for preventing the secretion of these enzymes from the cell (10). Although human liver microsomal carboxylesterases have been shown to convert CPT-11 to SN-38 (3), they appear to be among the least efficient enzymes studied in activating classical substrates of carboxylesterases (9). No known human enzyme converts APC to SN-38.

We have recently isolated the cDNA encoding a rabbit liver carboxylesterase that efficiently converts CPT-11 to SN-38

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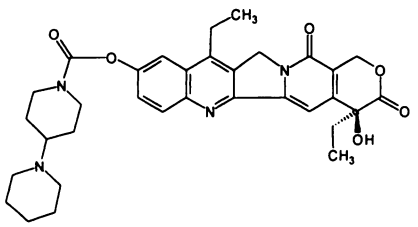
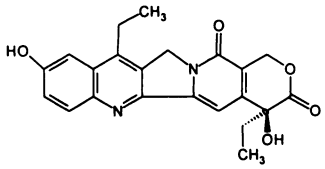
[3] The abbreviations used are: CPT-11, irinotecan, 7-ethyl-10-[4–1-piperidino)-1-piperidino]carbonylcamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; APC, 7-ethyl-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin; IC_{50}, concentration of drug that inhibits the growth of cells in tissue culture by 50%; HPLC, high-performance liquid chromatography.





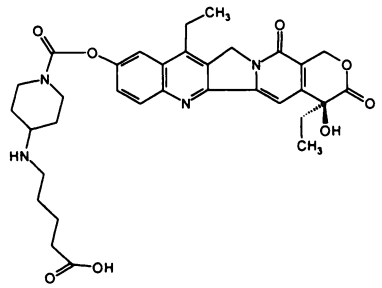


Fig. 1 Chemical structures of CPT-11, SN-38, and APC.

(11). Here, we investigate the possibility that the rabbit carboxylesterase might use APC, as well as CPT-11, as a substrate.

MATERIALS AND METHODS

Chemicals and Cell Lines. Partially purified rabbit liver carboxylesterase was obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents and chemicals were from sources published previously (12). APC, CPT-11, and SN-38 were a gift from Dr. Pat McGovren at Pharmacia Upjohn (Bridgewater, NJ). APC was dissolved in water at a concentration of 10 mM and diluted serially; solutions were stored at −20°C without loss of potency or change in HPLC profile for a maximum of 1 week. CPT-11 was dissolved in 100% methanol at a concentration of 10 mM and stored at −20°C for up to 2 weeks without loss of potency or change in HPLC profile. CPT-11 was diluted serially with water immediately prior to use. HPLC analysis of the stock solutions showed that the APC contained 0.2% SN-38.

U373 MG human glioblastoma cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in MEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 2 mM L-glutamine and kept at 37°C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in exponential growth.

Kinetics of APC Bioactivation. Comparison of the apparent K_m and V_{max} for the conversion of APC or CPT-11 to SN-38 by the rabbit liver carboxylesterase was determined under the following conditions. The reaction mixture contained 50 mM HEPES (pH 7.4), 6 units of rabbit enzyme, and 0–500 μM APC or 0–50 μM CPT-11 in a reaction volume of 200 μl. Reaction mixtures were prewarmed to 37°C, and reactions started with the addition of enzyme. Reactions were carried out for 2 min and stopped by the addition of one volume of ice-cold acidified methanol. Samples were vortexed for 10 s and centrifuged at 14,000 × *g* for 2 min, and supernatants were stored at −80°C for HPLC analysis within 24 h. K_m and V_{max} were determined by analyzing data using the GraphPad Prism program. Data were fitted to one-site hyperbolic nonlinear regression analysis.

Conversion of APC to SN-38 in Tissue Culture Medium. To confirm that the activity of the rabbit carboxylesterase was not inhibited in tissue culture medium and to estimate the extent to which APC was converted to SN-38 under tissue culture conditions, we resuspended the rabbit carboxylesterase to a concentration of 1000 units/ml in water and then mixed APC (1 nM to 10 μM) with increasing amounts of enzyme (50–500 units/ml) in medium containing 10% fetal bovine serum in final volumes of 200 μl. Reactions were carried out at 37°C for 24 h. Control samples contained APC and medium but no enzyme. Reactions were terminated, and samples were processed and analyzed by HPLC, as detailed below.

Mass Spectroscopy. MALDi-TOF mass spectroscopy was performed using a Perspective Biosystems Voyager DE-RP mass spectrometer. Two μl of a 20 mg/ml solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile, 0.1% trifluoroacetic acid was applied to the sample plate. Each sample (1 μl) was then mixed with the above matrix solution and allowed to dry before being placed in the spectrometer. A mixture of standard peptide and matrix ions in the appropriate mass range for the sample was used to calibrate the instrument.

Stable Transfection of U373 MG Human Glioblastoma Cells. U373 MG cells (10^7) were electroporated with 20 μg of pIRES plasmid DNA (pIRESneo) or plasmid containing the rabbit carboxylesterase cDNA (pIRESrabbit) in a volume of 200 μl of PBS using a Bio-Rad (Hercules, CA) electroporator and a capacitance extender. Optimized conditions for electroporation were achieved using 180 V and 960 μF. The cells were plated into 75-cm^2 flasks in fresh medium, and 400 μg of G418/ml were added 48 h after transfection to select for cells expressing the *neo* gene and the carboxylesterase. Cells were grown for a minimum of 10 days before use in growth inhibition experiments.

Growth Inhibition Assays. Two types of growth inhibition experiments were performed. U373 MG cells were plated in 35-mm dishes (Falcon, Franklin Lakes, NJ) at a density of 1.5 × 10^4 cells/dish. The following day, the cells were exposed to APC (1 nM to 10 μM in half-log increments) in the presence or absence of 50 units/ml of rabbit carboxylesterase in a total volume of 2 ml. After 24 h of incubation, the medium containing drug was replaced with fresh medium. After three cell

doubling times, nuclei were counted with a Coulter Multisizer II (Coulter Electronics, Luton, Bedfordshire, United Kingdom). Results were expressed as a percentage of cells in untreated control dishes.

In the second type of growth inhibition experiment, U373pIRES and U373pIRESrabbit cells were plated at a density of 1.5×10^4 cells/dish and allowed to attach. The cells were incubated with APC (1 nM to 10 μM) for 24 h. After the drug exposure, the medium was replaced with fresh medium, and cells were allowed to grow to a time equivalent to three cell doublings.

All determinations were performed in duplicate, and each experiment was repeated three times. Data were analyzed using the sigmoidal dose-response program of GraphPad Prism software. Results were expressed as the concentration of drug required to reduce cell growth to 50% of control cells (IC_{50}).

HPLC Identification and Quantitation of APC, SN-38, and CPT-11. APC, CPT-11, and SN-38 concentrations were determined according to Rivory and Robert (13). Acidification of the samples before HPLC analysis converted all molecules to a lactone form. Briefly, samples were diluted with acidified methanol (5 μl of 1 N HCl per ml of methanol) and centrifuged at 14,000 × *g* for 2 min, and the supernatant was applied to a 300 × 3.9-mm 4-μm Nova-Pak C_{18} column equilibrated in 75 mM ammonium acetate, 25% acetonitrile (pH 4.0). The flow rate was 1 ml/min. Under these conditions, APC, CPT-11, and SN-38 eluted at 4.0, 5.2, and 7.5 min, respectively. Products were detected with a Jasco 821-FP fluorescence detector at an excitation wavelength of 375 nm and emission wavelength of 550 nm. Data were analyzed using System Gold software. The limit of detection for APC, CPT-11, and SN-38 was 20, 20, and 2 pg/μl, respectively.

RESULTS

Kinetic Analysis of the Conversion of APC or CPT-11 to SN-38 by Rabbit Liver Carboxylesterase *in Vitro*. We recently identified a rabbit liver carboxylesterase that efficiently converts CPT-11 to SN-38 and sensitizes human tumor cells to CPT-11 (12). The experiment that follows was designed to determine whether APC might also be a substrate for this carboxylesterase and to compare the efficiency of conversion of APC to SN-38 with the metabolism of CPT-11 to SN-38 by this enzyme. Fig. 2 shows the amount of SN-38 produced when six units of enzyme and APC (0–500 μM; *top*) or CPT-11 (0–50 μM; *bottom*) were incubated for 2 min in HEPES buffer, and the SN-38 produced was quantitated by HPLC. The reaction was linear at the 2-min time point (data not shown) when substrate was in excess. The apparent K_m for APC was 37.9 ± 7.1 μM, and the V_{max} was 16.9 ± 0.9 pmol/units/min (r^2 = 0.91). Similar experiments were performed with CPT-11 (0–50 μM). These results show that for CPT-11, the rabbit enzyme had an apparent K_m of 3.9 ± 0.4 μM and a V_{max} of 15.8 ± 0.6 pmol/units/min (r^2 = 0.97). The 10-fold lower K_m for CPT-11 than for APC indicates a stronger affinity of CPT-11 for the enzyme, whereas the similarity of the V_{max} indicates a similar reaction velocity. These K_ms are in the same range as those reported for a purified human liver microsomal carboxylesterase for CPT-11; however, that human enzyme apparently did not convert APC to SN-38 (2).

Mass Spectrometry of the Enzymatic Reaction Product of Rabbit Carboxylesterase and APC. Confirmation of SN-38 as the final product of the reaction catalyzed by rabbit carboxylesterase from APC was assessed by mass spectrometry. Fig. 3 shows the mass spectra of the enzyme alone (*A*), SN-38 alone (*C*), and APC alone (*B*) or after a 24-h incubation with 500 units/ml rabbit (*D*). APC and SN-38 were identified in their individual spectra as having atomic masses of 619 and 393, respectively. SN-38 could not be detected when APC was incubated for 24 h at 37°C in the absence of enzyme (data not shown). The spectrum of APC incubated for 24 h with enzyme demonstrated peaks reflecting compounds having atomic masses of both 393 (SN-38) and 619 (APC). We conclude that the rabbit liver carboxylesterase converts APC to the active metabolite SN-38.

Conversion of APC to SN-38 in Tissue Culture Medium. Prior to assessing the effect of APC on human tumor cells in culture, we determined that in tissue culture medium the incubation of APC (10 nM to 10 μM) with increasing amounts of enzyme (50–500 units/ml) produced SN-38. The incubation of 10 μM APC with 500 units/ml of rabbit carboxylesterase produced 4 μM SN-38, meaning that 40% of APC was converted to SN-38 in 24 h at 37°C (Fig. 4).

Inhibition of Growth of U373 MG Cells by APC. The incubation of 1 μM APC with 50 units/ml of rabbit carboxylesterase produced a biologically active amount of SN-38; these reaction conditions were, therefore, used in the growth inhibition experiments below.

Two types of growth inhibition experiments were performed. Fig. 5*A* shows the growth inhibitory effect of a 24-h exposure of U373 MG cells to a range of concentrations of APC alone or in the presence of 50 units/ml of rabbit carboxylesterase in the tissue culture medium. No effect on cell growth was observed by incubating the U373 MG cells with APC alone at concentrations up to 1 μM. At higher drug concentrations, the cytotoxicity observed with APC was likely not due to the APC itself but more probably to the 0.2% of SN-38 contaminating the APC solution. The IC_{50} for SN-38 under the same experimental conditions was 4 ± 1 nM. The IC_{50} of APC for U373 MG cells in the presence of 50 units/ml of rabbit carboxylesterase was 0.27 ± 0.08 μM. Incubation of 100 nM APC and 50 units/ml of rabbit carboxylesterase for 24 h in tissue culture media resulted in the formation of 9 nM SN-38 (Fig. 4). We conclude that the cytotoxicity observed in U373 MG cells for APC in presence of the rabbit carboxylesterase is consistent with the amount of SN-38 formed during the incubation time.

The results of the growth inhibition experiments performed with U-373 MG cells stably transfected with pIRESneo vector (U373pIRES) or with the plasmid containing the rabbit carboxylesterase cDNA (U373pIRESrabbit) are depicted in Fig. 5*B*. The IC_{50} for APC after a 24-h exposure was 0.81 ± 0.07 μM in the cells expressing the rabbit carboxylesterase, whereas the IC_{50} for APC of cells transfected with vector alone was 2.3 ± 0.02 μM. Because no conversion of APC to SN-38 can be detected by cell sonicates of vector-transfected cells (data not shown), the toxicity of APC in these control cells is likely due to the 0.2% contamination of the APC preparation with SN-38 (see "Mate-

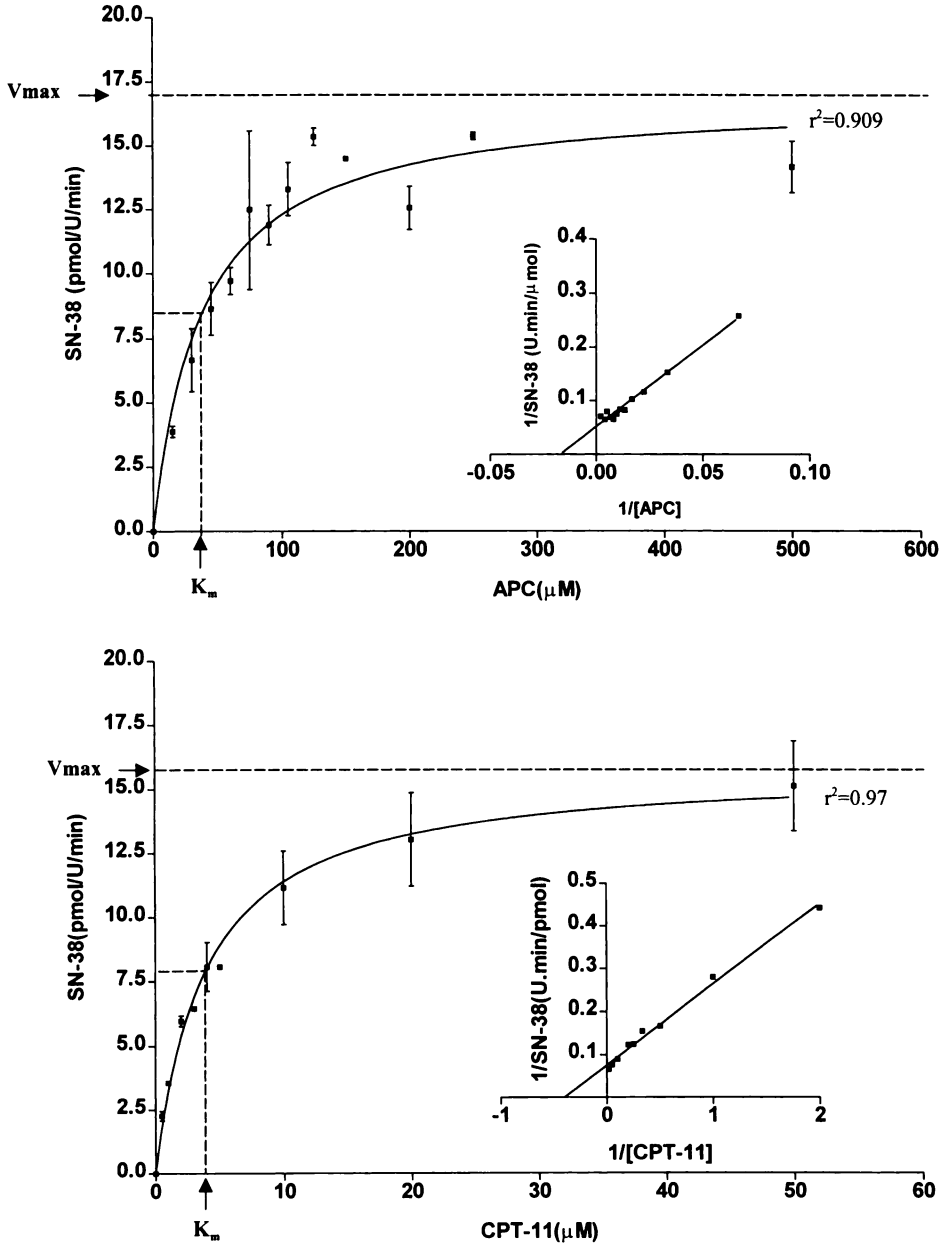


Fig. 2 Hydrolysis of APC and CPT-11 by the rabbit carboxylesterase. The assays were performed at 37°C for 2 min, using six units of enzyme in the presence of 10–500 μM APC or 0.5–50 μM CPT-11. *Top panel,* the rate of formation of SN-38 (pmol/units/min) as a function of APC concentration (μM). *Bottom panel,* the rate of formation of SN-38 (pmol/U/min) as a function of CPT-11 concentration (μM). *Bars,* SD. *Insets,* double reciprocal plots.

rials and Methods"). Consistently, the IC_{50} for APC in the transfected cells is in the same range as that observed for U373 MG cells with APC in presence of the rabbit enzyme in the culture medium. The amount of rabbit carboxylesterase expressed in the transfected cells is sufficient to convert APC to SN-38 to induce a cytotoxic effect.

DISCUSSION

This study reports the novel observation that both APC and CPT-11 are converted to SN-38 by a rabbit liver carboxylesterase. A growth-inhibitory effect was induced either by exposing the U373 MG cells to APC and rabbit carboxylesterase or by treating U373 cells transfected with the rabbit carboxylesterase cDNA with APC.

Carboxylesterases are ubiquitous enzymes, involved in the hydrolysis of endogenous and xenobiotic compounds. An interspecies comparison of carboxylesterases showed that a human liver enzyme had the lowest activity against a panel of substrates used commonly to describe carboxylesterase activity (9). Another study with a human microsomal carboxylesterase revealed that CPT-11 was a better substrate than APC, but that even CPT-11 bioactivation was an inefficient process (3). The low plasma concentrations of SN-38 observed after i.v. administration of CPT-11 to patients are consistent with the observation

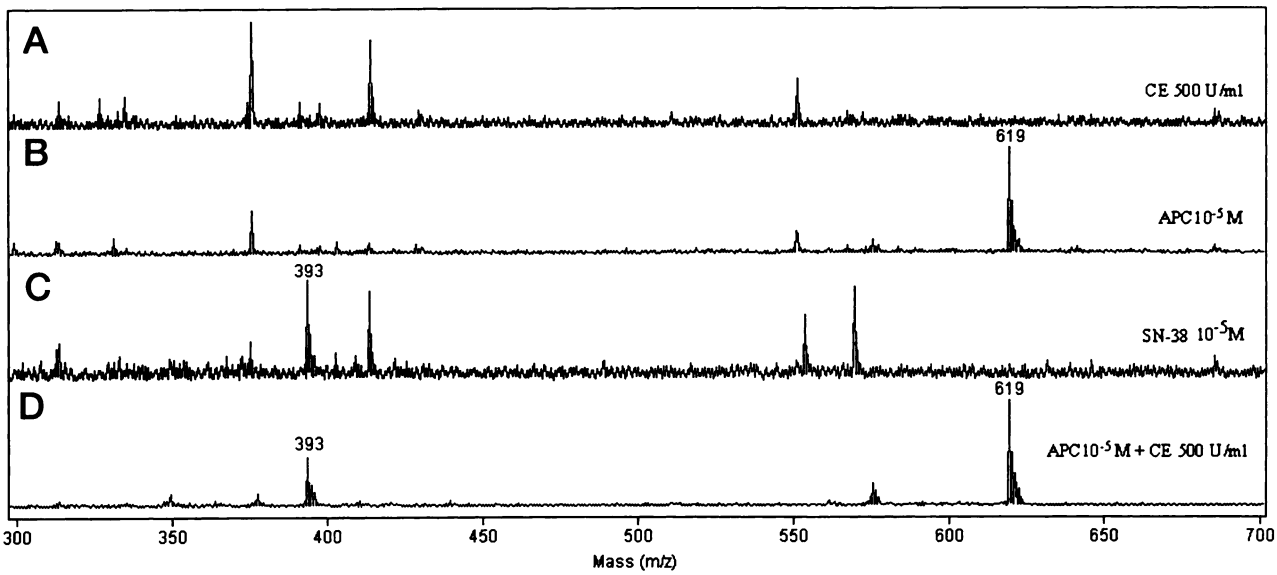


Fig. 3 Mass spectra (*m/z*) obtained with 500 units/ml rabbit carboxylesterase solution (*A*), 10 μM APC solution (*B*), 10 μM SN-38 solution (*C*), and a reaction mixture of 10 μM APC with 500 U/ml of rabbit carboxylesterase (*D*). The atomic masses for APC (619) and SN-38 (393) are indicated.

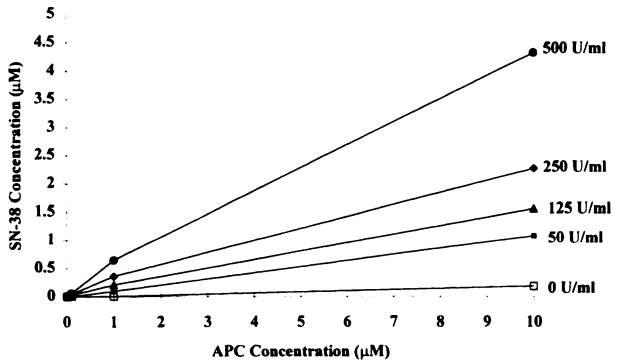


Fig. 4 Hydrolysis of APC by the rabbit carboxylesterase in culture media after a 24-h incubation at 37°C. The figure indicates the SN-38 concentrations as a function of APC concentrations in the presence of 0 (□), 50 (■), 125 (▲), 250 (◆), and 500 (●) units/ml of rabbit carboxylesterase.

that the human carboxylesterases are inefficient enzymes in the activation of CPT-11. Alternatively, an efficient converting enzyme may be expressed in humans but may represent a small fraction of the overall carboxylesterase activity.

Whereas CPT-11 is hydrolyzed by both the rabbit and the human carboxylesterases (2), APC can be converted to SN-38 only by the rabbit enzyme. Such a substrate specificity is a general feature of carboxylesterases; small modifications in substrate chemical structure can result in large changes in enzyme affinity. Two features of the substrate structure appear to influence the activity of individual carboxylesterases: the overall length of the acyl moiety and the presence of charged groups (8). The importance of the side chain size was observed with the CPT-11 converting enzyme isolated from rat serum by Tsuji *et al.* (14). These investigators demonstrated that the substrates with the highest affinity for the enzyme contained bulky and hydrophobic residues on the side chain, and that substitution of the terminal piperidine ring from CPT-11 by a toluene ring increased the esterolytic activity by a factor of 7 (14). The presence of a long acyl chain in the APC molecule instead of a piperidine ring in the CPT-11 molecule could be a factor restricting its binding to the human carboxylesterase (Fig. 1).

Treatment of solid tumors is unsuccessful in some cases, stressing the need to develop new therapeutic approaches. One of the long-range goals of our laboratories is to identify a carboxylesterase that can be used in a gene therapy/chemotherapy combination to activate prodrugs in a manner similar to that described for the *Herpes simplex* virus thymidine kinase and ganciclovir (15). The use of the rabbit carboxylesterase/CPT-11 combination is potentially useful. However, CPT-11 administration induces a cholinergic syndrome, as reported in the Phase I studies, attributed to CPT-11 and not to SN-38 (16). Moreover, individual variability in the expression of cytochrome P450 3A, which metabolizes CPT-11 to APC, may contribute to the interpatient variability in SN-38 concentration. Finally, CPT-11 induces limiting side effects, such as secretory diarrhea and myelosuppression. A rabbit carboxylesterase/APC combination may have a more favorable therapeutic index. Plasma concentrations of APC in the low micromolar range are readily achievable (5); conversion of APC to SN-38 would be limited to cells that express the rabbit carboxylesterase, because APC is not a substrate for human carboxylesterases. Finally, the rabbit carboxylesterase/APC combination may offer an advantage over that shown with other combinations such as *Herpes simplex*/ganciclovir in that bystander effects by SN-38 would likely not be limited to cells with gap junctions (17).

In summary, the treatment of patients with APC after transfection or transduction of tumor cells with the rabbit carboxylesterase cDNA might prove a favorable therapeutic com-

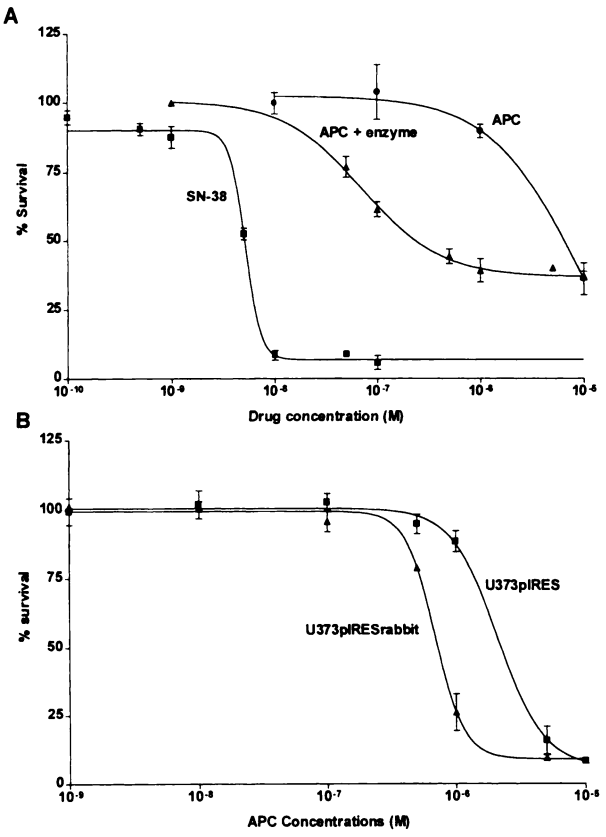


Fig. 5 *A*, growth inhibition curve of U373 MG cells with SN-38 (■) or APC in the absence (●) or in the presence (▲) of 50 units/ml of rabbit carboxylesterase. The drug exposure was for 24 h in a final volume of 2 ml, and the number of cells was evaluated after three doubling times. *B*, growth inhibition curves of U373pIRES (■) and U373pIRESrabbit (▲) cells with APC. The drug exposure was for 24 h, and the number of cells was evaluated after three doubling times. *Bars*, SD.

bination because of the low toxicity of APC, the prodrug activation limited to cells expressing the rabbit enzyme, and the bystander effect of SN-38. We are presently investigating this approach.

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