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Advances in Brief

The Role of Rat Serum Carboxylesterase in the Activation of Paclitaxel and Camptothecin Prodrugs

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Abstract

Paclitaxel-2-ethylcarbonate (PC) is a prototype for a family of paclitaxel prodrugs that have significant levels of antitumor activities in rodent models for human cancer. In this study, an enzyme responsible for the conversion of PC to paclitaxel was purified from rat serum. N-terminal amino acid sequence analysis indicated that the isolated enzyme was rat serum carboxylesterase. This enzyme was shown to significantly enhance the cytotoxic activities of both PC and 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), a water-soluble analogue of camptothecin, on lung carcinoma and melanoma cell lines. Rat serum carboxylesterase may have applications for the site-specific delivery of anticancer drugs to tumor masses.

Introduction

Paclitaxel (Taxol) is a microtubule-binding agent that has shown pronounced activity against advanced ovarian, breast, lung, and head and neck cancers (1). The broad-spectrum activity, together with a potentially unique mechanism of action displayed by paclitaxel, have prompted a large number of investigations to find less toxic, more active analogues (2). This, for the most part, has involved chemical modification of the hydroxyl groups at position 7 on the baccatin core or position 2′ on the paclitaxel side chain (Fig. 1). Several active paclitaxel-2′-esters and carbonates have been described (2–5), and animal studies have shown that many of these derivatives are metabolically converted to paclitaxel (4, 5). For example, PC,[2] (Fig. 1) is a prodrug of paclitaxel that is converted to paclitaxel by a previously unreported mechanism. Information regarding this transformation may be of significance, particularly in light of the fact that PC and several of its congeners have significant *in vivo* antitumor activities in animal models of human cancer. Here, we report an enzyme responsible for the conversion of PC to paclitaxel. In addition, we show that this enzyme may have therapeutic applications for the activation of other anticancer prodrugs.

Materials and Methods

Paclitaxel and PC were provided by Dr. John Kadow (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT). CPT-11 was provided by Dr. William Rose (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Protein concentrations were determined using the micro BCA assay (Pierce Chemical Co., Rockford, IL). The H2987 (human lung adenocarcinoma) and Clone 62 (mouse melanoma transfected with the p97 antigen) cell lines have been described previously (6, 7). Automated amino acid sequence analysis was performed using a pulsed liquid protein sequencer as described previously (8). The purified protein was recovered from a SDS-polyacrylamide gel by electroblotting onto a Problott membrane (Applied Biosystems, Inc., Foster City, CA).

Determination of Enzyme Activity

Total esterase activity was measured by adding aliquots of the protein samples to *p*-nitrophenylacetate [Sigma Chemical Co., St. Louis, MO; 0.5 mM in PBS (pH 7.4)] and measuring the change in absorbance at 405 nm ($\Delta\epsilon = 1.36 \times 10^4$). The ability of the various preparations to effect the hydrolysis of PC at 37°C was determined by combining 10 μl of the sample with 80 μl PBS (pH 7.4), containing 2 mg/ml BSA, and then adding 10 μl PC (1 mg/ml in DMSO). Serum stability studies were performed by adding PC (1 mg/ml in DMSO) directly to serum (1:10 dilution). At appropriate time intervals, 50-μl portions were withdrawn and added to 50 μl acetonitrile, and precipitated material was removed by centrifugation. HPLC analyses of 50-μl aliquots were carried out using a 4.6 × 250-mm cyanopropyl column with 50 mM sodium phosphate (pH 3.0):acetonitrile:methanol (5:4:1) as the mobile phase. The flow rate was 1 ml/min, and the eluant was monitored at 240 nm. The amount of paclitaxel and PC in each sample was determined using similarly treated standard solutions. Units of enzyme activity are defined as nmol PC hydrolyzed/min.

Purification of Carboxylesterase from Rat Serum

All steps in the enzyme purification were undertaken at ~4°C.

Step 1: Affinity Chromatography. Trace hemolyzed normal rat serum (20 ml; Pel-Freez, Rogers, AR) was applied to a 2.3 × 3.0-cm MBPFT-Sepharose affinity column (9). The column was washed with 25 mM Tris-HCl (pH 7.5) until the *A* at 280 nm returned to 0. Bound material was eluted by circulating ~50 ml 1 mM solution of PTFP (10) in 25 mM Tris-HCl (pH 7.5) through the column for 2 h and then collecting a single fraction of 150 ml PTFP solution (*A* at 280 nM, 0.053; 0.26 mg protein/ml).

Step 2: Anion-Exchange Chromatography. The entire PTFP-containing solution was applied to a 2.5 × 15-cm QAE-Sephadex (Pharmacia, Piscataway, NJ) column, and the column was washed with 1 liter 25 mM Tris (pH 7.5). A linear gradient was applied that consisted of 300 ml each of 25 mM Tris (pH 7.5) and 25 mM Tris containing 250 mM NaCl (pH 7.5). Fractions (8.5 ml each) that contained the majority of esterase activity (fractions 33–60) were pooled, concentrated to 3 ml by ultrafiltration through an Amicon (Danvers, MA) PM-30 filter, and then dialyzed against PBS (pH 7.4). A small amount of precipitated material was removed by centrifugation. The supernatant (3 ml) contained 0.14 mg protein/ml, having 3.3 units/mg activity.

Step 3: Size-Exclusion HPLC. A 0.4-ml portion of the material from step 2 was applied at 0.4 ml/min to a TSK-3000SW column (7.8 × 300 mm; TosoHaas, Philadelphia, PA) equilibrated with PBS (pH 7.4). Fractions (0.25 ml each) were collected and monitored by SDS-PAGE and esterase activity. The final purified material (0.25 ml) contained 43 μg protein/ml with a specific activity of 60 units/mg.

In Vitro Cytotoxicity

H2987 and Clone 62 cells in IMDM [Iscove's modified Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 60 μg/ml penicillin G] were plated into 96-well microtiter plates at 6000 (for

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[2] The abbreviations used are: PC, paclitaxel-2′-ethylcarbonate; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; HPLC, high-performance liquid chromatography; MBPFT, 3-[4′-mercaptobutane-1′-thio]-1,1,1-trifluoro-2-propanone; PTFP, 3-(pentane-1′-thio)-1,1,1-trifluoro-2-propanone; QAE, quarternary aminoethyl; SN-38, 7-ethyl-10-hydroxycamptothecin.

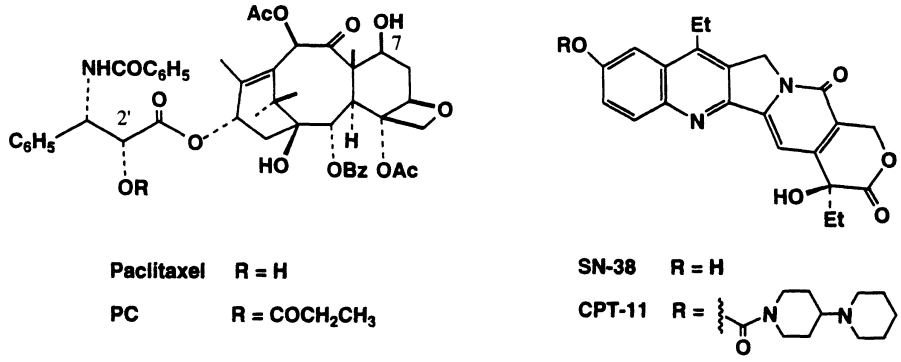

Fig. 1. Structures of paclitaxel and camptothecin derivatives.

camptothecin series) or 8000 (for paclitaxel series) cells/well and allowed to adhere overnight at 37°C. The medium was removed, and various drug dilutions followed by rat serum carboxylesterase (0.4 units for paclitaxel series, 0.1 units for camptothecin series) in medium were added. After 1 h (for paclitaxel series) or 24 h (for camptothecin series), the cells were washed, 0.2 ml IMDM was added, and incubation was continued for 24 h. The medium was removed, [^{3}H]thymidine (1 μCi in 0.2 ml IMDM) was added, and after 4 h, the cells were frozen, thawed, and harvested onto glass fiber filter mats. Radioactivity was counted with an LKB Wallac (Bromma, Sweden) β-plate scintillation counter.

Results

PC (Fig. 1) was prepared from paclitaxel as described previously (4). An HPLC assay was used to monitor the stability of PC in normal rat, mouse, and human sera at 37°C. It was found that PC was quite unstable in rat and mouse serum (half-lives, 7 and 53 min, respectively) and was converted in a quantitative manner to paclitaxel. In contrast, PC was found to be stable in human serum (0% hydrolysis in 240 min). Therefore, it seems that a factor is present in rat and mouse serum, but not in human serum, that is capable of effecting the conversion of PC to paclitaxel.

It was initially thought that an esterase might be responsible for PC hydrolysis. Consistent with this was the finding that the serine protease and esterase inhibitor phenylmethylsulfonyl fluoride at a concentration of 1 mM inhibited 90% of PC hydrolysis activity in rat serum. It was interesting to note, however, that there was no correlation between PC hydrolysis activity (see above) and total esterase activity using *p*-nitrophenylacetate as a substrate in rat (1.3 μmol/min/ml), mouse (4.4 μmol/min/ml), and human (0.51 μmol/min/ml) sera. This suggested that if an esterase was involved in PC hydrolysis, the enzymes differed significantly in their specificity profiles. Before embarking on the isolation of the active serum enzyme, commercially available enzymes, such as porcine liver esterase, acylase, carbonic anhydrase, papain, chymotrypsin, Pronase, elastase, bromolain, ficin, carboxypeptidases A, G, and Y, thermolysin, subtilisin, and various lipases, phosphodiesterases, and phosphatases, were tested for their abilities to convert PC to paclitaxel. None of these enzymes displayed any hydrolytic activity with PC, even after 17 h at high enzyme concentrations (0.1 mg/ml).

Some insight into the nature of the enzyme responsible for PC hydrolysis was gained by subjecting rat serum to a purification procedure in which serum albumin was first adsorbed onto blue Sepharose, and the unbound material was subjected to anion-exchange chromatography. The fractions obtained were monitored for PC hydrolysis activity as well as for their activities on *p*-nitrophenylacetate. The fact that the two activities coeluted (Fig. 2) suggested that the enzyme responsible for PC hydrolysis was a rat serum esterase. This provided the basis for using a trifluoroketone affinity resin for enzyme purification, because previous studies have reported the use of such supports for the purification of insect blood esterases (9).

Purification of the rat serum enzyme responsible for PC hydrolysis was achieved in three steps. The serum was applied to an affinity column consisting of a trifluoroketone derivative bound to a Sepharose support (MBPFT-Sepharose; Ref. 9). Bound material was eluted off the column with PTFP, a trifluoroketone esterase inhibitor (10), and was then subjected to anion-exchange chromatography followed by gel filtration. Analysis of the purified preparation by SDS-PAGE indicated the presence of mainly a single protein having an apparent M_r of 73,000 (Fig. 3). The specific activity of this material with PC as the substrate was 60 nmol PC hydrolyzed/min/mg protein. N-terminal amino acid sequence analysis gave the sequence HPSSPPVVDTT-KGKVL, which is identical to that of rat serum carboxylesterase, a protein with a reported M_r of 70,000 (11). These results lead to the conclusion that carboxylesterase is the enzyme in rat blood responsible for PC hydrolysis.

Carboxylesterases are known to be involved in the activation of a number of anticancer prodrugs (12–14). Two such prodrugs, PC and

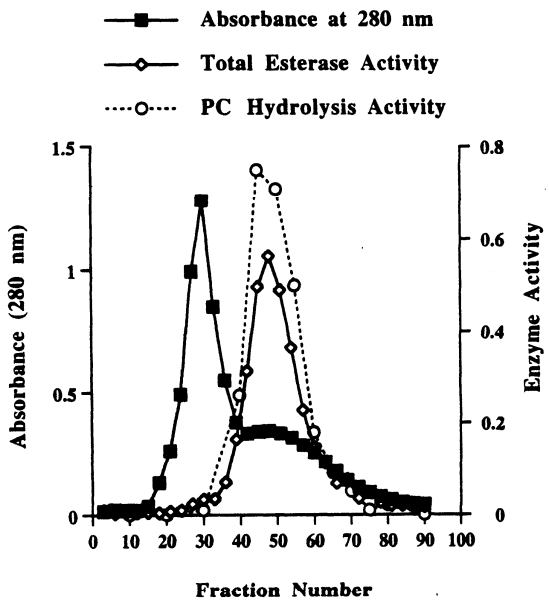

Fig. 2. Coelution of esterase and PC hydrolysis activities off a QAE-Sephadex anion-exchange column. Fractions were monitored for *A* at 280 nm, total esterase activity using *p*-nitrophenylacetate as a substrate (units on the *right Y-axis* correspond to the increase of *A* at 405 nm resulting from the hydrolysis of *p*-nitrophenyl acetate), and hydrolysis of PC by HPLC (units on the *right Y-axis* × 100 correspond to percentage of PC hydrolysis after 3 h at 37°C).

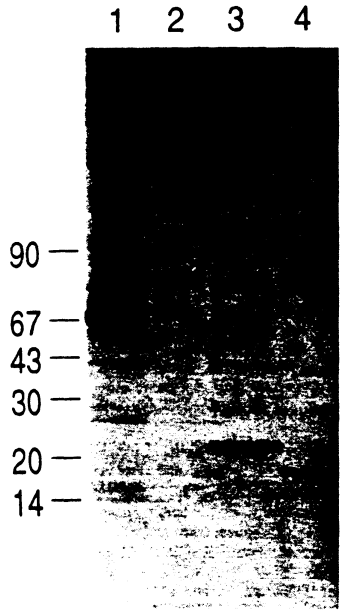

Fig. 3. SDS-PAGE analyses (4–20%, nonreducing) of samples obtained during enzyme purification. *Lane 1*, rat serum; *Lane 2*, after MBPFT-Sepharose chromatography; *Lane 3*, after QAE Sephadex anion exchange chromatography; *Lane 4*, after TSK-3000SW size-exclusion chromatography.

CPT-11 (Fig. 1), were tested for antitumor activity both in the presence and absence of rat serum carboxylesterase. These particular prodrugs were chosen for the studies described here, because the drugs from which they are derived represent important new chemotypes in cancer therapy (15). The cytotoxic activities of PC and PC combined with purified rat serum carboxylesterase were determined on the H2987 human lung adenocarcinoma (6) and Clone 62 mouse melanoma (7) cell lines and were compared with those obtained from the parent drug paclitaxel (Fig. 4, *A* and *B*). PC was ~300-fold less toxic to H2987 cells compared with paclitaxel and was fully activated in the presence of rat serum carboxylesterase (Fig. 4*A*). A similar trend was observed using Clone 62 cells, but the cytotoxic differential between the drug and prodrug was only 35-fold (Fig. 4*B*). In contrast, the combination of CPT-11 and purified rat serum carboxylesterase was 10^4 times more toxic to Clone 62 cells than CPT-11 alone (Fig. 4*C*). These results demonstrate the utility of rat serum carboxylesterase for the activation of two clinically relevant anticancer prodrugs.

Discussion

2′-Carbonate derivatives of paclitaxel were originally designed as paclitaxel prodrugs that would undergo hydrolysis when administered *in vivo* (4). The *in vivo* activity displayed by PC (4) prompted the development of a further series of such derivatives that were water soluble and at least as active as paclitaxel in a lung carcinoma tumor model (5). Here, we establish that rat serum carboxylesterase is capable of effecting carbonate hydrolysis of PC, one of the most active of the paclitaxel derivatives, in a mouse model of human cancer. Carbamate hydrolysis seems to be selective, based on the relative stability of PC in human compared with rat and mouse serum. These results might lead to the prediction that the *in vivo* activities obtained from PC in rodent studies (4, 5) would not reflect the outcome in humans.

Carboxylesterases have been implicated in the activation of a number of anticancer agents (12–14). Clinically, one of the most important of these agents is CPT-11, a water-soluble analogue of the topoisomerase I inhibitor camptothecin (16). Several recent studies have demonstrated that CPT-11 is hydrolyzed by serum carboxylesterases to form the active counterpart SN-38 (12, 17, 18). We have shown that rat serum carboxylesterase effects a 10^4-fold increase in the cytotoxic activity of CPT-11, which is most likely due to carbamate hydrolysis. These results are consistent with those of Kaneda and coworkers (17), who have reported a similar difference in cytotoxic activities between CPT-11 and SN-38.

It may be possible to use rat serum carboxylesterase for prodrug activation *in vivo* by targeting the enzyme to tumors with an appropriate monoclonal antibody and then administering a prodrug such as PC or CPT-11 (19). An alternative means for rat serum carboxylesterase delivery would be to target the gene encoding the enzyme to tumor cells (20). There is reason to believe that these targeting

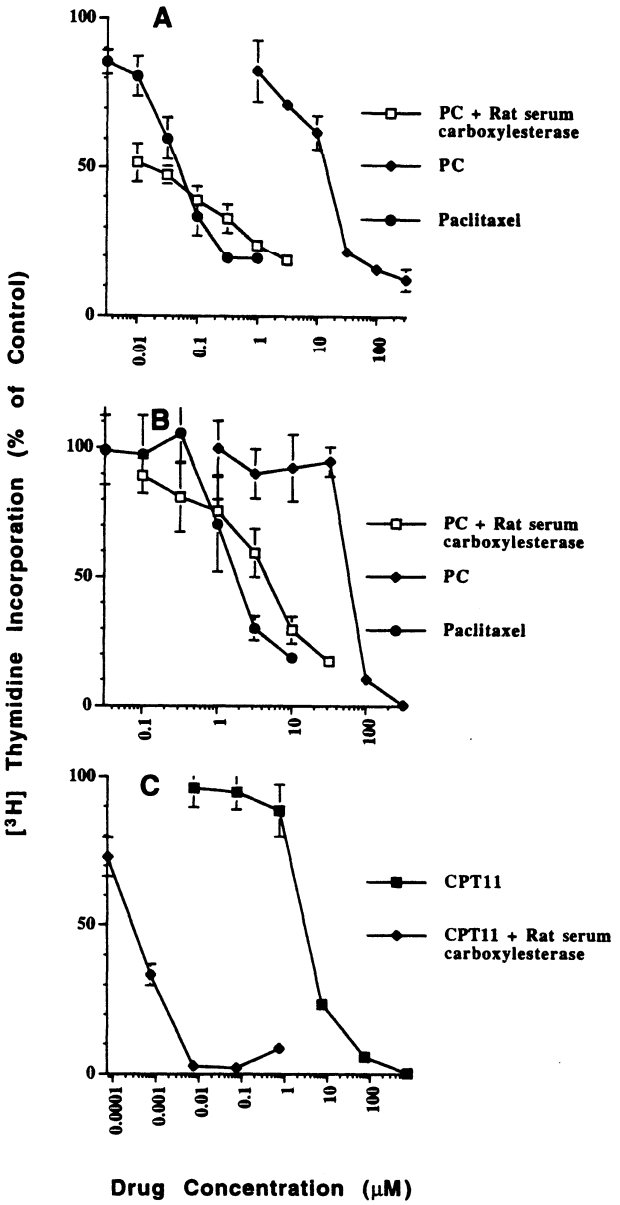

Fig. 4. Cytotoxic effects of PC in combination with rat serum carboxylesterase on H2987 lung adenocarcinoma (*A*) and Clone 62 melanoma cells (*B*). *C*, cytotoxic effects of CPT-11 with rat serum carboxylesterase on Clone 62 melanoma cells.

strategies would be particularly applicable for the activation of CPT-11, because pharmacokinetic studies have shown that only a small portion of the total administered dose of CPT-11 is converted to SN-38 in humans (21). In addition, tumor cell sensitivity to CPT-11 has been reported to be related to carboxylesterase activity (22).

One interesting aspect regarding rat serum carboxylesterase lies in its high degree of homology (72% identity and 83% similarity) with human serum carboxylesterase (11, 23). On this basis, the enzyme may not be as immunogenic as other enzymes used for targeted prodrug activation (24). A chimeric form of rat serum carboxylesterase, in which human sequences are substituted for rat sequences in areas of low homology, might be even less immunogenic and would also provide basic information for further prodrug design. The data reported here suggest that such studies are warranted.

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