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Advances in Brief

Overexpression of a Rabbit Liver Carboxylesterase Sensitizes Human Tumor Cells to CPT-11[1]

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Abstract

CPT-11 [7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin] is a prodrug that is converted to the active metabolite SN-38 by carboxylesterases. In its active form, the drug inhibits topoisomerase I, causes DNA damage, and induces apoptosis. Data in this study show metabolism of CPT-11 to SN-38 (7-ethyl-10-hydroxycamptothecin) by a rabbit liver carboxylesterase *in vitro* and growth-inhibitory activity of the products of the reaction. Additionally, stable expression of the cDNA encoding this protein in Rh30 human rhabdomyosarcoma cells increased the sensitivity of the cells to CPT-11 8.1-fold. We propose that this prodrug/enzyme combination can be exploited therapeutically in a manner analogous to approaches currently under investigation with the combinations of ganciclovir/herpes simplex virus thymidine kinase and 5-fluorocytosine/cytosine deaminase.

Introduction

SN-38,[3] the active metabolite of the chemotherapeutic agent CPT-11, inhibits topoisomerase I (1, 2). Inhibition of this enzyme can result in DNA damage and induction of apoptosis (3). The specific enzyme responsible for activation *in vivo* of CPT-11 has not been identified, although serum or liver homogenates from several mammalian species have been shown to contain activities that convert CPT-11 to SN-38 (4–6). Uniformly, these activities have characteristics of CEs (4–6). Recently, Potter *et al.*[4] isolated from rabbit liver RNA the cDNA encoding a CE and demonstrated that this protein had CE activity *in vitro* and in transiently transfected Cos7 cells.

Results from preclinical studies in which mice bearing xenografts of human tumors were treated with CPT-11 demonstrated a steep dose-response relationship between dose of drug administered and induction of complete tumor regressions (7). In these studies, 20 mg of CPT-11/kg/dose given daily for 5 days for 2 consecutive weeks produced complete regressions of Rh18 rhadomyosarcoma xenografts, whereas 10 mg/kg/dose given on the same schedule produced only transient partial tumor regressions. Similar effects were seen when mice bearing SJG_3A colon adenocarcinoma xenografts were treated with 40 mg of CPT-11/kg/dose compared to 20 mg/kg/dose. These results suggest that attempts to increase either the tolerated doses of CPT-11 or the sensitivity of tumors by as little as 2-fold to this drug might be beneficial therapeutically.

In patients, the tolerated exposure to CPT-11 has already been increased by the use of loperamide and octreotide (8–10) to minimize the diarrhea associated with CPT-11 administration. Our long-range goal is to determine whether expression of a CE capable of efficient conversion of CPT-11 to SN-38 *in situ* expressed in tumor cells can sensitize tumors to tolerated levels of CPT-11.

Materials and Methods

Enzymes and Drugs. Rabbit liver CE was purchased from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN). As defined in the product literature, 1 unit of CE is the amount of enzyme required to convert 1 pmol of *o*-nitrophenyl butyrate to nitrophenol and butyrate per min at pH 7.5 at 25°C. CPT-11 and SN-38 were generous gifts from Dr. J. P. McGovern (Pharmacia and Upjohn Co., Kalamazoo, MI).

Cell Lines and Plasmids. The pediatric rhabdomyosarcoma cell line Rh30 was derived from the bone marrow aspirate of a patient at St. Jude Children's Research Hospital (11). The tumor specimen was obtained in accordance with guidelines from the St. Jude Internal Review Board. The cell line was grown in DMEM containing 10% FCS and 2 mM glutamine, under an atmosphere of 10% CO_2 at 37°C. The pIRES plasmid was obtained from Clontech (Palo Alto, CA). This bicistronic mammalian expression vector generates one transcript encoding both the cDNA of interest and the *neo* gene (12). An internal ribosome entry site allows translation of both genes; hence, any transfectants that are G418 resistant should also express the desired protein.

CPT-11 Conversion Activity *in Vitro*. The conversion of CPT-11 to SN-38 was monitored by fluorometry. Increasing amounts of partially purified rabbit liver CE (Sigma) were incubated with 1 μM CPT-11 in a final volume of 500 μl of 30 mM HEPES (pH 7.4) at 37°C for 18 h. Reactions were terminated by the addition of 5 volumes of methanol. The samples were then vortexed for 10 s and centrifuged at 14,000 × *g* for 2 min (13). The methanolic supernatant was analyzed in a Hitachi F2000 spectrofluorometer using an excitation wavelength of 377 nm. Because the emission maxima for CPT-11 and SN-38 occur at 440 and 538 nm, respectively (14), the amounts of each species can readily be quantitated by the F2000 software, which calculates area under the curve of fluorescence emission. Routinely, 50 μl of rat serum were used a positive control. Comparison of this assay with a high-pressure liquid chromatography method indicated that the sensitivity of detection of SN-38 was approximately 10 ng/ml.[5]

Carboxylesterase Activity. Whole-cell extracts were prepared by sonication for 10 s in 50 mM HEPES (pH 7.4), using a Cole Parmer Ultrasonic Homogenizer 4710. CE activity was determined spectrophotometrically by incubation of sonicates in 1 ml of 30 mM HEPES (pH 7.4) containing 3 mM *o*-nitrophenyl acetate and monitoring at 420 nm. Data were plotted using Kaleidograph software. ϵ_m for *o*-nitrophenol at 420 nm was 13,600. Protein concentrations were determined with the Bio-Rad (Hercules, CA) protein assay reagent using BSA as a standard.

Stable Transfection of Rh30 Rhabdomyosarcoma Cells. Rh30 cells (10^7) were electroporated with 20 μg of pIRES plasmid DNA or plasmid containing the CE cDNA in a volume of 200 μl of PBS using a Bio-Rad 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 500 μg G418/ml were added 48 h following transfection to select for cells expressing the *neo* gene and the CE.

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[3] The abbreviations used are: SN-38, 7-ethyl-10-hydroxycamptothecin; CPT-11, irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; CE, carboxylesterase;

[4] P. M. Potter, C. A. Pawlik, C. L. Morton, and M. K. Danks, Isolation and characterization of a cDNA encoding a rabbit carboxylesterase that converts CPT-11 to SN-38, submitted for publication.

[5] M. K. Danks and C. F. Stewart, unpublished data.

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. Initially, CPT-11 was preincubated with rabbit liver CE *in vitro* to produce SN-38 prior to exposure of the cells to drug. Specifically, 0.5 to 5 units of CE were incubated with 1 μM CPT-11 at 37°C in DMEM for 2 h. Each reaction mixture was then filter sterilized, and Rh30 cells were exposed to drug for 1 h, after which the medium was replaced with drug-free medium containing serum. Enzyme that had been inactivated by boiling for 5 min prior to incubation with drug and CPT-11 to which no enzyme had been added were used as negative controls. Cells were allowed to grow for three cell-doubling times, and cell numbers were determined with a Coulter Multisizer II (Coulter Electronics, Luton, Bedfordshire, UK).

In the second type of growth inhibition assay, Rh30 cells that had been transfected with either pIRES parent plasmid DNA or the plasmid containing the rabbit CE cDNA were exposed to different concentrations of CPT-11. Drug was added to the tissue culture medium of each of the stably transfected cell lines for 2 h, after which the medium was replaced with drug-free medium. Cells were then allowed to grow for three cell-doubling times as in the protocol above. All determinations were performed in duplicate or triplicate, and data were analyzed using the sigmoidal dose-response program of GraphPad Prism software. Results are expressed as the concentration of drug required to reduce cell growth to 50% of control cells (IC_{50}).

Results

Concentration-dependent Conversion of CPT-11 to SN-38 by Partially Purified Rabbit Liver CE *in Vitro*. No conversion of CPT-11 to SN-38 was seen in the absence of enzyme (Fig. 1) or with inactivated CE (not shown). In contrast, 1–100 units of rabbit CE incubated with 1 μM CPT-11 produced a concentration-dependent conversion of CPT-11 (emission maximum, 428 nm) to SN-38 (emission maximum, 540 nm). The cDNA that encodes this enzyme[4] was used for all subsequent transfection and growth inhibition experiments with Rh30 cells.

The Metabolite Produced by Rabbit Liver CE *in Vitro* Is Biologically Active. CPT-11 (1μM) was incubated for 2 h without enzyme or with 0.5–5 units of heat-inactivated or active CE. Rh30 cells were then exposed to the products of each reaction for 1 h, and the percentage of growth inhibition was determined after three cell doublings of control cells. As expected, Rh30 cells exposed to 1–5 units of CE that had been inactivated by heating produced no inhibition of cell growth (Fig. 2). In contrast, reaction products of CPT-11 incubated with 1–5 units of active CE produced a 30–60% inhibition of cell growth. A representative experiment of three is shown. The data are consistent with the conclusion that the CE converted CPT-11 to SN-38.

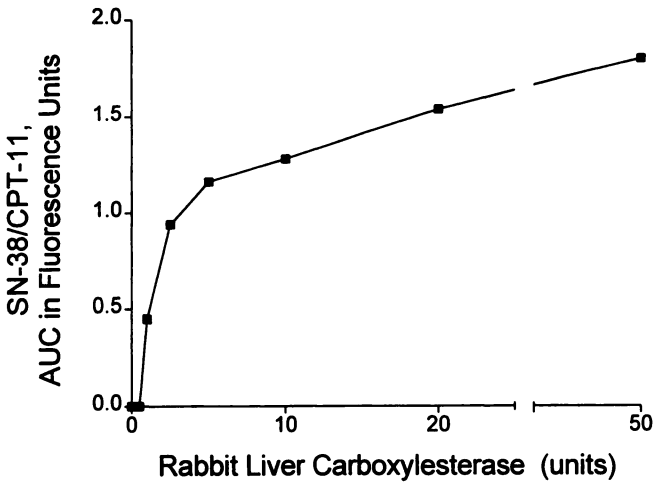

Fig. 1. The indicated amounts (units) of partially purified rabbit liver CE were incubated with 1 μM CPT-11 for 18 h. Methanolic extracts were made of the reaction products, and fluorometric scans were done to determine the relative concentrations of CPT-11 and SN-38. Data are reported as the area under the fluorescence emission curve of SN-38/CPT-11. Experimental details are described in "Materials and Methods."

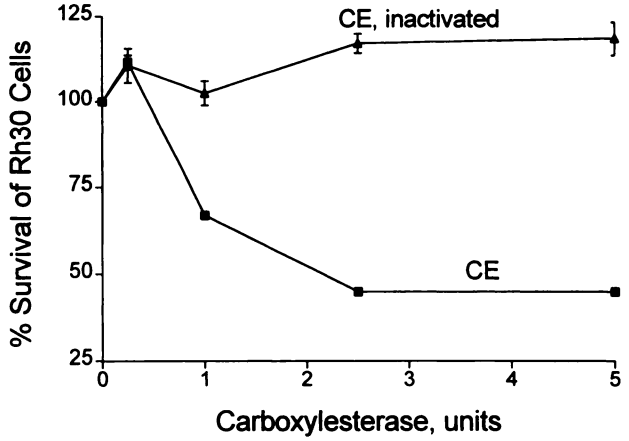

Fig. 2. CPT-11 (1 μM) was incubated for 2 h with 0, 0.5, 1, 2.5, or 5 units of rabbit liver CE. The products of each reaction were then filter sterilized, and Rh30 cells were exposed to the sterilized reaction products for 1 h. After three cell-doubling times, cells were counted, and results are presented as mean number of drug-treated cells/number of untreated control cells (bars, SE).

Table 1 *CE activity in sonicates from transfected Rh30 cell lines*

Cell line	Activity (μmol/min/mg protein)
Rh30pIRES	4.1 ± 0.69
$Rh30pIRES_{rabbit}$	256.2 ± 17.7

Increased Sensitivity of Rh30 Cells Stably Transfected with the Rabbit CE cDNA to CPT-11. We next determined CE activity of extracts of the transfected cells, as well as the IC_{50}s of CPT-11 for Rh30 rhabdomyosarcoma cells that had been stably transfected with the rabbit liver CE cDNA or the pIRES vector alone. Table 1 demonstrates that the cells transfected with the CE cDNA contained ~60-fold more CE activity, as determined by conversion of *o*-nitrophenyl acetate to *o*-nitrophenol.

Fig. 3 shows growth inhibition data from an experiment representative of five analyses performed with Rh30pIRES and $Rh30pIRES_{rabbit}$ cell lines. The IC_{50} of CPT-11 for the Rh30pIRES cells was $4.33 \times 10^{-6} \pm 2.29 \times 10^{-6}$ M compared to the IC_{50} for the $Rh30pIRES_{rabbit}$ cells of $5.76 \times 10^{-7} \pm 3.28 \times 10^{-7}$ M. The $Rh30pIRES_{rabbit}$ cells were 8.1-fold more sensitive to CPT-11. The data are consistent with increased conversion of CPT-11 to SN-38 in the $Rh30pIRES_{rabbit}$ cell line.

Discussion

To confirm previous observations that CEs were responsible for the metabolism of CPT-11 to SN-38 (4–6), we incubated the prodrug with a commercially available rabbit liver CE and added the reaction products to exponentially growing cells. Significant inhibition of cell growth was observed (Fig. 2), consistent with the activation of CPT-11 by the CE. Additionally, when the cDNA encoding this enzyme was expressed in Rh30 rhabdomyosarcoma cells, the IC_{50} value for CPT-11 decreased 8-fold compared to the parental cell line (Fig. 3). This is the first report that demonstrates the increased sensitivity of mammalian cells to CPT-11 following transfection with a cDNA encoding a CE.

CEs are a diverse family of enzymes that react with many "classic" substrates, such as *o*-nitrophenyl acetate, nitrophenyl butyrate, or

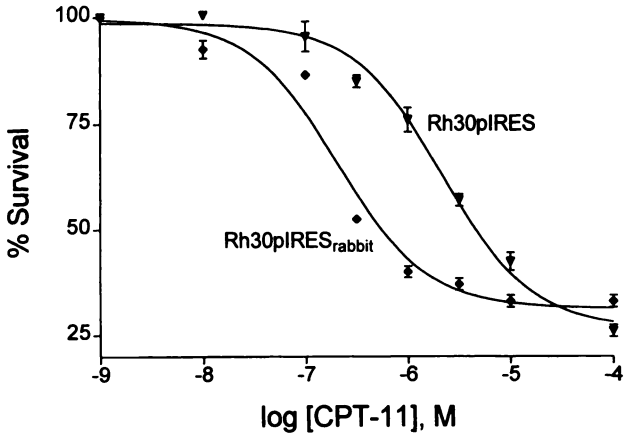

Fig. 3. Rh30 cells were transfected with the pIRES vector (Rh30pIRES) or the pIRES vector containing the cDNA that encoded the rabbit liver CE used in experiments shown in Figs. 1 and 2 (Rh30pIRES$_{rabbit}$). Each cell line was exposed to the indicated concentration of CPT-11 for 2 h in serum-free medium. Cell numbers were determined following three doublings of untreated control cells. Results are expressed as the mean number of drug-treated cells/number of untreated control cells (*bars*, SE).

4-methylumbelliferone butyrate and are inhibited by such compounds as bis(4-nitrophenyl) phosphate. CEs are classified by the substrates for which they have high affinity and the specific compounds that inhibit their activity (15, 16). Many CEs that metabolize classic substrates are ineffective or inefficient at converting CPT-11 to SN-38. For example, the cDNA encoding human alveolar macrophage CE, the sequence for which is in the GenBank database, does not confer sensitivity to CPT-11 when expressed in Rh30 cells.[6] Therefore, it was important to use the substrate of interest, *i.e.*, CPT-11, to identify CEs that were candidate enzymes for conversion of this prodrug to its active metabolite, SN-38.

SN-38, however, represents only 1 of at least 16 metabolites of CPT-11 that have been identified (17), and the fluorescence excitation and emission properties of each of these metabolites have not yet been characterized. In our study, identification of potentially useful CEs was based on a fluorometric assay. Therefore, it was necessary to show that the product of the reactions of CPT-11 with candidate enzymes inhibited cell growth. Data in Fig. 2 show that preincubation of CPT-11 with the rabbit CE generated a product that inhibited cell growth. Additionally, expression of this enzyme in Rh30 cells increased the sensitivity of the cells to CPT-11.

The approach of combining overexpression of an activating enzyme with the appropriate prodrug to improve therapeutic outcome is currently under investigation for several other prodrug/enzyme combinations. Ganciclovir/herpes simplex virus thymidine kinase; 5-fluorocytosine/cytosine deaminase (18); and, in this study, CPT-11/CE have all been proposed as potentially beneficial combinations. One of the factors that will determine the success of these approaches is the degree to which tumor sensitivity can be increased with each system and the achievable plasma concentrations of the drug used. Preclinical studies suggest that the dose-response curve of CPT-11 is very steep, such that increasing the dose of CPT-11 by 2-fold results in complete tumor regressions as compared to transient partial regressions. The cloning of the human homologue of the described cDNA is ongoing, and preclinical studies are being performed to determine whether CPT-11 administered to mice bearing xenografts that express the CE are sensitized to this drug.

[6] P. M. Potter and M. K. Danks, submitted for publication.

Acknowledgments

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References

1. Tanizawa, A., Fujimori, A., Fujimori, Y., and Pommier, Y. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. J. Natl. Cancer Inst., *86:* 836–842, 1994.
2. Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H., and Sato, K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res., *51:* 4187–4194, 1991.
3. Hsiang, Y-H., Lihou, M. G., and Liu, L. F. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res., *49:* 5077–5082, 1989.
4. Tsuji, T., Kaneda, N., Kado, K., Yokokura, T., Yoshimoto, T., and Tsuru, D. CPT-11 converting enzyme from rat serum: purification and some properties. J. Pharmacobio-Dyn., *14:* 341–349, 1991.
5. Senter, P. D., Marquardt, H., Thomas, B. A., Hammock, B. D., Frank, I. S., and Svensson, H. P. The role of rat serum carboxylesterase in the activation of paclitaxel and camptothecin prodrugs. Cancer Res., *56:* 1471–1474, 1996.
6. Satoh, T., Hosokawa, M., Atsumi, R., Suzuki, W., Hakusui, H., and Nagai, E. Metabolic activation of CPT-11,7-ethyl-10-[4-(-piperidino)-1-piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. Biol. Pharm Bull *17:* 662–664, 1994.
7. Houghton, P. J., Cheshire, P. J., Hallman, J. C., Bissery, M. C., Mathieu-Boue, A., and Houghton, J. A. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance *in vivo* in tumors with acquired resistance to the topoisomerase I inhibitor 9-dimethylaminomethyl-10-hydroxycamptothecin. Cancer Res., *53:* 2823–2829, 1993.
8. Armand, J. P. Irinotecan (CPT11): recent clinical development and future direction. Ann. Oncol., *5:* (Suppl. 5): 163, 1994.
9. Cascinu, S. Management of diarrhea induced by tumors or cancer therapy. Curr. Opin. Oncol., *7:* 325–329, 1995.
10. Harris, A. G., O'Dorisio, T. M., Woltering, E. A., Anthony, L. B., Burton, F. R., Geller, R. B., Grendell, J. H., Levin, B., and Redfern, J. S. Consensus statement: octreotide dose titration in secretory diarrhea: Diarrhea Management Consensus Development Panel. Dig. Dis. Sci., *40:* 1464–1473, 1995.
11. Douglass, E. C., Valentine, M., Etcubanas, E., Parham, D. M., Webber, B. L., Houghton, P. J., Houghton, J. A., and Green, A. A. A specific chromosomal abnormality in rhabdomyosarcoma. Cytogenet. Cell Genet., *45:* 148–155, 1987.
12. Rees, S., Coote, J., Stables, J., Goodson, S., Harris, S., and Lee, M. G. Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. Biotechniques, *20:* 102–110, 1996.
13. Stewart, C. F., Zamboni, W. C., Crom, W. R., and Houghton, P. J. Disposition of irinotecan and SN-38 following oral and intravenous irinotecan dosing in mice. Cancer Chemother. Pharmacol., *40:* 259–265, 1997.
14. Barilero, I., Gandia, D., Armand, J. P., Mathieu-Boue, A., Re, M., Gouyette, A., and Chabot, G. G. Simultaneous determination of the camptothecin analogue CPT-11 and its active metabolite SN-38 by high-performance liquid chromatography: application to plasma pharmacokinetic studies in cancer patients. J. Chromatogr., *575:* 275–280, 1992.
15. Miller, S. B., Main, A. R., and Rush, R. S. Purification and physical properties of oligomeric and monomeric carboxylesterases from rabbit liver. J. Biol. Chem., *255:* 7161–7167, 1980.
16. Satoh, T., and Hosokawa, M. Molecular aspects of carboxylesterase isoforms in comparison with other esterases. Toxicol. Lett., *82/83:* 439–445, 1995.
17. Lokiec, F., Monegier du Sorbier, B., and Sanderink, G-J. Irinotecan (CPT-11) metabolites in human bile and urine. Clin. Cancer Res., *2:* 1943–1949, 1996.
18. Trinh, O. T., Austin, E. A., Murray, D. M., Knick, V. C., and Huber, B. E. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine *versus* thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. Cancer Res., *55:* 4808–4812, 1995.

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