Int. J. Cancer: **70,** 335–340 (1997)
© 1997 Wiley-Liss, Inc.

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

CPT-11 IN HUMAN COLON-CANCER CELL LINES AND XENOGRAFTS: CHARACTERIZATION OF CELLULAR SENSITIVITY DETERMINANTS

Willy J.M. JANSEN, Bas ZWART, Saskia T.M. HULSCHER, Giuseppe GIACCONE, Herbert M. PINEDO and Epie BOVEN*
Department of Medical Oncology, Academic Hospital Vrije Universiteit, Amsterdam, The Netherlands

CPT-11, a new semisynthetic derivative of camptothecin, is active in a number of tumor types in the clinic, including colon cancer. CPT-11 is a drug that is converted into the active metabolite SN-38 by a carboxylesterase. Experiments were performed to obtain more insight in the cellular characteristics in 5 unselected human colon-cancer cell lines that account for the differential sensitivity to CPT-11 and SN-38. *In vitro*, the sensitivity to CPT-11 and SN-38 was highest in LS174T and COLO 320 cells, intermediate in SW1398 cells and lowest in COLO 205 and WiDr cells. SN-38 was 130 to 570 times more active than CPT-11. CPT-11 induced complete remissions in 6 out of 12 COLO 320 tumors grown as subcutaneous xenografts, but was not effective in WiDr tumors. The cellular carboxylesterase activity did not relate to the sensitivity to CPT-11. The enzyme activity was higher in normal mouse tissues, *i.e.*, serum and liver, than in COLO 320 or WiDr xenografts, indicating that tumor carboxylesterase is of minor importance for CPT-11 efficacy. The topoisomerase-I mRNA expression in tumor cells was not predictive of the antiproliferative effects of CPT-11 or SN-38. We observed a positive relationship between the DNA topoisomerase-I activity and the cellular sensitivity to carboxylesterase-activated CPT-11 ($r = 0.75$, $p < 0.1$) as well as to SN-38 ($r = 0.89$, $p < 0.05$). The higher topoisomerase-I activity in COLO 320 cells and tumors when compared with that in WiDr cells and tumors reflected the differences in sensitivity to the drug(s). In conclusion, the DNA topoisomerase-I activity was the best determinant for CPT-11/SN-38 sensitivity in this panel of unselected human colon-cancer cell lines. *Int. J. Cancer*, 70:335–340, 1997.
© 1997 Wiley-Liss, Inc.

Camptothecin is a naturally occurring anti-tumor agent, which was isolated from the Chinese tree *Camptotheca acuminata.* It has a broad spectrum of activity against experimental animal tumor models in the ascites as well as the solid form. Its clinical development has been hampered by severe side-effects, consisting of unpredictable hemorrhagic cystitis, diarrhea and myelosuppression, and because of its poor solubility. More recent studies have revealed that camptothecin inhibits the resealing of single-strand DNA breaks mediated by topoisomerase I, which was identified as the specific intracellular enzyme target for the drug. Camptothecin stabilizes cleavable complexes, resulting in single-strand DNA breaks that cannot be religated in the presence of the drug. The cytotoxicity of camptothecin occurs mainly in the S-phase of the cell cycle (reviewed by Slichenmyer *et al.,* 1993; Creemers *et al.,* 1994).

With the increasing knowledge of the function of topoisomerase I and the potent cytotoxic activity of camptothecin, semi-synthetic analogues were developed aiming at better water solubility and fewer side-effects. 7-Ethyl-10-(4-[1-piperidino]-1-piperidino)carbonyloxycamptothecin (CPT-11) is a camptothecin analogue which was shown to have excellent anti-tumor activity against a variety of human tumor xenografts, when administered by the intravenous, intraperitoneal or oral route (Kawato *et al.,* 1991; Houghton *et al.,* 1995). The drug was only marginally active *in vitro* and it was discovered that metabolic activation of CPT-11 by a carboxylesterase was essential for its activity. The active metabolite was identified to be 7-ethyl-10-hydroxycamptothecin (SN-38) (Tsuji *et al.,* 1991). Clinical studies have shown that CPT-11 has significant activity against a broad range of tumor types, including colon cancer. Moreover, CPT-11 has less unpredictable side-effects than the parent compound (Slichenmyer *et al.,* 1993; Creemers *et al.,* 1994).

Although much information is available concerning the cytotoxic mechanism of camptothecins, we performed experiments to gain more insight into the differences between tumor cells that account for the differences in sensitivity to CPT-11. Therefore, an *in vitro* analysis of 5 human colon-cancer cell lines was carried out to determine the anti-proliferative effects of CPT-11 and SN-38. CPT-11 activity was also studied in nude mice bearing subcutaneous human colon-cancer xenografts grown from 2 of these cell lines. We investigated the role of endogenous carboxylesterase as well as the addition of exogenous carboxylesterase in the cellular sensitivity to CPT-11. Carboxylesterase activity was also assessed in normal mouse organs and in human tumor xenografts to identify the sites primarily involved in CPT-11 activation. The topoisomerase-I gene expression and activity were also determined. The various cellular characteristics were analysed for the possible presence of a relationship with the *in vitro* and *in vivo* sensitivity to the drugs.

MATERIAL AND METHODS

Reagents

CPT-11 (Irinotecan) as a solution of 20 mg/ml and SN-38 as a powder were kindly provided by Rhône-Poulenc Rorer (Vitry sur Seine, France). SN-38 was dissolved in DMSO to a final concentration of 10 mM. Carboxylesterase (EC 3.1.1.1), isolated from porcine liver, was purchased from Sigma (Zwijndrecht, The Netherlands). Drugs were further diluted in tissue culture media when investigated for their anti-proliferative effects *in vitro.*

Cell lines and xenografts

The human colon-cancer cell lines COLO 205, COLO 320, SW1398, WiDr and LS174T were cultured in Dulbecco's modified Eagle's medium (GIBCO, Breda, The Netherlands), 10% heat-inactivated FCS (Sebak, Aidenbach, Germany), 50 IU/ml penicillin and 50 µg/ml streptomycin (Flow, Irvine, UK) in an incubator with a humified atmosphere containing 5% CO_2 at 37°C (Jansen *et al.,* 1995). All cell lines were free from *Mycoplasma* contamination as tested regularly with the Mycoplasma T.C. rapid detection system with a ^{3}H-labeled DNA probe from Gene-Probe (San Diego, CA).

Female nude mice (Hsd: athymic nude-*nu*) were purchased at the age of 6 weeks (Harlan CPB, Zeist, The Netherlands). The animals were maintained in cages with paper filter covers under controlled atmospheric conditions. Cages, covers, bedding, food and water were changed and sterilized weekly. Animals were handled in a sterile manner in a laminar down-flow hood. The COLO 320 and WiDr xenografts were established from cell lines grown in tissue-culture medium. Mice were inoculated subcutaneously with 1×10^7 cells in both flanks. The solid tumors arising at the inoculation site (passage 1) were transferred as tissue fragments with a diameter of 2–3 mm through a small skin incision into both flanks of 8- to 10-week-old mice.

*Correspondence to: Academic Hospital Vrije Universiteit, Department of Medical Oncology, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Fax: 31 20-4444355. E-mail: e.boven.oncol@med.vu.nl

Received 26 August 1996; revised 28 October 1996.

In vitro *sensitivity*

For growth-inhibition experiments, colon-cancer cells were plated in 96-well microtiter plates (5,000 cells/well) as described (Jansen *et al.,* 1995). Briefly, after 24 hr cells were exposed continuously for 96 hr to varying concentrations of the drugs CPT-11, SN-38, or CPT-11, in combination with an excess of carboxylesterase (1 μg/ml). The anti-proliferative effects were determined by the MTT assay. The cells were stained for 4 hr with the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,6-dimethyl-morpholino)-2,5-diphenyl-tetrazolium bromide; Sigma, St Louis, MO) in PBS (0.4 mg/ml). MTT was reduced to a dark-colored formazan by living cells only, as measured on a Labsystems Multiscan Bichromatic plate reader (Helsinki, Finland) at 540 nm. The results were expressed as the IC_{50}, which is the concentration of the drug(s) inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells. In control cultures, cell growth was exponential during the assay period. All concentrations were tested in 4 replicate wells and each experiment was performed at least 3 times.

In vivo *sensitivity*

Treatment experiments in mice bearing subcutaneous colon-cancer xenografts were carried out in passage 2 or higher. The tumors were measured twice a week in 3 dimensions with Vernier calipers. The volume was calculated by the equation length × width × thickness × 0.5, and expressed in mm^3. At the start of treatment (designated as day 0), groups of 5 to 6 tumor-bearing mice were formed to provide a mean tumor volume of approximately 150 mm^3 in each group (Boven *et al.,* 1988).

For *in vivo* use, CPT-11 was diluted in NaCl 0.9% to 2 mg/ml and 20 mg/kg were administered intraperitoneally on days 0, 1, 2, 3, 4. The selected dose of CPT-11 in the daily ×5 schedule was the maximum tolerated dose as established in non-tumor-bearing nude mice. This maximum tolerated dose was based on the occurrence of a mean weight loss of approximately 10% of the initial weight within the first 2 weeks after the start of the treatment. For the evaluation of drug efficacy, the tumor volume was expressed by the formula V_T/V_0, where V_T is the volume on any given day and V_0 is the volume on day 0. The ratio of the mean relative volume of treated tumors over that of control tumors multiplied by 100% (T/C%) was assessed on each day of measurement (Boven *et al.,* 1988). Anti-tumor effects were expressed as the percentage of growth inhibition (100%-T/C%).

Carboxylesterase activity

The determination of the carboxylesterase activity in cells, mouse organs or xenografts was adapted from an assay based on the conversion of colorless *para*-nitrophenylacetate (pNPA) to yellow *para*-nitrophenol (Tsuji *et al.,* 1991). Cells (1×10^7) were lysed on ice in 1 ml 20 mM TRIS-HCl buffer pH 7.5 for 1 hr, followed by freeze-thawing. Organs and tumors were weighed and 20 mM TRIS-HCl buffer were added. Tissues were homogenized thoroughly with a Polytron homogenizer and lysed on ice for 1 hr. Cells and homogenates were then centrifuged at 4°C for 30 min at 14,000 *g*, then the supernatants were centrifuged again and stored at −20°C. Before analysis, samples were diluted in TRIS-HCl buffer to obtain measurable carboxylesterase activity. The diluted sample (180 μl) was transferred to a 96-well microtiter plate and the reaction was started by adding 20 μl 10 mM pNPA (Sigma) as a substrate. After an incubation period of 10 min at 37°C, the extinction of the liberated *para*-nitrophenol was monitored at 405 nm on the Labstems Multiscan Bichromatic plate reader. To correct the data for the spontaneous conversion of the substrate, 20 μl 10 mM pNPA was added to 180 μl TRIS-HCl buffer. The enzyme activity was expressed in units, one unit of enzyme activity being 1 μmol liberated *para*-nitrophenol per min at 37°C. Three batches of each cell line and 3 different specimens of the various tissues were tested. The samples were tested in 3 replicate wells and each experiment was performed 3 times.

Topoisomerase-I expression

Total cellular RNA was isolated from exponentially growing cells or from frozen xenograft tissue sections with RNAzol B (Campro Scientific, Veenendaal, The Netherlands). [α-^{32}P]labeled RNA complementary to the topoisomerase-I cDNA 703-bp sequence (nucleotides 835-1538) (Juan *et al.,* 1988), inserted into pGEM3, was transcribed from *Fok*I-linearized DNA using T7 polymerase. RNase protection was carried out as described (Giaccone *et al.,* 1995). In all experiments, a probe for γ-actin was included to control for RNA loading. The hybridized probe was visualized after gel electrophoresis through a denaturing 6% acrylamide gel. For autoradiography, the gel was exposed at −70°C to a Kodak BIOMAX MR film for 3 days. The amount of topoisomerase-I mRNA relative to the amount of γ-actin was calculated by densitometric scanning of autoradiograms. Topoisomerase-I expression was determined at least 3 times in each cell line and at least twice in 3 separate tumors of a cell line.

Topoisomerase-I activity

DNA topoisomerase-I activity was determined using the DNA relaxation assay (Liu and Miller, 1981). Briefly, at least 1×10^7 human colon-cancer cells or 50–100 mg of fresh xenograft tissue were lysed for 10 min on ice in nuclear buffer supplemented with Triton-X, 1 nM phenylmethanesulfonylfluoride (PMSF) and 0.2 μM dithiothreitol (DTT). Nuclear enzymes were extracted from cell nuclei by incubation with nuclear buffer containing 0.4 M NaCl for 30 min on ice. The enzyme solution was diluted with an equal volume of 87% glycerol and stored at −70°C for a maximum of one week. Topoisomerase-I activity was determined by measuring the relaxation of supercoiled pBR329 plasmid DNA by incubation of serial dilutions of the nuclear extracts at 37°C for 30 min. Supercoiled and relaxed DNA were separated in a 1% agarose gel by electrophoresis and visualized by ethidium bromide staining. The enzyme activity was expressed in units. One unit of enzyme activity was defined as the total relaxation of 1 μg pBR329 plasmid DNA per min at 37°C. DNA topoisomerase-I activity was measured at least 4 times in each cell line and at least 3 times in 3 separate tumors of a cell line.

Statistics

Linear regression analysis was used to analyze the sensitivity of the cells in relation to the cellular carboxylesterase activity, topoisomerase-I mRNA expression and DNA topoisomerase-I activity.

RESULTS

In vitro *sensitivity*

The anti-proliferative effects of CPT-11 without or with the addition of carboxylesterase and of SN-38 in the 5 human colon-cancer cell lines, expressed as IC_{50} values, are summarized in Table I. The sensitivity to CPT-11 alone varied between 2.5 and 6.1 μM. The addition of carboxylesterase in an excess of 1 μg/ml to CPT-11 resulted in IC_{50} values between 0.021 μM and 0.21 μM which were 30 to 150 times lower than those measured for CPT-11 alone. The sensitivity to CPT-11 plus carboxylesterase was highest in LS174T and COLO 320 cells, intermediate in SW1398 cells and lowest in COLO 205 and WiDr cells. The anti-proliferative effects of the active metabolite SN-38 showed IC_{50} values between 5.6 and 38 nM, 130 to 570 times lower than those measured for CPT-11 alone. The sensitivity to SN-38 was highest in LS174T and COLO 320 cells, intermediate in SW1398 cells and lowest in COLO 205 and WiDr cells. The degree of sensitivity to CPT-11 and to SN-38 was similar for the 5 colon-cancer cell lines.

In vivo *sensitivity*

The WiDr and COLO 320 cell lines were selected for *in vivo* experiments, because of their difference in sensitivity to SN-38. *In vitro,* WiDr cells were approximately 7-fold less sensitive than COLO 320 cells. In the nude mouse, the volume doubling times

TABLE I – ANTIPROLIFERATIVE EFFECTS OF CPT-11 WITHOUT/WITH CARBOXYLESTERASE AND OF SN-38 IN HUMAN COLON-CANCER CELL LINES AFTER DRUG EXPOSURE FOR 96 HR

Cell line	CPT-11[1] M (±SD)	CPT-11 + CE[1] M (±SD)	SN-38[1] M (±SD)
COLO 205	$4.3 (\pm 0.9) \times 10^{-6}$	$5.7 (\pm 2.1) \times 10^{-8}$	$2.2 (\pm 1.8) \times 10^{-8}$
COLO 320	$3.2 (\pm 0.7) \times 10^{-6}$	$2.1 (\pm 2.0) \times 10^{-8}$	$5.6 (\pm 4.8) \times 10^{-9}$
SW1398	$2.8 (\pm 1.1) \times 10^{-6}$	$4.8 (\pm 6.1) \times 10^{-8}$	$1.2 (\pm 1.5) \times 10^{-8}$
WiDr	$6.1 (\pm 3.3) \times 10^{-6}$	$2.1 (\pm 2.7) \times 10^{-7}$	$3.8 (\pm 2.6) \times 10^{-8}$
LS174T	$2.5 (\pm 2.1) \times 10^{-6}$	$3.4 (\pm 3.3) \times 10^{-8}$	$6.7 (\pm 5.9) \times 10^{-9}$

[1]Mean in M (±SD) of at least 3 separate experiments.

(T_D) of the COLO 320 and WiDr xenografts were 15 and 7.5 days, respectively. In COLO 320 xenografts, CPT-11 induced a maximum growth inhibition of 92% on day 42 (Fig. 1). Six out of 12 animals with COLO 320 tumors showed a complete remission, which was sustained beyond the end of the experiment (day 105). Regrowth in the 6 other mice occurred after day 60 of the experiment. Because of the low percentage of growth inhibition (52%) in WiDr xenografts, CPT-11 activity testing was repeated twice in these tumors. Maximum growth inhibition was even lower, being 21% and 11%, respectively. In WiDr tumors no complete remissions were observed. The weight loss (±SD) induced by CPT-11 did not differ between mice bearing WiDr and COLO 320 xenografts and was 4.8% (±2.8%) and 7.3% (±5.9%), respectively.

Carboxylesterase activity

Using pNPA as a substrate to determine carboxylesterase activity, we found a slight variation in endogenous carboxylesterase activity among the different cell lines (Fig. 2). LS174T and WiDr cells showed the highest enzyme activity of 65 and 64 mU/mg protein, respectively. COLO 205 cells had an intermediate activity of 48 mU/mg protein, whereas the enzyme activity in SW1398 and COLO 320 cells was relatively low, amounting to 38 and 29 mU/mg protein, respectively. The protein contents of the various colon-cancer cell lines differed substantially, for which reason we also estimated the carboxylesterase activity per 10^7 cells. The carboxylesterase activity was lowest in COLO 320 cells (23 mU/10^7 cells), and highest in COLO 205 and WiDr cells (42 and 54 mU/10^7 cells, respectively). Only in the case of LS174T cells was the carboxylesterase activity expressed per 10^7 cells lower than when expressed per mg protein. The endogenous carboxylesterase activity, expressed either per mg protein or per 10^7 cells, was not related to CPT-11 sensitivity ($r = 0.35$; $p < 0.5$ and $r = 0.33$; $p < 0.5$, respectively). Remarkably, the level of carboxylesterase activity found in WiDr cells (64 mU/mg) was higher than that in COLO 320 cells (29 mU/mg), despite COLO 320 cells showing a higher sensitivity to CPT-11 than WiDr cells. FCS contained <10 mU/mg protein carboxylesterase activity.

The carboxylesterase activity was also determined in COLO 320 and WiDr xenografts and in normal mouse organs (Fig. 2). The enzyme activity in COLO 320 (45 mU/mg) and WiDr (90 mU/mg) xenografts reflected the degree of activity in the COLO 320 (29 mU/mg) and WiDr (64 mU/mg) cell lines. The 1.4-fold higher enzyme activity in tumor tissue may be due to the presence of minimal amounts of mouse serum in the assay, as mouse serum contained 150 mU/mg protein carboxylesterase activity. Normal organs contained a higher carboxylesterase activity than mouse serum; this activity was highest in small intestine, stomach and liver, being 900, 675 and 525 mU/mg, respectively. Kidney and colon expressed a lower activity, *i.e.* 300 and 275 mU/mg, respectively.

DNA topoisomerase-I gene expression and activity

The expected 84-bp transcript size for topoisomerase-I mRNA was detected in all cell lines (Table II). Small variations were seen among the 5 cell lines, in contrast with the wider range of sensitivity to CPT-11 plus carboxylesterase or to SN-38. Topoisomerase-I mRNA expression did not correlate either with the sensitivity to CPT-11 plus carboxylesterase ($r = 0.34$; $p < 0.5$) or to SN-38 ($r = 0.36$; $p < 0.5$).

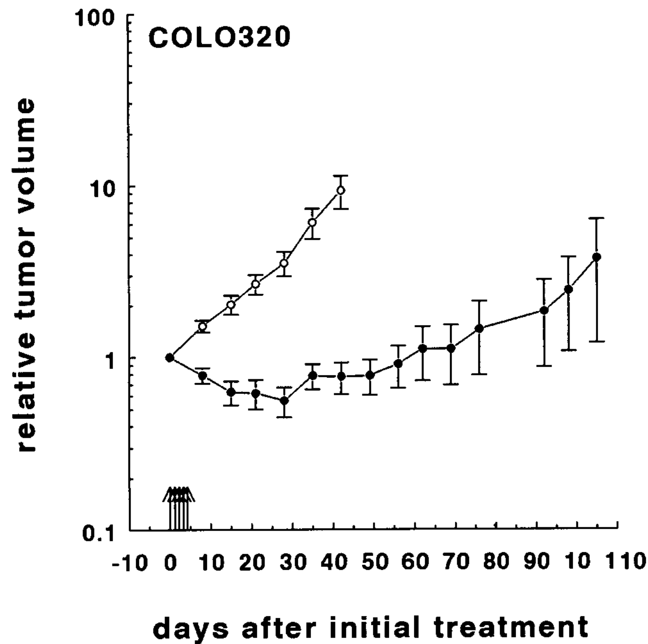
Topoisomerase-I activity was measured in nuclear extracts from cell lines by ATP-independent relaxation of supercoiled DNA. The highest relaxation activity was found in LS174T cells and amounted to 113 mU/μg nuclear protein (Table II). The other cell lines showed a lower DNA topoisomerase-I activity, between 38 and 85 mU/μg nuclear protein, the enzyme activity of which was lowest in WiDr cells. Figure 3 demonstrates that a low IC_{50} value for CPT-11 plus carboxylesterase or for SN-38 was associated with a high DNA topoisomerase-I activity. A positive relationship was present between the sensitivity to CPT-11 plus carboxylesterase and the extent of DNA topoisomerase-I activity ($r = 0.75$; $p < 0.1$). The relationship between the sensitivity to SN-38 and the enzyme activity was even better and statistically significant ($r = 0.89$; $p < 0.05$).

The DNA topoisomerase-I activity was also determined in COLO 320 and WiDr xenografts. The relaxation assay indicated that the DNA topoisomerase-I activity was significantly higher in COLO 320 xenografts than in WiDr xenografts and amounted to 77 and 50 mU/μg protein, respectively. The topoisomerase-I activity in COLO 320 and WiDr xenograft tissue was comparable with that in the same cells *in vitro.*

DISCUSSION

The cellular determinants for sensitivity to topoisomerase-I inhibitors, and CPT-11 in particular, are only partially known. Topoisomerase I is the intracellular target for camptothecins and the sensitivity to these compounds may be related to the topoisomerase-I gene expression, topoisomerase-I protein levels, the activity of the enzyme, and/or the formation of drug-stabilized cleavable complexes. Other cellular factors of importance for sensitivity to camptothecins may be the uptake of the drugs, the cell-cycle distribution and, for CPT-11, the endogenous carboxylesterase activity. In our study in 5 human colon-cancer cell lines and 2 human colon-cancer xenografts, we found the DNA topoisomerase-I activity to be a better determinant for CPT-11 and SN-38 sensitivity than the carboxylesterase activity or the topoisomerase-I mRNA expression.

A carboxylesterase is required for the conversion of CPT-11 into the active metabolite SN-38 to exert its cytotoxicity (Tsuji *et al.,* 1991). The anti-proliferative effects of CPT-11 *in vitro* are, therefore, most likely caused by its conversion into SN-38, either by cellular (endogenous) carboxylesterase or by carboxylesterase present in FCS (<10 mU/mg protein). Addition of an excess of (exogenous) carboxylesterase increased the anti-proliferative effects of CPT-11 30- to 150-fold in the 5 colon-cancer cell lines tested. This demonstrates that the amount of cellular carboxylesterase is not sufficient for inducing maximal CPT-11 activation. The anti-proliferative effects of CPT-11 plus an excess of carboxylesterase were less marked, however, than those of SN-38, but the degree of sensitivity was similar in the 5 colon-cancer cell lines tested. The activation of CPT-11 by exogenous carboxylesterase *in vitro* may not have been complete. A possible explanation may be the action of the enzyme under suboptimal conditions, as a result of which the various nutrients present in the tissue-culture medium might



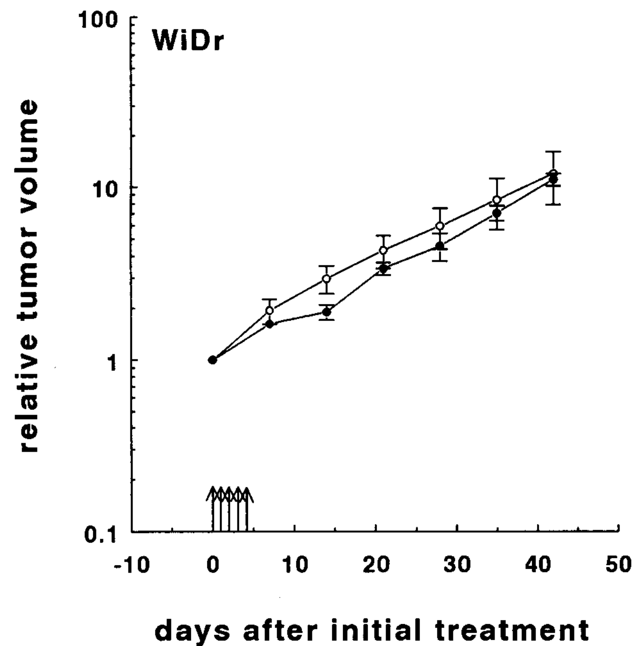


FIGURE 1 – Growth curves of COLO 320 and WiDr xenografts in nude mice. The mean relative volumes of untreated tumors (○) and of tumors treated with CPT-11 20 mg/kg i.p. daily ×5 (●) are shown. Arrows indicate the days of treatment and bars represent SEM.

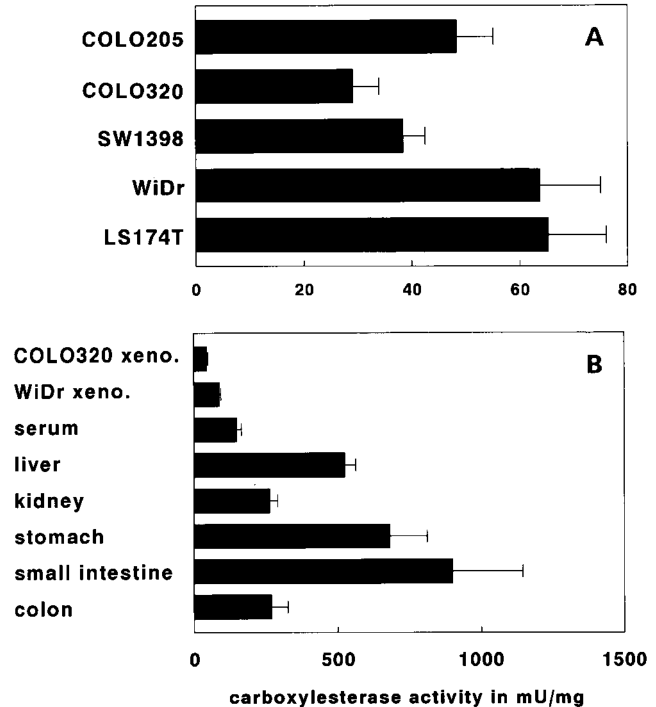


FIGURE 2 – *(A)* Endogenous carboxylesterase activity (mean ± SD) in various human colon cancer cell lines expressed in mU/mg protein. (B) Endogenous carboxylesterase activity (mean ± SD) in xenograft tissue and normal mouse organs expressed in mU/mg protein.

interfere with the conversion to SN-38. In addition, it is uncertain whether the carboxylesterase extract from porcine liver, which we used in our experiments, is a good substitute for the endogenous carboxylesterase activity that converts CPT-11 in other species. One should also consider the possibility that CPT-11 activation *in vitro* at neutral pH will partly generate the less active open ring form of SN-38 (Rivory and Robert, 1995). An interesting finding, in pharmacokinetic studies in patients, was that only a proportion of the CPT-11 administered was converted into SN-38 (Rothenberg *et al.,* 1993; Abigerges *et al.,* 1995). By comparing the area under the curve concentrations for total CPT-11 and SN-38 in these patients, the amount of SN-38 measured varied between 3% and 8% of that of the parent compound.

As CPT-11 is converted into SN-38 by a carboxylesterase, a correlation might be expected between the carboxylesterase activity in tumor cells and the sensitivity to CPT-11. In our study we found no relation between the intracellular enzyme activity and CPT-11 sensitivity. As an illustration, WiDr cells were less sensitive to CPT-11 than the more sensitive COLO 320 cells that contained a lower level of enzyme activity. Our cell lines were not selected for resistance to camptothecins and exhibited natural differences in sensitivity to these compounds. However, a role for cellular carboxylesterase has been described in a number of cell lines with acquired resistance to CPT-11, such as PC-7/CPT cells with reduced capacity to form SN-38 (Kanzawa *et al.,* 1990) or HAC2/0.1 cells with decreased activity of the enzyme (Ogasawara *et al.,* 1995).

Although there is local activation of CPT-11 in tumor tissue, carboxylesterases are ubiquitous in all vertebrates and conversion of CPT-11 is likely to occur in the major pharmacological sites, *i.e.* blood and liver. We found that mouse serum and liver contained higher enzyme activity than human colon-cancer xenografts. This appears to indicate that the low endogenous carboxylesterase activity present in colon-cancer tissue has only a minor influence on CPT-11 efficacy. Further support for this hypothesis has been provided by Kawato *et al.* (1991), demonstrating an almost equal conversion of CPT-11 into SN-38 by homogenates of 4 different human tumor xenografts, whereas the sensitivity of these tumors to CPT-11 *in vivo* was independent of their ability to produce SN-38.

TABLE II – TOPOISOMERASE-I EXPRESSION AND ACTIVITY IN HUMAN COLON-CANCER CELL LINES

Material	Topo-I Expression[1] Mean (±SD)	Topo-I activity[2] Median (range)	Topo-I activity[2] Mean (±SD)
Cell line			
COLO 205	1.48 (±0.63)	47 (34–95)	55 (±25)
COLO 320	1.00	85 (56–91)	79 (±16)
SW1398	0.86 (±0.30)	63 (47–95)	66 (±18)
WiDr	1.36 (±0.46)	38 (30–61)	41 (±12)
LS174T	1.48 (±0.42)	113 (55–170)	111 (±46)
Xenografts			
COLO 320	0.97 (±0.04)	77 (61–96)	78 (±18)
WiDr	0.79 (±0.18)	50 (38–65)	53 (±8)

[1]Topoisomerase-I gene expression relative to the γ-actin gene measured in at least 3 different samples. Values are expressed relative to COLO 320 cells or to one of the 3 COLO 320 xenografts.–[2]Topoisomerase-I activity (mU/μg) measured in at least 4 different samples.

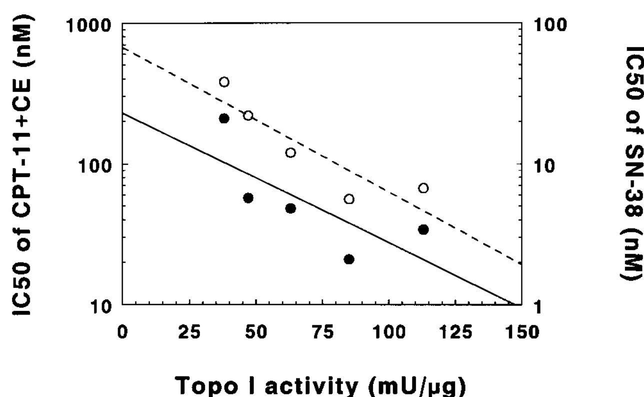


FIGURE 3 – Linear regression between the topoisomerase-I activity (mU/μg) and the IC_{50} values of CPT-11 (●, r = 0.75; $p < 0.1$) or of SN-38 (○, r = 0.89; $p < 0.05$) in the 5 human colon-cancer cell lines.

A relationship may be present between topoisomerase-I gene expression and sensitivity to CPT-11 or to SN-38. A decreased gene expression has indeed been found in camptothecin-resistant cell lines selected after exposure to the drug (Woessner *et al.,* 1992). In our panel of unselected human colon-cancer cell lines, we found no correlation between the extent of topoisomerase-I mRNA expression and the anti-proliferative effects of CPT-11 without or with an excess of carboxylesterase or of SN-38. Similar observations have been described in another panel of 7 unselected human colon-cancer cell lines (Goldwasser *et al.,* 1995) and in 7 unselected cell lines of different tumor origin (Perego *et al.,* 1994).

Reduced levels of cellular topoisomerase-I protein measured by Western blotting in nuclear extracts have been reported to relate with a lower sensitivity to camptothecin (Sugimoto *et al.,* 1990; Chang *et al.,* 1992), and can be associated with decreased topoisomerase-I mRNA expression (Woessner *et al.,* 1992). In unselected cell lines, however, Goldwasser *et al.* (1995) have demonstrated that there was no relation between topoisomerase-I protein expression and camptothecin sensitivity. Apart from Western blotting, one can also use a monoclonal antibody to detect the topoisomerase-I protein by immunocyto- or histochemistry (Negri *et al.,* 1992). In our hands, this method was less useful for quantification of the protein contents.

Our study indicated a positive correlation between the DNA topoisomerase-I activity and the cellular sensitivity to activated CPT-11 as well as to SN-38. The more sensitive cells showed a higher enzyme activity, and this was also found in the human colon-cancer xenografts. A reduction in the topoisomerase-I activity has also been described in a number of cell lines with acquired drug resistance to camptothecins (Chang *et al.,* 1992; Woessner *et al.,* 1992).

The 2-fold difference in topoisomerase-I activity between COLO 320 and WiDr xenografts would probably not explain the much higher sensitivity of COLO 320 tumors to CPT-11 *in vivo.* This suggests that other cell-specific factors, *e.g.* proliferation rate, cell-cycle distribution, SN-38 uptake or drug-resistance mechanisms, might contribute to the sensitivity to CPT-11. Non-proliferating tumor cells are generally less sensitive to camptothecins, since DNA replication is essential for the cytotoxicity of these topoisomerase-I inhibitors (Gallo *et al.,* 1971). Camptothecins are specifically cytotoxic to S-phase cells, although cellular levels of topoisomerase I are relatively constant during the phases of the cell cycle (Heck *et al.,* 1988). The growth rate of the xenografts, however, did not account for the difference in CPT-11 sensitivity as COLO 320 tumors had a slow T_D of 15 days when compared with the T_D of 7.5 days in WiDr tumors. We cannot be sure whether or not the uptake of CPT-11 or SN-38 in the tumor cells played a role in the different drug effects. Earlier observations have revealed that the uptake of camptothecins *in vitro* was unaltered in cells that differed in sensitivity (Kanzawa *et al.,* 1990; Goldwasser *et al.,* 1995). Some studies have suggested a role for P-glycoprotein (Pgp) in resistance against camptothecins. For example, Mattern *et al.* (1993) have shown that the expression of Pgp may affect the cytotoxicity of topotecan, 9-aminocamptothecin, CPT-11 and SN-38 *in vitro. In vivo,* however, the various camptothecins were equally effective against the multidrug-resistant tumors and the parental tumors. Unlike WiDr cells, the COLO 320 cells express Pgp (Jansen *et al.,* 1995), which does not explain the difference in sensitivity to CPT-11.

Although we found a positive correlation between the DNA topoisomerase-I activity and cellular sensitivity to activated CPT-11 and SN-38, the correlation may even be stronger when measuring drug-induced topoisomerase-I-cleavable complexes. The formation of stable complexes is the initial step leading to drug-induced cell death (Tanizawa *et al.,* 1995). Rubin *et al.* (1994) and Fujimori *et al.* (1995) have described cell lines with acquired resistance to camptothecins containing functional topoisomerase I, but with reduced formation of drug-induced topoisomerase-I-cleavable complexes. Goldwasser *et al.* (1995) have indeed reported a positive correlation between camptothecin-induced topoisomerase-I-cleavable complexes and camptothecin sensitivity. Whether this assay will better explain the difference in *in vivo* sensitivity between COLO 320 and WiDr tumors remains to be investigated. The technique, however, is cumbersome and most reliable when cellular DNA is labeled. It can, therefore, not be easily transferred to the clinic (Goldwasser *et al.,* 1995).

In conclusion, of the various cellular characteristics responsible for sensitivity to CPT-11, we found the activity of DNA topoisomerase I to correlate best in this panel of unselected colon-cancer cell lines. The usefulness of this assay as a potential predictive test in the daily management of cancer patients to be treated with CPT-11 will be the subject of further studies.

REFERENCES

ABIGERGES, D., CHABOT, G.G., ARMAND, J.P., HERAIT, P., GOUYETTE, A. and GANDIA, D., Phase-I and pharmacologic studies of the camptothecin analog irinotecan administered every 3 weeks in cancer patients. *J. clin. Oncol.,* **13,** 210–221 (1995).

BOVEN, E., WINOGRAD, B., FODSTAD, O., LOBBEZOO, M.W. and PINEDO, H.M., Preclinical phase-II studies in human tumor lines: a European multicenter study. *Europ. J. Cancer clin. Oncol.,* **24,** 567–573 (1988).

CHANG, J-Y., DETHLEFSEN, L.A., BARLEY, L.R., ZHOU, B. and CHENG, Y., Characterization of camptothecin-resistant Chinese hamster lung cells. *Biochem. Pharmacol.,* **43,** 2443–2452 (1992).

CREEMERS, G.J., LUND, B. and VERWEIJ, J., Topoisomerase I inhibitors: topotecan and irinotecan. *Cancer Treat. Rev.,* **20,** 73–96 (1994).

FUJIMORI, A., HARKER, W.G., KOHLHAGEN, G., HOKI, Y. and POMMIER, Y., Mutation at the catalytic site of topoisomerase I in CEM/C2, a human leukemia cell line resistant to camptothecin. *Cancer Res.,* **55,** 1339–1346 (1995).

GALLO, R.C., WHANG-PHENG, J. and ADAMSON, R.H., Studies on the antitumor activity, mechanism of action, and cell cycle effects of camptothecin. *J. nat. Cancer Inst.,* **48,** 1722–1726 (1971).

GIACCONE, G., VAN ARK-OTTE, J., SCAGLIOTTI, G., CAPRANICO, G., VAN DER VALK, P., RUBIO, G., DALESIO, O., LOPEZ, R., ZUNINO, F., WALBOOMERS, J. and PINEDO, H.M., Differential expression of DNA topoisomerases in non-small-cell lung cancer and normal lung. *Biochim. biophys. Acta,* **1264,** 337–346 (1995).

GOLDWASSER, F., BAE, I., VALENTI, M., TORRES, K. and POMMIER, Y., Topoisomerase-I-related parameters and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen. *Cancer Res.,* **55,** 2116–2121 (1995).

HECK, M.M.S., HITTELMAN, W.N. and EARNSHAW, W.C., Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. *Proc. nat. Acad. Sci. (Wash.),* **85,** 1086–1090 (1988).

HOUGHTON, P.J., CHESHIRE, P.J., HALLMAN, J.D., LUTZ, L., FRIEDMAN, H.S., DANKS, M.K. and HOUGHTON, J.A., Efficacy of topoisomerase-I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother. Pharmacol.,* **36,** 393–403 (1995).

JANSEN, W.J.M., PINEDO, H.M., VAN DER WILT, C.L., FELLER, N., BAMBERGER, U. and BOVEN, E., The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine *in vitro* and in human tumour xenografts. *Europ. J. Cancer,* **31,** 2313–2319 (1995).

JUAN, C.C., HWANG, J., LIU, A.A., WHANG-PENG, J., KNUTSEN, T., HUEBNER, K., CROCE, C.M., ZHANG, H., WANG, J.C. and LIU, L.F., Human DNA topoisomerase I is encoded by a single-copy gene that maps to chromosome region 20q12-13.2. *Proc. nat. Acad. Sci. (Wash.),* **85,** 8910–8913 (1988).

KANZAWA, F., SUGIMOTO, Y., MINATO, K., KASAHARA, K., BUNGO, M., NAKAGAWA, K., FUJIWARA, Y., LIU, L.F. and SAIJO, N., Establishment of a camptothecin analogue (CPT-11)-resistant cell line of human non-small-cell lung cancer: characterization and mechanism of resistance. *Cancer Res.,* **50,** 5919–5924 (1990).

KAWATO, Y., FURUTA, T., AONUMA, M., YASUOKA, M., YOKOKURA, T. and MATSUMOTO, K., Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother. Pharmacol.,* **28,** 192–198 (1991).

LIU, L.F. and MILLER, K.G., Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc. nat. Acad. Sci. (Wash.),* **78,** 3487–3491 (1981).

MATTERN, M.R., HOFMANN, G.A., POLSKY, R.M., FUNK, L.R., MCCABE, F.L. and JOHNSON, R.K., *In vitro* and *in vivo* effects of clinically important camptothecin analogues on multidrug-resistant cells. *Oncol. Res.,* **5,** 467–474 (1993).

NEGRI, C., CHIESA, R., CERINO, A., BESTAGNO, M., SALA, C., ZINI, M., MARALDI, N.M. and ASTALDI RICOTTI, G.C.B., Monclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170- and 180-kDa isozymes. *Exp. Cell Res.,* **200,** 452–459 (1992).

OGASAWARA, H., NISHIO, K., KANZAWA, F., LEE, Y-S., FUNAYAMA, Y., OHIRA, T., KURAISHI, Y., ISOGAI, Y. and SAIJO, N., Intracellular carboxyl esterase activity is a determinant of cellular sensitivity to the antineoplastic agent KW-2189 in cell lines resistant to cisplatin and CPT-11. *Jap. J. Cancer Res.,* **86,** 124–129 (1995).

PEREGO, P., CAPRANICO, G., SUPINO, R. and ZUNINO, F., Topoisomerase-I gene expression and cell sensitivity to camptothecin in human cell lines of different tumor types. *Anti-Cancer Drugs,* **4,** 645–649 (1994).

RIVORY, L.P. and ROBERT, J., Molecular, cellular, and clinical aspects of the pharmacology of 20(S)camptothecin and its derivatives. *Pharmacol. Ther.,* **68,** 269–296 (1995).

ROTHENBERG, M.L., KUHN, J.G., BURRIS, H.A., NELSON, J., ECKARDT, J.R., TRISTAN-MORALES, M., HILSENBECK, S.G., WEISS, G.R., SMITH, L.S., RODRIGUEZ, G.I., ROCK, M.K. and VON HOFF, D.D., Phase-I and pharmacokinetic trial of weekly CPT-11. *J. clin. Oncol.,* **11,** 2194–2204 (1993).

RUBIN, E., PANTAZIS, P., BHARTI, A., TOPPMEYER, D., GIOVANELLA, B. and KUFE, D., Identification of a mutant topoisomerase I with intact catalytic activity and resistance to 9-nitro-camptothecin. *J. biol. Chem.,* **269,** 2433–2439 (1994).

SLICHENMYER, W.J., ROWINSKY, E.K., DONEHOWER, R.C. and KAUFMANN, S.H., The current status of camptothecin analogues as antitumor agents. *J. nat. Cancer Inst.,* **85,** 271–291 (1993).

SUGIMOTO, Y., TSUKAHARA, S., OH-HARA, T., ISOE, T. and TSURUO, T., Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res.,* **50,** 6925–6930 (1990).

TANIZAWA, A., KOHN, K.W., KOHLHAGEN, G., LETEURTRE, F. and POMMIER, Y., Differential stabilization of eukaryotic DNA topoisomerase I cleavable complexes by camptothecin derivatives. *Biochemistry,* **34,** 7200–7206 (1995).

TSUJI, K., KANEDA, N., KADO, K., YOKOKURA, T., YOSHIMOTO, T. and TSURU, D., CPT-11 converting enzyme from rat serum: purification and some properties. *J. pharmacobiol. Dyn.,* **14,** 341–349 (1991).

WOESSNER, R.D., ENG, W.K., HOFMANN, G.A., RIEMAN, D.J., MCCABE, F.L., HERTZBERG, R.P., MATTERN, M.R., TAN, K.B. and JOHNSON, R.K., Camptothecin hyper-resistant P388 cells: drug-dependent reduction in topoisomerase I content. *Oncol. Res.,* **4,** 481–488 (1992).