tivation of T cells without clonal deletion has been demonstrated recently as a mechanism for establishing peripheral T cell tolerance in vivo (*23*). Thus, during T cell development, cells with high-affinity self-reactive TCR that escape clonal deletion in the thymus might nevertheless be tolerized peripherally by the induction of clonal energy. Experiments with $V_\beta$-specific activating antibodies would directly address this possibility.


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# DNA Topoisomerase I–Targeted Chemotherapy of Human Colon Cancer in Xenografts


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**Drug development is needed to improve chemotherapy of patients with locally advanced or metastatic colon carcinoma, who otherwise have an unfavorable prognosis. DNA topoisomerase I, a nuclear enzyme important for solving topological problems arising during DNA replication and for other cellular functions, has been identified as a principal target of a plant alkaloid 20(*S*)-camptothecin. Significantly increased concentrations of this enzyme, compared to that in normal colonic mucosa, were found in advanced stages of human colon adenocarcinoma and in xenografts of colon cancer carried by immunodeficient mice. Several synthetic analogs of camptothecin, selected by tests with the purified enzyme and tissue-culture screens, were evaluated in the xenograft model. Unlike other anticancer drugs tested, 20(*RS*)-9-amino-camptothecin (9-AC) induced disease-free remissions. The overall drug toxicity was low and allowed for repeated courses of treatment.**


THE ANTITUMOR ACTIVITY OF 20(*S*)-camptothecin, a plant alkaloid isolated from *Camptotheca acuminata* (*1*), was studied in the early 1970s (*2*). Its water-soluble sodium salt, substantially less effective than the lactone form (*3*), was briefly tested in phase I clinical trials. Leukopenia was the dose-limiting toxic effect, and hemorrhagic cystitis was the most prominent nonhematological complication. Since the purpose of phase I trials is to establish drug toxicity, therapeutic responses were evaluated only in some patients. Partial remissions were noted in patients with advanced gastrointestinal cancer, which had been refractory to other treatments. Further development of camptothecins was hampered by the unavailability of compounds with better antitumor efficacy and by the lack of understanding of its mechanism of action. The recent demonstration that DNA topoisomerase I is the main, if not exclusive, target of camptothecin (*4*) has revived interest in research on camptothecin analogs as anticancer drugs. Human topoisomerase I, a monomeric protein of 100 kD (*5*), acts by relaxing supercoiled DNA. Its activity is likely to be important for semiconservative replication of double-helical DNA and for other DNA functions such as transcription, recombination, and chromosomal decondensation (*6*). The 20(*S*)-camptothecin interferes with the DNA breakage-reunion reaction catalyzed by topoisomerases I, by trapping a key covalent enzyme-DNA intermediate termed the "cleavable complex" (*4, 7*). Topoisomerase I levels are lower in normal cells than in cells of chronic lymphocytic leukemia as well as in several types of lymphoma (*8*).

Human colon cancer was selected as a model for solid tumors of epithelial origin, because colonic cancer is a major problem in clinical oncology. One of 25 Americans will develop this disease during their lifetime (*9*).


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Progress in treatment has come mainly through improved operative and perioperative care. However, 20 to 50% of the patients appear for initial treatment with locally advanced or metastatic disease and often have an extremely unfavorable prognosis. The principal therapeutic drug used in treatment, 5-fluorouracil, is only marginally effective, but no other single agent or combination treatment has been shown to perform better (*10*).

Our studies revealed that the concentrations of DNA topoisomerase I in surgical specimens obtained from patients with colon cancer were elevated in comparison to the concentrations in normal colon mucosa. The results of immunoblot analysis in primary colon adenocarcinomas of 38 untreated patients is shown in Fig. 1. The disease was evaluated at (i) an early stage, with tumors confined to the primary site and with various depth of colonic wall penetration (stages B1 to B3, modified Duke's classification of colorectal cancer) (*9, 11*); (ii) an advanced stage, with various depths of wall penetration and lymph node involvement (stages C1 to C3); and (iii) with tumor metastases to distant organs (stages C1 to C3, D). On average, topoisomerase I levels (normalized for DNA content) were 14-fold (patients at stage C1 to C3, $P < 0.01$) or 16-fold (stage C1 to C3, D, $P < 0.01$; $t$ test with Bonferroni correction used in both cases) higher in cancerous tissue than in normal colonic mucosa. Topoisomerase I was also measured in 10- to 14-day xenograft implants of NCI-1 immunodeficient (nude) mice (Fig. 2) (*12*). Topoisomerase I levels in the four xenograft lines examined were equivalent to enzyme concentrations in the cancer of advanced clinical stages (Fig. 1).

Three xenograft lines were selected to test the hypothesis that topoisomerase I in human colon cancer presents a suitable target for chemotherapy. Earlier, several new analogs of camptothecin (*13*) had been investigated in vitro, and the most active were selected for this study: 20(*RS*)-9-aminocamptothecin (9-AC), 20(*RS*)-10-aminocamptothecin, 20(*RS*)-10,11-methylenedioxy-camptothecin and its sodium salt (*3*). Representative results of xenograft treatments are shown in Fig. 2, A and B. A moderate to poorly differentiated human colon cancer (HT-29) and poorly differentiated colon cancer lines [designated CASE and SW 48(*12*)] were implanted on day 0. The drugs were injected subcutaneously, with the exception of doxorubicin, which was injected intravenously. The treatment started on day 7 and continued twice a week for 5 to 6 weeks. Drug doses were based on an established median toxic dose for the schedule applied. Among the nine anticancer agents tested, marginal growth retardation of tumor implants was noticed in some cases (for example, 80 mg of 5-fluorouracil per kilogram of body weight per dose or 160 mg of methotrexate per kilogram of body weight per dose). This confirmed earlier results that showed the ineffectiveness of these drugs against 14 human colorectal xenograft lines (*14*). However, mice treated with camptothecin analogs showed marked inhibition of tumor growth. Five of six mice with HT-29 tumors, injected subcutaneously twice a week with 10 mg of 9-AC per kilogram of body weight per dose for 6 weeks (Fig. 2A), had no evidence of disease (NED) or minimal disease (tumor volume

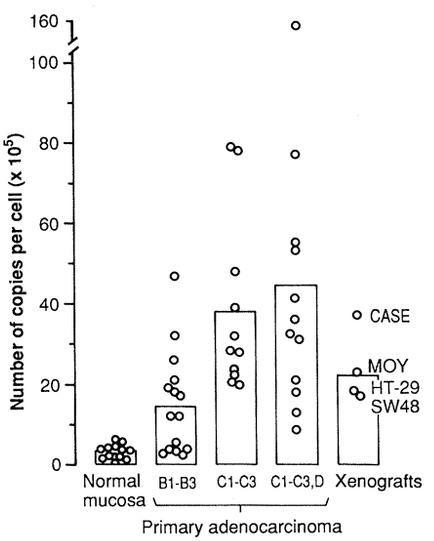


**Fig. 1.** DNA topoisomerase I levels in surgical specimens of normal human mucosa of the colon, primary adenocarcinoma of the colon, and in human colon cancer xenografts. The enzyme was purified to homogeneity from HeLa cells, and the preparation of antisera to the enzyme followed an established procedure (*16*). Under the supervision of a pathologist, tissues of colonic tumors and of normal mucosa were frozen in liquid nitrogen within 1 hour after the surgery and stored in multiple fractures at −80°C for further analysis. Specimens were also obtained from four lines of colon cancer xenografts, which had grown 10 to 14 days in NCI-1 immunodeficient mice. The immunoblot analysis for topoisomerase I was performed as described previously (*8, 16*). DNA content of tissue homogenate was estimated by an established procedure (*17*). DNA content was used for the normalization of enzyme levels, which were expressed as number of copies per cell with DNA content of a HeLa cell line. Circles show enzyme levels in individual specimens, and the histograms indicate the means of the number of topoisomerase I copies per cell.

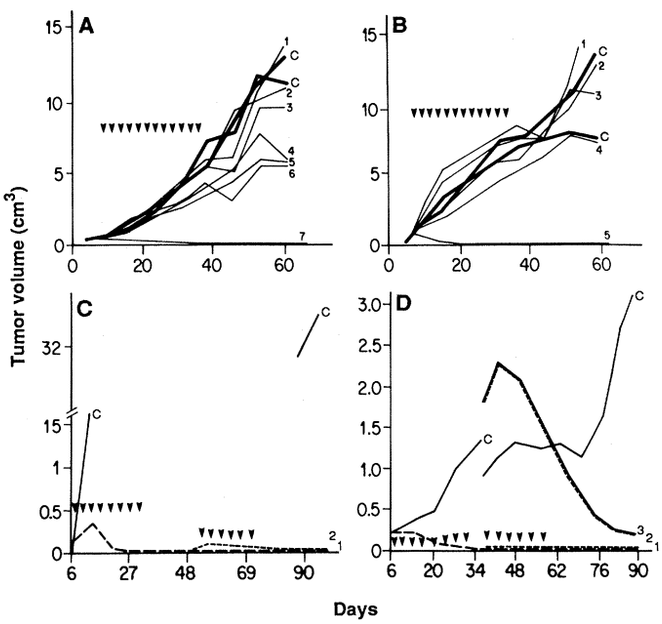


**Fig. 2.** Treatment of human colon cancer xenografts carried by immunodeficient NIH-1 mice. Each control or drug-treated group included six males; tumor fragments were implanted on day 0. For an implant, 50 mg wet weight of finely minced tumor tissue in 0.5 ml of Eagle's minimum essential medium (Gibco) was injected under the skin over the right dorsal chest region. The treatments started on day 7 and continued twice a week for 3 to 6 weeks. The drugs were formulated in Tween 80:0.15 NaCl and injected subcutaneously, except for doxorubicin, which was injected intravenously. Controls were treated with the solvent only. The tumors were measured in three dimensions with a caliper, and the tumor volumes were calculated. Means of tumor volumes in centimeters cubed were plotted against time; SD of the means was less than 15% of the value. The arrowheads indicate the time of injections. All mice were treated at the same time; two control groups are shown in (A) and (B) to indicate variability. Variation between the two groups was statistically insignificant ($P > 0.1$, $t$ test) at any point of the measurements. All indicated drug doses are per a simple treatment. (**A**) HT-29: C, control; 1, doxorubicin (5 mg/kg); 2, methyl-1(2-chlorethyl)-3-cyclohexyl-1-nitrosourea (3 mg/kg); 3, 1,3-bis(2-chlorethyl-1-nitrosurea (3 mg/kg); 4, Alkeran (9 mg/kg); 5, 5-fluorouracil (80 mg/kg); 6, methotextrate (160 mg/kg); 7, 9-AC (10 mg/kg). (**B**) CASE: C, control; 1, vincristine (1.5 mg/kg); 2, methyl-1(2-chlorethyl)-3-cyclohexy-1-nitrosourea (3 mg/kg); 3, vinblastine (1.5 mg/kg); 4, 5-fluorouracil (80 mg/kg); 5, 9-AC (12.5 mg/kg). (**C**) CASE: C, control; 1, 9-ACI (one course of treatment) (12.5 mg/kg); 2, 9-ACII (subset of 9-ACI, retreated). (**D**) SW 48: C, control; 1, 9-ACI; 2, 9-ACII; 3, 9-AC (subset of control treated).

$<0.1$ cm$^3$). In another group, six of six mice treated with 12.5 mg of 9-AC per kilogram of body weight per dose had NED or minimal disease (data not shown). The mean ± SD remission was 62 ± 20 and 56 ± 15 days, respectively. The appearance of a palpable nodule followed by continuous tumor regrowth signaled the end of a remission, and no signs of drug toxicity were detected. The first course of treatment was also well tolerated in mice with CASE or SW 48 human colon cancer. The latter tumor line is extremely unresponsive to any kind of therapy (*12*). The first course of treatment of CASE (Fig. 2, B and C) or SW 48 tumors (Fig. 2D) (12.5 mg of 9-AC per kilogram of body weight, respectively) induced NED or minimal disease in six of six mice of either group.

On day 51, mice with CASE tumors and minimal disease received the second course of therapy, which was a total of six injections, 12.5 mg of 9-AC per kilogram of body weight per dose (Fig. 2C). After the first course of treatment, the second course (75 mg of 9-AC per kilogram of body weight, total dose) was also administered to mice with SW 48 tumors and minimal disease (Fig. 2D). In either case, five of six mice had NED and the other had minimal disease. Long-term survival (in excess of 7 months) was observed in seven of eight treated mice with CASE tumors and in six of six mice with SW 48 tumors.

The first course of treatments of the CASE and SW 48 lines started with well-established tumors, averaging 0.2 to 0.25 cm$^3$ in volume. In another experiment, day 35 SW 48 tumors (average size, 2.5 cm$^3$) were selected for the first treatment with 9-AC. The disparity of tumor sizes between the control and treated groups is intentional (Fig. 2D): The largest tumors available were selected for treatment with six doses of 9-AC (12.5 mg per kilogram of body weight per dose, injected twice a week). There was a 91% reduction in the volume of the tumors in treated mice. A second course of treatment of the HT-29 line was administered to mice with an average tumor size of 8.0 cm$^3$. A total dose of 60 mg of 9-AC per kilogram of body weight reduced the tumors by 46% (*15*).

The 20(*RS*)-10,11-methylenedioxy-camptothecin sodium salt, a water-soluble compound, is highly active in topoisomerase I–directed screens and was selected for in vivo tests. Five injections of this drug (10 or 12.5 mg per kilogram of body weight per dose) stopped the growth of day 7 CASE tumors. The overall toxicity of the higher dose, however, resulted in animal deaths, whereas the toxicity of the lower dose was still acceptable (loss of <30% of body weight).

The overall toxicity was evaluated in all experiments. The first course of treatment with 150 mg of 9-AC per kilogram of body weight (total dose) had no detectable toxic effects. The response of treated animals would have allowed either higher doses or a treatment course delivered over a longer time period. Although 50 mg of 20(*S*)-camptothecin, 25 mg of 20(*RS*)-10-amino, and 62.5 mg of 20(*RS*)-10,11-methylenedioxy-camptothecin sodium salt led to toxic deaths of all animals, 50 mg of 20(*RS*)-10,11-methylenedioxy-camptothecin sodium salt per kilogram of body weight only resulted in 26% body weight loss, followed by rapid recovery and gains in body weight.

The second course of treatment with 50 to 75 mg of 9-AC per kilogram of body weight appeared to be more toxic than the first. The body weight dropped by 19% on the average (CASE and SW 48 tumor lines), but again recovered rapidly. The poorer performance of mice receiving the second course of treatment can be explained by a cumulative toxicity of the two treatments. There were, however, no signs of gastrointestinal toxicity or sterile hemorrhagic cystitis, which had been observed among patients treated with 20(*S*)-camptothecin sodium salt (*2*).

In conclusion, 9-AC, a synthetic analog of 20(*S*)-camptothecin, is a potent anticancer agent, highly effective against three lines of human colon cancer carried by immunodeficient mice and exerts considerably low overall toxicity. Its high efficacy, however, is not completely understood. The increased levels of topoisomerase I in colon cancer xenografts, which reflects the findings in surgical specimens obtained from patients with advanced colon carcinoma, may be a contributing factor (*6*). Further studies of camptothecin analogs are necessary to evaluate their clinical usefulness.

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