Biological Properties of IDN5174, a New Synthetic Camptothecin with the Open Lactone Ring

Giovanni L. Beretta,[1] Giovanna Petrangolini,[1] Michelandrea De Cesare,[1] Graziella Pratesi,[1] Paola Perego,[1] Stella Tinelli,[1] Monica Tortoreto,[1] Massimo Zucchetti,[2] Roberta Frapolli,[2] Ezia Bello,[2] Carla Manzotti,[3] Gabriele Fontana,[3] Ezio Bombardelli,[3] Arturo Battaglia,[4] Cristian Samorì,[4] and Franco Zunino[1]

[1]Department of Experimental Oncology and Laboratories, Istituto Nazionale Tumori; [2]Department of Oncology, Istituto di Ricerche Farmacologiche Mario Negri; [3]Indena S.p.A., Milan, Italy; and [4]Istituto Consiglio Nazionale delle Ricerche-Istituto per la Sintesi Organica e la Fotoreattività, Bologna, Italy

Abstract

A series of water-soluble camptothecins obtained by linking a spermidine moiety to the 21-position of the open form through an amidic bond have been tested for their biochemical and biological activities. Growth inhibition assay on the human non–small cell lung cancer carcinoma NCI-H460 cell line revealed that the camptothecin analogues were less potent than topotecan and SN38 after 1 hour of treatment. The potency increased after 72 hours of exposure, being similar to that of reference camptothecins. The analysis of topoisomerase I–mediated DNA cleavage using the purified enzyme indicated that the novel camptothecin analogues retained ability to poison topoisomerase I and displayed the same cleavage pattern of SN38. Persistence of the DNA cleavage was comparable with that of SN38. Stabilization of the cleavable complex was not the result of hydrolysis of the N-C bond between polyamine and the drug because no free camptothecin was recovered at the end of DNA cleavage in presence of IDN5174, the analogue selected for detailed studies. IDN5174 exhibited an antitumor activity comparable with that of topotecan and irinotecan against NCI-H460 tumor xenograft. The pharmacokinetics in mice showed a favorable disposition in tumor tissue with low amount of camptothecin detectable in plasma and tumor (around 5-10%), thus supporting the efficacy of intact IDN5174. In conclusion, we found that IDN5174 maintained the biological and antitumor properties, in spite of lack of the closed E ring. The available results support the interpretation that the polyamine linked at the 21-position may allow a favorable drug interaction in the ternary complex. (Cancer Res 2006; 66(22): 10976-82)

Introduction

Among clinically effective antitumor agents, camptothecins are a unique class because they specifically inhibit topoisomerase I. Camptothecins bind to and reversibly stabilize the covalent topoisomerase I-DNA complex, thus slowing the religation phase of the enzyme catalytic cycle and prolonging the lifetime of the covalent protein-DNA complex. Collision between trapped topoisomerase I-DNA complex and moving replication forks during DNA synthesis results in double-stranded DNA breaks and cell death (1).

The clinical success of the water-soluble camptothecins topotecan and irinotecan has stimulated efforts to optimize the efficacy of this class of agents. In an attempt to synthesize new analogues, the chemical structure of camptothecin has been extensively dissected, and structure-activity relationship studies have revealed critical aspects for drug activity (2). Among these, the structure of the lactone E ring and its 20(*S*)-hydroxyl configuration are considered essential for the antitumor action (1, 3, 4).

The α-hydroxylactone E ring is unstable in water solution at physiologic pH, and camptothecins yield two molecular species, the lactone and the ring-opened carboxylate forms. The opened form is almost inactive and substantially less potent than the lactone form (5, 6). At physiologic pH, the equilibrium between the two species is shifted toward the carboxylate form. The equilibrium is also dependent on the presence of specific binding proteins (i.e., human serum albumin, which binds the opened ring shifting the equilibrium towards the carboxylate form; refs. 5, 7).

Chemical modifications of the lactone ring, such as transformation into lactam ring, reduction/removal of the carbonyl oxygen, or substitution of the 20(S)-hydroxyl for hydrogen, inactivate the molecule (6, 8, 9), thus implicating the presence of an intact 20-hydroxy lactone ring as an essential requirement for the cytotoxic activity. However, some natural glycoside analogues are characterized by a ring-opened form (10). In particular, one natural constituent of *Nothapodytes foetida*, named Foetidin I, is a di-coumaroyl spermidine ester of the carboxylate form of camptothecin. The biological significance of these camptothecin-like molecules is still unknown.

Among the ways thus far devised to improve the efficacy of DNA-interactive agents, conjugation of a cytotoxic drug to natural polyamines seems an attractive strategy. The polyammonium cations have proved to favor the interaction with the negatively charged phosphates of the DNA backbone maintaining a high degree of translational freedom along the polyanion backbone, thus conferring a high DNA affinity without modifying the capability of the drug to locate appropriate sites of DNA (11). Conjugation of a cytotoxic drug to polyamines gave successful conjugates with chlorambucil (12–14), nitroimidazole (15, 16), aziridines (17–19), acridine (20), and enediynes (21), with enhanced cytotoxicity and/or increased drug selectivity for tumor cells.

Based on these observations, we have prepared a number of camptothecin analogues containing spermidine linked to the 21-position of the open form through a stable amidic bond. In the present article, we report that a water-soluble camptothecin

Requests for reprints: Franco Zunino, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-2-23902267; Fax: 39-2-23902692; E-mail: franco.zunino@istitutotumori.mi.it.

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(IDN5174) containing such a polyamine linked to the carboxylate function of the opened E ring maintained the ability to stabilize the cleavable complex and showed an antitumor activity comparable to that of classic camptothecins.

Materials and Methods

Drugs

Topotecan and irinotecan are products of Glaxo-Smith-Kline (Uxbridge, United Kingdom) and Sanofi-Aventis (Paris, France), respectively.

Chemistry

IDN5975. N^2,N^3-di-*t*-butoxycarbonylspermidine (2.8 g, 8.1 mmol) was added to a suspension of camptothecin (0.70 g, 2.01 mmol) in dry pyridine (50 mL) under nitrogen flow. The reaction was stirred at 80°C, affording a yellow and limpid solution. After 72 hours, the solution was cooled to room temperature, washed with 5% HCl, and extracted with $CHCl_3$. Organic layers were dried on sodium sulfate, concentrated in vacuum. The crude intermediate was dissolved in pyridine (10 mL) and cooled at 0°C. Acetic anhydride (1.54 mL) was then added, and the mixture was stirred at room temperature for 24 hours under nitrogen atmosphere. The solution was then concentrated under vacuum, and the oily residue obtained was poured into ice water (50 mL). The separated solid was dried under vacuum and purified on a flash silica gel column using 3% methanolic DCM to afford IDN5975 as a yellow solid (0.49 g, yield = 81%).

^{1}H-nuclear magnetic resonance (^{1}H-NMR; $CDCl_3$): δ 1.15 (t, 3H, J = 6.9), 1.2 (6H), 1.45 (18H), 2.05 (s, 3H), 2.35 (m, 2H), 3.05 (4H), 3.2 (4H), 5.25 (s, 2H), 5.4 (2H), 7.5 to 8.4 (6H).

^{13}C-NMR ($CDCl_3$): δ 7.87 (q), 20.97 (q), 25.66 (t), 2x27.39 (t), 6x28.37 (q), 32.93 (t), 40.15 (t), 3x46.58 (t), 50.2 (t), 58.98 (t), 78.74 (s), 79.64 (s), 100.6 (d), 125.0 (s), 127.72 (d), 127.89 (d), 2x128.35 (s), 129.32 (d), 130.19 (d), 130.61 (d), 2x144.47 (s), 2x148.45 (s), 2x155.97 (s), 161.58 (s), 2x171.15 (s).

High-performance liquid chromatography (HPLC; RP 8; 75% H_2O, 25% CH_3CN): 18.47 minutes.

IDN5174. IDN5975 (0.49 g, 0.66 mmo1) was dissolved in dry CH_2Cl_2 and cooled to 0°C. Trifluoroacetic acid (0.8 mL) was then added dropwise. The mixture was left under stirring at 0°C for 1.5 hours. The solution was concentrated under vacuum, and the crude residue was dissolved in $CHCl_3$. Crude IDN5174 was precipitated by addition of *n*-hexane. Pure IDN5174 (0.50 g, yield: 98%, camptothecin content ≤0.1% as HPLC area %) was obtained by multiple recrystallization from methanol.

^{1}H-NMR (DMSO-d_6/D_2O): δ 8.63 (s, 1H, arom.), 8.12 (d, 1 H, J = 8.4 Hz, arom.), 8.05 (d, 1H, J = 8.4 Hz arom.), 7.86 to 7.81 (m, 1H, arom.), 7.70 to 7.65 (m, 1H, arom.), 7.50 (s, 1H, arom.), 5.29 (d, 1H, CH_2-O, J = 11.2 Hz), 5.24 (d, 1H, CH_2-O, J = 11.2 Hz), 5.21 (s, 2H, CH_2-N), 3.20 to 3.00 (m, 2H), 2.88-2.80 (m, 4H), 2.80 to 2.72 (m, 2H), 2.18 to 2.28 (m, 1H), 2.06 to 2.18 (m, 1H), 1.95 (s, 3H, Me, OAc), 1.76 to 1.66 (m, 2H), 1.60 to 1.48 (m, 4H), 0.88 (t, 3H, J = 7.8 Hz, Me).

^{13}C-NMR (DMSO-d_6): δ 173.9, 171.0, 161.3, 158.0 (q, CO_2H of trifluoroacetic acid); 156.5, 153.4, 148.7, 144.9, 132.3, 131.1, 130.6, 129.7, 1292, 128.7, 128.3, 124.0, 99.5, 79.4, 59.5, 51.0, 46.8, 45.3, 38.9, 36.7, 32.3, 26.5, 24.8, 23.3, 21.4, 8.6.

IDN6080 and IDN6083. N^2,N^3-dibenzyloxycarbonyl spermidine (6.5 g, 15 mmol) was added to a suspension of 7-ethylcamptothecin (3 g, 7.9 mmol) in dry pyridine (8.6 mL). The solution was sonicated at 55°C for 12 hours. The solvent was then removed by distillation under vacuum, and the residue was dissolved in CH_2Cl_2 (20 mL), washed with a solution of 5% citric acid (2 × 50 mL), and then with water (20 mL). The combined organic layers were dried on sodium sulfate, filtered, and concentrated in vacuum. The crude (7.5 g, 9.6 mmol) was suspended again in dry pyridine (8.6 mL). Acetic anhydride (9.12 g) was then added. The reaction was left to room temperature and under nitrogen until disappearance of starting material and then concentrated to small volume under vacuum, poured into ice water (40 mL), and extracted with CH_2Cl_2 (2 × 30 mL). The solvent was then removed under reduce pressure, and the residue was purified on a flash silica gel column (ethyl acetate/methanol, 98:2) to afford 3.5 g of intermediate for IDN6080 e IDN6083.

(*a*) IDN6080. To a solution of intermediate (0.17 g, 0.2 mmol) in acetonitrile (20 mL), 0.10 g Pd/C 5% and L-(+)-tartaric acid (0.06 g, 0.4 mmol) were added. The mixture was hydrogenated for 3 hours (4 atm of H_2). After the mixture was filtered, the solvent was removed under reduce pressure to give the desired product (0.14 g, 98.5%).

^{1}H-NMR (DMSO-d_6/D_2O): δ 8.26 (d, 1H, J = 8.4 Hz, arom.), 8.17 (d, 1H, J = 8.4 Hz, arom.), 8.10 to 8.05 (m, 1H, NHC=O), 7.83 (m, 1H, arom.), 7.71 (m, 1H, arom.), 7.48 (s, 1H, arom.), 5.38 (d, 1H, CH_2O, J = 11.0 Hz), 5.32 (d, 1H, CH_2O, J = 11.0 Hz), 5.27 (s, 2H, CH_2N), 3.97 (s, 4H of tartaric acid, 2 CH_2), 3.24 to 3.10 (m, 4H, CH_2 of C7-Et and CH_2 of CH_2N-C=O), 2.90 to 2.70 (m, 6H, 3 CH_2), 2.30 to 2.00 (m, 2H, CH_2 of C19-H_2), 1.97 (s, 3H, Me, OAc), 1.80 to 1.68 (m, 2H, CH_2), 1.68 to 1.50 (m, 4H, 2 CH_2), 1.30 (t, 3H, Me of C7-Et), 0.87 (t, 3H, Me at C18).

(*b*) IDN6083. To a solution of intermediate II (0.50 g, 0.6 mmol) in acetonitrile (20 mL), 0.16 g Pd/C 5% and succinic acid (0.14 g, 1.2 mmol) were added. The mixture was hydrogenated for 3 hours (4 atm of H_2). After the mixture was filtered, the solvent was removed under reduce pressure to give the desired product.

^{1}H-NMR (DMSO-d_6/D_2O): δ 8.19 (d, 1H, J = 8.5 Hz, arom.), 8.09 (d, 1H, J = 8.5 Hz, arom.), 7.82 to 7.77 (m, 1H, arom.), 7.69 to 7.64 (m, 1H, arom.), 7.45 (s, 1H, arom.), 5.18 (s, 2H, CH_2N), 3.10 to 3.2 (m, 4H, CH_2 of C7-Et and CH_2-NHC=O), 2.80 to 2.70 (m, 6H, 3 CH_2), 2.28 (s, 4H, 2 CH_2 of succinic acid), 2.23 (s, 3H, Me), 2.30 to 2.10 (m, 2H, CH_2 of C20-Et), 1.70 to 1.50 (m, 6H, 3 CH_2), 1.27 (t, 3H, Me of C7-Et), 0.87 (t, 3H, Me at C18).

^{13}C-NMR (DMSO): δ 175.8 (C of succinic acid), 173.7 (C), 161.8 (C), 153.4 (C), 152.1 (C), 149.2 (C), 146.0 (C), 142.1 (C), 130.5 (CH), 128.4 (CH), 128.0 (C), 127.8 (2 CH), 127.0 (C), 124.7 (CH), 99.9 (CH), 78.8 (C), 50.0 (CH_2N), 48.0 (CH_2), 46.3 (CH_2), 38.8 (CH_2) 36.6 (CH_2), 32.1 (CH_2), 31.5 (CH_2), 26.8 (CH_2), 24.8 (CH_2), 23.7 (CH_2), 22.9 (CH_2), 20.3 (Me), 14.6 (Me), 8.6 (Me).

Growth Inhibition Assay

Human non–small cell lung cancer NCI-H460 cells were cultured in RPMI 1640 containing 10% FCS. Cell sensitivity was assessed by growth inhibition assay after 1 or 72 hours of drug exposure. Cells in the logarithmic phase of growth were seeded in duplicates into six-well plates. Twenty-four hours later, cells were exposed to the drug and counted with a Coulter counter 72 hours after the beginning of drug exposure. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.

Topoisomerase I–Dependent DNA Cleavage Assay

A gel-purified 751-bp *Bam*HI-*Eco*RI fragment of SV40 DNA was used for the cleavage assay (22). DNA fragments were uniquely 3′-end labeled. Topoisomerase I-DNA cleavage reactions (20,000 cpm per sample) were done in 20 μL of 10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L KCl, 5 mmol/L $MgCl_2$, 15 μg/mL bovine serum albumin, 0.1 mmol/L DTT, and the human recombinant enzyme (full-length topoisomerase I) for 30 minutes at 37°C. Reactions were stopped using 0.5% SDS and 0.3 mg/mL of proteinase K for 45 minutes at 42°C. Persistence of DNA cleavage at different time points was examined by adding 0.6 mol/L NaCl after 30 minutes of incubation with 10 μmol/L drug. After precipitation, DNA was resuspended in denaturing buffer (80% formamide, 10 mmol/L NaOH, 0.01 mol/L EDTA, and 1 mg/mL dyes) before loading on a denaturing 7% polyacrylamide gel in Tris-borate EDTA buffer. Overall, DNA cleavage levels were measured with a PhosphoImager 425 model (Molecular Dynamics, Sunnyvale, CA; ref. 23). HPLC analysis was used to evaluate the amount of IDN5174 and total camptothecin (carboxylate + lactone forms) after DNA cleavage. Topoisomerase I-DNA cleavage reactions were done with 50 μmol/L IDN5174 as described, using unlabeled gel-purified 751-bp DNA fragment. Reactions were stopped by adding acetonitrile. Then, an aliquot of 50 μL was added with 10 μL of 0.6 N trichloroacetic acid and 50 μL of PBS and analyzed by HPLC (see below).

Antitumor Activity Studies

All the antitumor activity and pharmacokinetic studies were carried out using female athymic Swiss nude mice, 8 to 10 weeks old (Charles River,

Calco, Italy). Mice were kept in laminar flow rooms at constant temperature and humidity with free access to food and water. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori, Milan, according to the U.K. Coordinating Committee on Cancer Research Guidelines (24).

The human lung carcinoma NCI-H460 was maintained *in vivo* by serial s.c. passages of tumor fragments (about 2 × 2 × 6 mm) in healthy mice, as described previously (25).

For antitumor activity studies, the experimental groups included four to five mice bearing bilateral s.c. tumors. Tumor fragments were implanted on day 0, and tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor weight was calculated according to the formula: tumor weight (mg) = tumor volume (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameter, respectively. Treatment started at day 2, when tumor weight was around 50 mg. All the compounds were dissolved in sterile, distilled water. Topotecan and irinotecan were delivered i.v. at the dose of 15 and 50 mg/kg, respectively. IDN5174 was delivered i.v. at the dose of 30 mg/kg split in two consecutive injections (15 + 15 mg/kg), according to a every 4th day schedule (q4d) for four times. For oral (p.o.) delivery, IDN5174 was given at the dose of 40 mg/kg, q4d for 12 times.

The efficacy of drug treatment was assessed as (*i*) percent tumor weight inhibition (TWI%) in drug-treated versus control mice expressed as TWI% = 100 − (mean tumor weight treated / mean tumor weight control × 100) and (*ii*) $\log_{10}$ cell kill (LCK) calculated by the formula LCK = $(T - C)/3.32 \times$ DT, where T and C are the mean time (days) required for treated (T) and control (C) tumors, respectively, to reach the same weight (1.0 g), and DT is the doubling time of control tumors. Tumor growth curves were obtained by plotting tumor weights versus time in treated and control groups.

The toxicity of drug treatment was assessed as (*i*) body weight loss percent during treatment and (*ii*) lethal toxicity (i.e., any death in treated groups occurring before any control death).

For statistical analysis, tumor weight was compared in treated versus control mice by the unpaired Student's t test (two tailed).

Pharmacokinetic Studies

To assess the pharmacokinetic profile of IDN5174, the compound was given i.v. at the dose of 15 mg/kg in mice bearing the NCI-H460 tumor xenograft. Four mice were sacrificed at different time points after treatment (5, 15, 30, and 45 minutes and 1, 2, 4, 6, and 10 hours), to collect blood and tumor samples. The plasma fraction was immediately separated by centrifugation (3,000 rpm, 15 minutes, 4°C) and stored at −20°C until analysis. Tumors were weighed and stored.

IDN5174 and total camptothecin (carboxylate + lactone forms) concentrations were determined by HPLC. Fifty microliters of plasma samples were added with 10 ng of irinotecan (as internal standard), 10 μL of 0.6 N trichloracetic acid, and 50 μL of acetonitrile. Tumor samples were homogenized in 1 mL of acetonitrile, added with 50 ng of irinotecan and 100 μL trichloracetic acid, and extracted with 4 mL acetonitrile. For both plasma and tumor, after vortex mixing for 10 seconds, the samples were centrifuged at 12,000 rpm for 10 minutes. A volume of 10 μL of the supernatant was injected into the HPLC instrument for quantitative analysis. A calibration curve was prepared in plasma in the range 2.5 to 500 ng/mL and in tumor in the range of 5 to 500 μg/g, for both IDN5174 and camptothecin, respectively. HPLC separation was carried out on Luna Phenyl-Hexyl column (5 μm, 150 × 4.6 mm; Phenomenex, Torrence, CA) and using a mobile phase constituted of formic acid and acetonitrile (CH_3CN) under gradient conditions. The flow rate was 1 mL/min, and peaks were detected by fluorescence detector (λ_{ex} = 370, λ_{em} = 510). The retention time of IDN5174, irinotecan, and camptothecin were of 10, 13, and 15 minutes, respectively. The limits of quantification for both the analyses were 2.5 and 10 ng/mL for plasma and tumor, respectively.

The experimental area under the curve (AUC) of the concentration versus time points was calculated by the linear trapezoidal rule extrapolated to infinite by the K_e. C_{max} values were obtained from experimental data. Clearance (Cl), volume of distribution (V_d), and half-life ($T_{1/2}$) were calculated by using Win Nonlin Pro Node 4.1 pharmacokinetic software (Pharsight Co., Mountain View, CA), according to the following relation: Cl = dose/AUC, V_d = Cl/K_e, $T_{1/2}$ = 0.693/K_e, where K_e is the constant of elimination of the drug obtained by the fitting.

Results

Growth inhibition studies. The polyamine camptothecin analogues (Fig. 1) were tested for growth inhibition ability against the human non–small cell lung cancer carcinoma NCI-H460 cell line (Table 1), which expresses high level of topoisomerase I (26). Topotecan and SN38, the active metabolite of irinotecan, were used as reference drugs. With one exception (IDN6083, which lacks the possibility to have a free hydroxyl group at the 17-position), all the compounds efficiently inhibited the proliferation of NCI-H460 cells after 1 hour of drug exposure. The reduced potency of IDN5174 is likely related to its formulation as water-soluble salt because the free charged amino groups could limit drug uptake. Although camptothecin derivatives were less potent than topotecan and SN38 after 1 hour of treatment, the antiproliferative effects increased after 72 hours of exposure. Indeed, during long-term exposure, growth inhibition potency of IDN5174 and IDN6080 was found similar to that of topotecan.

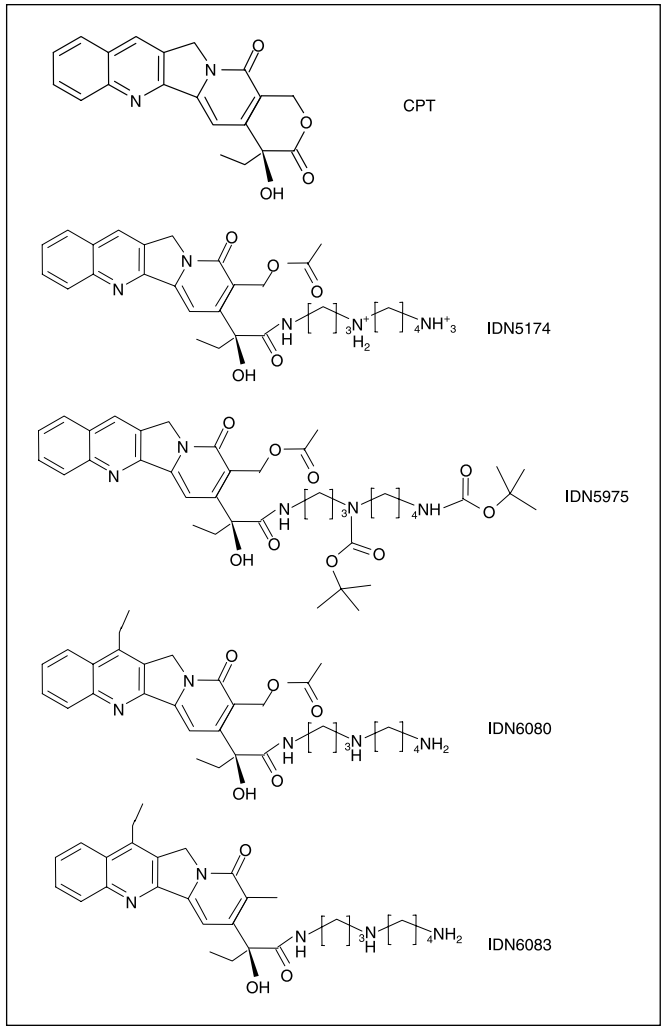

Figure 1. Chemical structures of camptothecin (*CPT*), IDN5174, IDN5975, IDN6080, and IDN6083.

Table 1. Sensitivity of NCI-H460 cells to camptothecin derivatives

	IC_{50} (μM)	
	1 h	72 h
Topotecan	1.18 ± 0.24	0.015 ± 0.0027
SN38	0.22 ± 0.013*	0.012 ± 0.001
IDN5174	3.66 ± 0.34*,†	0.048 ± 0.0075 *,†
IDN5975	1.12 ± 0.144 †	0.26 ± 0.0077 *,†
IDN6080	1.54 ± 0.01*,†	0.039 ± 0.0088 *,†
IDN6083	34.5 ± 14*,†	0.89 ± 0.132 *,†

NOTE: Sensitivity was assessed by growth inhibition assay after 1 or 72 hours of drug exposure. Cells were counted 72 hours after drug exposure for 1 hour of treatment and at the end of drug exposure in the case of 72 hours of treatment. The reported values are the mean ± SD of three independent experiments.
*P < 0.05 versus topotecan (ANOVA).
†P < 0.05 versus SN38 (ANOVA).

An analysis of the possible growth inhibition of spermidine indicated that the polyamine per se (0.1-10 μmol/L) did not exhibit any antiproliferative effect after 72 hours of exposure. Growth inhibition ability of IDN5174 was not affected by long-term preincubation with spermidine (data not shown).

Topoisomerase I–mediated DNA cleavage. Topoisomerase I–mediated DNA cleavage assay was used to investigate the ability of the compounds to stimulate the DNA damage. Purified human topoisomerase I was used with SN38 as reference compound (Fig. 2*A*). IDN5174 and IDN6080 revealed an intensity of DNA cleavage only slightly inferior to SN38, whereas IDN6083 and IDN5975 were substantially less efficient. The reduction was more evident for IDN5975, which exhibited low activity even at the highest concentration. The cleavage pattern was found identical to that of SN38 for all the camptothecins. The stabilization of the ternary cleavable complex was also evaluated after the addition of a high salt concentration (0.6 mol/L NaCl), which favors the dissociation of the ternary drug-enzyme-DNA complex. As shown in Fig. 2*B*, the compounds revealed a DNA damage persistence similar to that of topotecan and SN38. The stability of the polyamine-camptothecin conjugate IDN5174 during the cleavage reaction was examined by HPLC analysis. During the cleavage assay (30 minutes of incubation at 37°C), IDN5174 (50 μmol/L) was found unchanged, both in the presence and absence of topoisomerase I with no evidence of formation of free camptothecin.

***In vivo* antitumor activity studies.** IDN5174 was selected for *in vivo* studies as a representative compound of the novel series. The antitumor efficacy of IDN5174 was investigated in mice bearing s.c. growing NCI-H460 tumor, using both i.v. and p.o. administration (Table 2; Fig. 3). Topotecan and irinotecan were chosen as reference drugs. When delivered i.v., at the maximum tolerated dose 15 + 15 mg/kg, q4d for 4 times, IDN5174 achieved a TWI% and LCK comparable with those of topotecan and irinotecan. Moreover, a better antitumor activity was reached after p.o. delivery of 40 mg/kg (q4dx12 times), a substantial increase of the LCK value was observed without any evidence of toxicity (Fig. 3*B*).

Pharmacokinetic studies. Figure 4 shows the plasma (*A*) and the tumor (*B*) levels of IDN5174 in mice treated i.v. with 15 mg/kg of the compound. The main derived pharmacokinetic variables are listed in Table 3. IDN5174 achieved a C_{max} of 21.7 μg/mL; it was rapidly distributed and cleared from plasma with a Cl and an elimination $T_{1/2}$ of 1,106 mL/h/kg and 2.2 hours, respectively. In plasma, we found the formation of total camptothecin at all the investigated time points. AUC_{exp} of camptothecin was ~30 times lower than that of IDN5174. In the tumor, both IDN5174 and camptothecin were quantifiable up to 10 hours, and the ratio between the AUC_{exp} of IDN5174 and camptothecin was about 10.

Overall, the pharmacokinetic profile indicated that the compound achieved and maintained a favorable distribution in the tumor, being its C_{max} = 3.4 μg/g. Moreover, during distribution and elimination phases, the i.t. concentrations resulted 2 to 10 times higher than those in plasma.

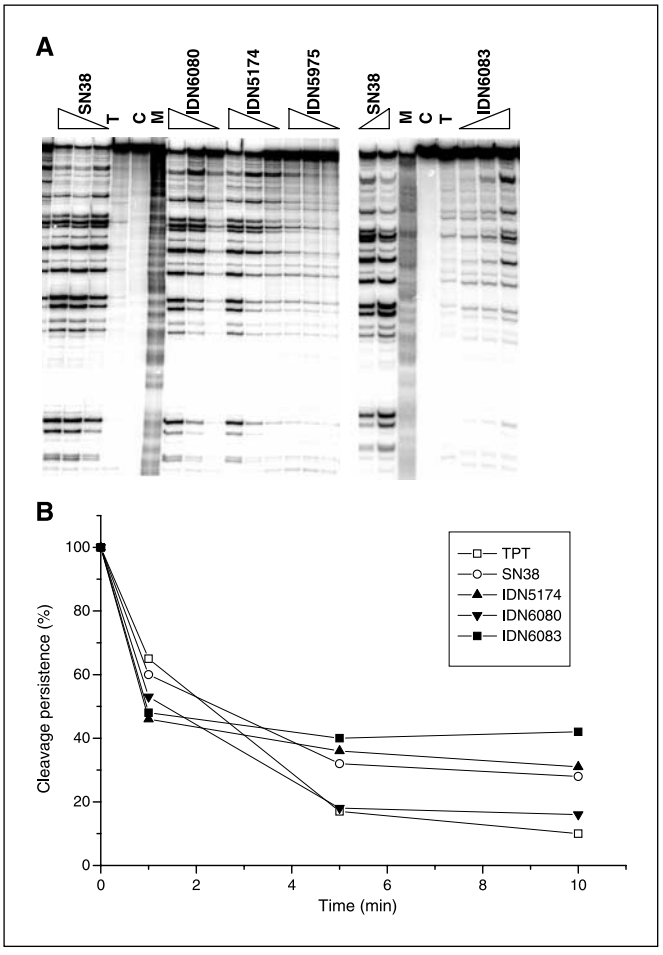

Figure 2. *A,* topoisomerase I–mediated DNA cleavage by SN38, IDN5174, IDN5975, IDN6080, and IDN6083. Samples were reacted with 1, 10, and 50 μmol/L drug at 37°C for 30 minutes. Reaction was then stopped by adding 1% SDS and 0.3 mg/mL proteinase K and incubating for 45 minutes at 42°C before loading on a denaturing 8% polyacrylamide gel. *C,* control DNA; *T,* reaction without drug; *M,* purine markers. *B,* persistence of topoisomerase I–mediated DNA cleavage in the presence of different compounds. The samples were reacted for 30 minutes with 10 μmol/L drug. DNA cleavage was then reversed by adding 0.6 mol/L NaCl. The 100% value is referred to the extent of DNA cleavage at 30 minutes of incubation.

Table 2. Antitumor effects of IDN5174 (q4d) against NCI-H460 tumor xenograft

Drug	Route	No. treatment*	Dose (mg/kg)	TWI% † (d)	LCK ‡	BWL%§	Toxicity‖
TPT	i.v.	4	15	91 (20)	2	10	0/5
Irinotecan	i.v.	4	50	91 (20)	1.8	3	0/5
IDN5174	i.v.	4	10 + 10	73 (20)	1.2	5	0/5
	i.v.	4	15 + 15	89 (20)	1.8	3	1/5
	i.v.	4	20	ND	ND	ND	5/5
	p.o.	12	40	85 (30)	3.3	0	0/5

Abbreviations: BWL%, body weight loss percent; ND, not done.
*Treatments started at day 2, with tumor weight about 50 mg.
†TWI% in treated over control mice. In parentheses, day of evaluation. All treated groups significantly differed ($P < 0.005$) from control tumors.
‡Gross LCK to reach 1 g of tumor.
§Maximal body weight loss percent of the group during treatment period.
‖Number of dead mice/number of treated mice.

Discussion

Opening of the lactone ring of camptothecin is known to cause partial drug inactivation. However, the open drug form still retains toxic properties. A number of chemical modifications and drug delivery approaches have been reported in an attempt to stabilize the lactone ring and to improve the therapeutic index of the drug (27, 28). An alternative approach to overcome the drawbacks of the camptothecins is the design of E ring–modified analogues characterized by activity independent of the closed lactone.

The present study indicates that the introduction of a suitable substituent in the open carboxylate form results in a molecule active at the cellular target and effective as antitumor agent. The water-soluble camptothecins containing a spermidine moiety linked as amide to the carboxylate function of the opened E ring exhibited antiproliferative activity and maintained the ability to stabilize the topoisomerase I-DNA cleavable complex. A growth inhibition potency, similar to that of topotecan and SN38, was recovered after long-term exposure, and *in vivo* studies indicated that IDN5174 exhibits antitumor activity similar to that of topotecan and

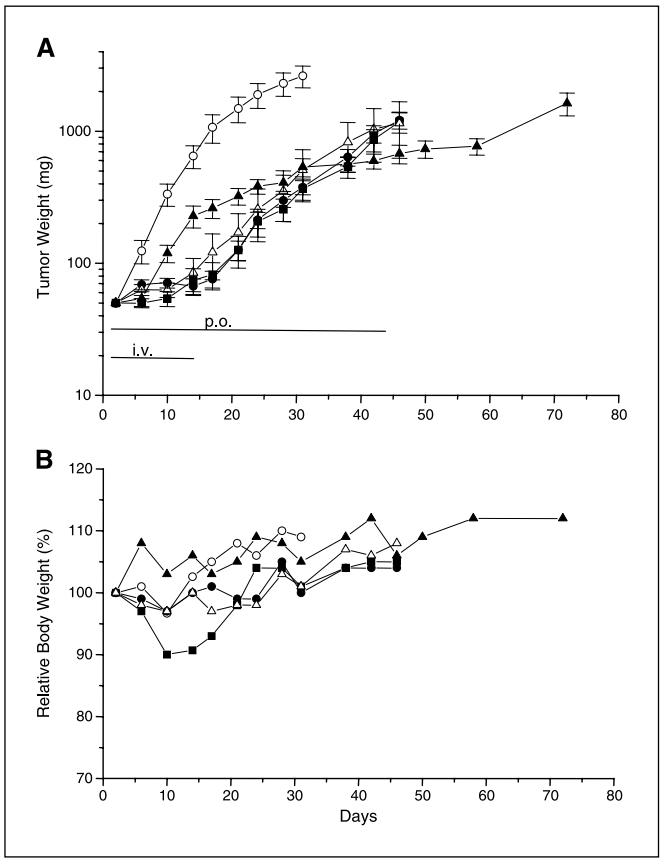

Figure 3. *A,* antitumor activity of camptothecins against NCI-H460 tumor xenograft. Mice were treated i.v., q4dx4, with solvent (○), 15 mg/kg topotecan (■), 50 mg/kg irinotecan (●), 15 + 15 mg/kg IDN5174 (△) or p.o., q4dx12, with 40 mg/kg IDN5174 (▲). *Points,* mean value of 8 to 10 tumors; *bars,* SE. *B,* changes in percent in mice weight during treatment. Symbols as in (*A*). *Points,* mean from five mice per group.

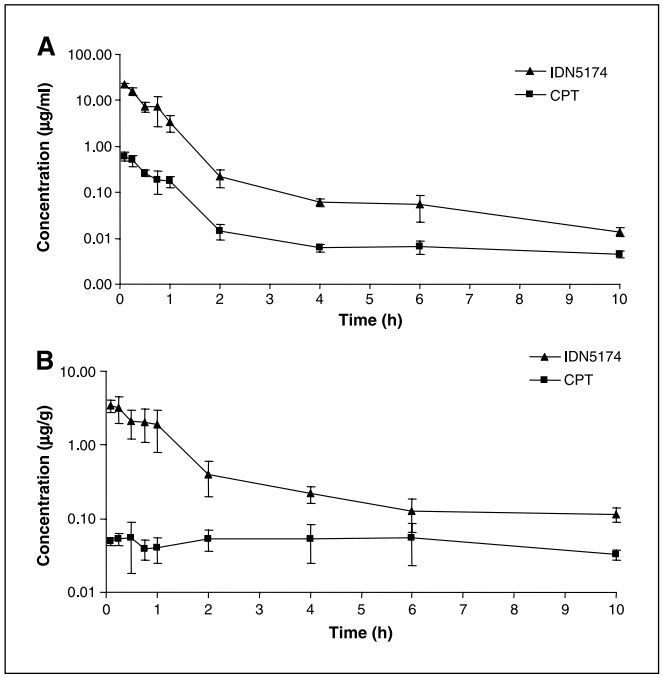

Figure 4. Levels of IDN5174 and camptothecin after i.v. administration of 15 mg/kg IDN5174 in female nude mice bearing NCI-H460 tumor. *A,* plasma levels. *B,* tumor levels.

Table 3. Pharmacokinetic variables of IDN5174 and camptothecin in nude mice bearing NCI-H460 tumor after i.v. administration of 15 mg/kg IDN5174

Drug	C_{max}*	AUC_{exp} †	AUC_{inf} † (μg/mL h)	$T_{1/2}$ (h)	Cl (mL/h/kg)	V_d (mL/kg)
Plasma						
IDN5174	21.70 μg/mL	13.52 μg/mL h	13.56	2.2	1,106	3,471
Camptothecin	0.61 μg/mL	0.50 μg/mL h	0.53	5.6		
Tumor						
IDN5174	3.36 μg/g	4.96 μg/g h				
Camptothecin	0.05 μg/g	0.48 μg/g h				

*C_{max} = maximum plasma concentration.
†AUC, experimental AUC of the concentration versus time points.

irinotecan. Stabilization of the cleavable complex was not the result of the hydrolysis of the N-C bond between polyamine and the drug because no free camptothecin was recovered after DNA cleavage assay. This finding is also supported by pharmacokinetic studies in mice, which revealed that only low amount of camptothecin was detected in plasma and tumor, thus indicating the efficacy of camptothecins in the ring-opened form as topoisomerase I poisons. This conclusion is also supported by an appreciable ability of IDN6083 to induce the DNA damage observed in cleavage assay. This analogue lacks the hydroxyl group at the 17-position; therefore, the formation of the closed lactone ring and the intramolecular interactions are impossible even following release of the polyamine. The reduced ability of IDN6083 to poison topoisomerase I compared with other analogues of the series likely reflects the loss of the 17-hydroxyl group. Indeed, the crystal structure has revealed that a hydrogen bond is formed through a water molecule, between Asn^{722} of the enzyme and 17-OH of the drug (6). Our results, documenting the ability of camptothecins with open E ring to inhibit topoisomerase I, are somewhat surprising because it was generally believed that only the lactone form of camptothecins is active. On the other hand, crystal structure studies have revealed that both the closed lactone ring and the open carboxylate topotecan can bind within the same intercalation pocket (6) and, in spite of what believed for long time, support a potential activity of the opened ring form. A plausible explanation for the recognized inactivity of the open carboxylate form of the conventional camptothecins is a more favorable accommodation for the closed lactone form in the binary topoisomerase I-DNA complex. Thus, it is likely that the polyamine linked at the 21-position confers a specific recognition or favorable interactions with DNA in the ternary complex, which could compensate the loss of interactions of the closed lactone. This interpretation is consistent with the observation that the *t*-butoxycarbonyl (Boc)–substituted analogue IDN5975 was less effective as topoisomerase I poison than the unsubstituted polyamine analogues IDN5174 and IDN6080. Indeed, the bulky Boc substituents may sterically hinder optimal DNA interaction. The marked ability of IDN5975 to inhibit cell growth after 1 hour of exposure could reflect its lipophilic nature likely allowing a faster/easier intracellular accumulation compared with other hydrophilic analogues of this series. This interpretation is also supported by a reduced growth inhibitory effect of IDN5975 in comparison with other hydrophilic camptothecin analogues of this series (e.g., IDN5174).

Some chemical modifications of camptothecins, which shift the carboxylate/lactone equilibrium, support a critical role of the opened form in the mechanism of action of camptothecin. Indeed, the sodium salt carboxylate form of camptothecin induces accumulation of topoisomerase I-DNA covalent adducts (8, 29, 30), whereas camptothecin analogues containing a lactam E ring, which shifts the equilibrium in favor of the closed E ring form, are not active (8). Among a series of lactone-free camptothecin bearing a five-membered E ring, the activity was markedly dependent on the position of the carbonyl function (31). Thus, specific interactions in the intercalation pocket of the DNA-enzyme complex are likely involved as critical determinants of the stabilization of the cleavable complex. Again, this observation supports our interpretation that the closed E ring could be replaced by an alternative pharmacophore able to provide critical interactions. Analogues obtained by opening of the lactone ring to form water-soluble amide derivatives have been described as prodrugs of camptothecin (32). However, the topoisomerase I inhibition *in vitro* and the stability and antitumor efficacy *in vivo* of such analogues were not reported. Our study indicating the IDN5174 was effective per se as topoisomerase I inhibitor, and the evidence that a substantial amount of *in vivo* given IDN5174 is still present as unchanged drug strongly supports that the *in vivo* efficacy reflects the activity of IDN5174 itself, although a partial contribution of released camptothecin could not be ruled out.

In conclusion, the introduction of a polyamine residue at the carboxylic function of the open E ring of camptothecin resulted in novel water soluble effective analogues. Our data indicate that IDN5174 maintained the biological and antitumor properties of the established camptothecins. Although free camptothecin was detectable in IDN5174-treated animals (around 10%), this amount did not account for the observed efficacy. Thus, IDN5174 could represent a prototypical compound of a new series of lactone opened camptothecins. To our knowledge, this analogue is the first example of an effective camptothecin with open lactone ring. Our finding provides a promising approach to design a novel series of camptothecins as useful tools to better understand the drug interaction at the level of cellular target.

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