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Novel Stable Camptothecin Derivatives Replacing the E-Ring Lactone by a Ketone Function Are Potent Inhibitors of Topoisomerase I and Promising Antitumor Drugs

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ABSTRACT

The E-ring lactone is the Achilles' heel of camptothecin derivatives: although it is considered necessary for the inhibition of the enzyme topoisomerase I (topo1), the opening of the lactone into a carboxylate abolishes the generation of topo1-mediated DNA breaks. S38809 is a novel camptothecin analog with a stable 5-membered E-ring ketone; therefore, it lacks the lactone function. DNA relaxation and cleavage assays revealed that S38809 functions as a typical topo1 poison by stimulating DNA cleavage at T ↓ G sites. The activity was strongly dependent on the stereochemistry of the C-7 carbon atom that bears the hydroxy group. S38809 proved to be a potent cytotoxic agent, with a mean IC_{50} of 5.4 nM versus 11.6 nM for topotecan and 3.3 nM for SN38 (the active metabolite of irinotecan) on a panel of 31 human tumor cell lines. The cytotoxicity of S38809 and its ability to stabilize cleavable complexes was considerably reduced in camptothecin-resistant cells that express a mutated topo1, confirming that topo1 is its primary target. Cell death induced by topo1 poisoning requires the conversion of DNA single-strand breaks into double-strand breaks that can be detected by the formation of phosphorylated histone H2AX. In HCT116 cells, topotecan, SN38, and S38809 induced histone H2AX phosphorylation in S phase of the cell cycle, with S38809 being as potent as SN38 and 5-fold more potent than topotecan. In vivo, S38809 showed a marked antitumor activity against HCT116 xenografts. These findings open a new route for improving the pharmacological properties of camptothecin derivatives.

Inhibition of human topoisomerase I (topo1) represents the primary mechanism of action for a series of small molecules derived from the plant alkaloid camptothecin (CPT; Fig. 1) (Wall et al., 1966; Hsiang et al., 1985; Pommier, 2006). Among these, topotecan (TPT) and irinotecan are used in cancer chemotherapy. CPT derivatives exhibit no significant affinity for purified DNA or topo1 alone but bind tightly to the topo1-DNA complex stabilizing a covalent intermediate through a phosphotyrosyl linkage. The high selectivity of CPT derivatives for topo1 is due to the targeting of the protein-DNA interface of the topo1-DNA complex (Pommier, 2005). At the cellular level, the poisoning of topo1 causes single-strand breaks in DNA that are converted into double-strand breaks (DSBs), which are the lethal DNA lesion (Pourquier et al., 2001).

The indolizinoquinoline moiety of CPT (A–D rings, Fig. 1) provides the necessary framework for DNA interaction, whereas the lactone E-ring interacts essentially with the enzyme through Arg364 and Asp533 residues of human topo1 (Fan et al., 1998; Redinbo et al., 1998; Kerrigan et al., 2001). For this reason, the lactone E-ring was considered to

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ABBREVIATIONS: topo1, topoisomerase I; CPT, camptothecin; DSB, double-strand break; hCPT, homocamptothecin; PAEC, primary aortic endothelial cell; SN38, 7-ethyl-10-hydroxycamptothecin; TPT, topotecan; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TBE, Tris borate-EDTA; PI, propidium iodide; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)-fluoromethyl ketone; S36272, five-membered E-ring ketone methylene dioxy derivative of CPT.

be the key element of all CPT derivatives. The pH-dependent opening of the lactone leading to a carboxylate derivative abolishes the capacity of the drug to stimulate topo1-mediated DNA breaks. The lactone ↔ carboxylate interconversion of CPT is reversible with an equilibrium shifted toward the inactive open form in human plasma, due to both plasma pH and the preferential binding of the carboxylate form to human serum albumin (Burke et al., 1993). The conversion of the open form into the closed lactone form at the acidic pH of the urinary tract was considered to be responsible for the severe hemorrhagic cystitis observed in the early clinical trials of CPT. For these reasons, different strategies have been developed to design CPT analogs with a stabilized lactone E-ring.

The structural modifications of CPT skeleton, grouped into two categories, aimed at reinforcing the lactone ring and/or at reducing the binding of the carboxylate form to serum albumin. The first includes the introduction of a side chain on the indolizinoquinoline moiety. For example, the 9-dimethylaminomethyl group of topotecan decreases the protein binding of the carboxylate to human serum albumin (Burke et al., 1993, 2000), thus increasing the concentration of the active lactone form. The second includes modification of the lactone E-ring to decrease its rate of hydrolysis. The modification of the E-ring α-hydroxy lactone function always resulted in less active compounds. Early work (Hertzberg et al., 1989) had shown that the removal of the 20-OH group on the E-ring lactone or the conversion of the lactone into a lactam was detrimental to the inhibition of topo1. Likewise, E-ring-modified CPT analogs containing a lactol, an α-halo, α-azido-, α-amino, or an α-exomethylene-lactone were found to be poorly active if not inactive (Bailly 2003). These observations led to the dogma that the E-ring lactone of CPT is necessary to preserve the anti-topo1 activity. In 1997, this dogma was challenged by the discovery of homocamptothecins (hCPT), a novel family of potent CPT analogs with a more stable seven-membered lactone obtained by incorporating a methylene unit between the C=O and the carbon atom at the 20-position that bears the hydroxy function. The hCPT derivatives revealed potent activities against topo1 with promising cytotoxic and antitumor effects (Lavergne et al., 1998; Lansiaux et al., 2003). One of these derivatives, difluoro diflomotecan, is undergoing phase II clinical trials (Bonneterre et al., 2000; Osheroff, 2004; Scott et al., 2007).

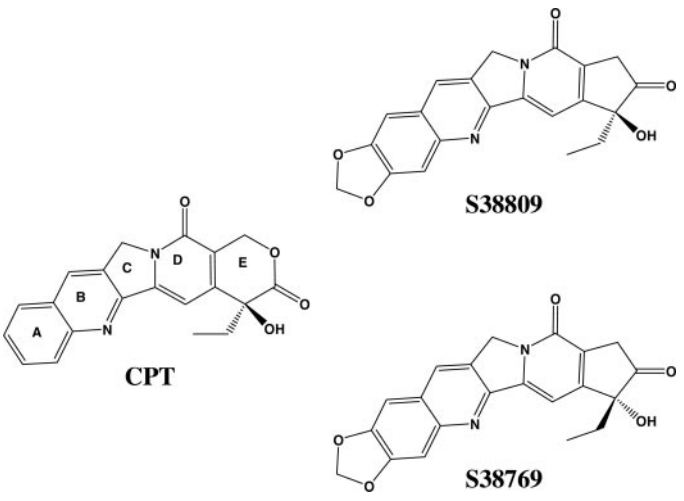

Fig. 1. Structure of camptothecin and the nonlactone camptothecin series with enantiomers S38809 (*S*) and S38769 (*R*).

If topo1 inhibition is preserved with a larger seven-membered E-ring, what happens when the E-ring is reduced and when there is no lactone function ? We recently addressed these questions through the design of a novel series of modified CPT analogs incorporating a five-membered E-ring ketone. The synthesis and preliminary biological evaluation of different A-ring substituted congeners were reported recently (Hautefaye et al., 2003), and the methylenedioxy derivative S36272, synthesized as a racemate, was identified as one of the most potent inhibitors of topo1 in this series.

The aim of this study was to determine whether an E-ring ketone derivative such as S36272 functions as a typical topo1 inhibitor, in a manner similar to topotecan and SN38 (irinotecan active metabolite). Because the activity of CPT derivatives is strongly dependent on the stereochemistry at C-20, we synthesized the two enantiomers of S36272 and showed that only the *S* enantiomer S38809 is active (Fig. 1). DNA relaxation and cleavage assays revealed that S38809 stabilized covalent enzyme-DNA complexes. The tight relationship between topo1 poisoning, formation of cleavable complex in cells, and induction of apoptosis demonstrates that topo1 is the main intracellular target of S38809. In vivo, S38809 showed a marked antitumor activity against HCT116 xenografts.

Materials and Methods

Drugs. CPT was purchased from Sigma Chemicals (St. Louis, MO), SN38 was from Sanofi-Aventis (Paris, France), and TPT was from GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). The racemate S36272 was synthesized as described previously (Hautefaye et al., 2003). The asymmetric synthesis of the two enantiomers S38769 (*R*) and S38809 (*S*) was performed using chiral intermediates and will be published elsewhere. The enantiomeric excess was >98% as measured by analytical chiral chromatography on a Chiralpak AS column in methanol (flow rate: 1 ml/min). The drugs were dissolved in dimethyl sulfoxide at 5 mM (except for TPT, which was dissolved in water) and kept at −20°C.

DNA Relaxation. Experiments were performed as described previously (Bailly, 2001). Plasmid pLAZ DNA (130 ng) was incubated with 4 U of human topo1 (TopoGen, Columbus, OH) at 37°C for 45 min in relaxation buffer (10 mM Tris, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1% bovine serum albumin, 0.1 mM spermidine, and 5% glycerol) in the presence of the tested compounds. Reactions were terminated by adding 0.25% SDS and 250 μg/ml proteinase K. DNA samples were then electrophoresed on a 1% agarose gel containing 1 μg/ml ethidium bromide. Gels were washed and photographed under UV light.

Purification and Radiolabeling of DNA Restriction Fragments. Plasmids were isolated from *Escherichia coli* by NaOH-SDS lysis and purified by banding in CsCl-EB gradients. The 117-base pair DNA fragment was prepared by 3′-^{32}P-end labeling at the EcoRI site of the PvuII and EcoRI double-digest of the plasmid pBS using [α-^{32}P]dATP (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and avian myeloblastosis virus reverse transcriptase (Roche, Neuilly/Seine, France). The labeled digestion products were separated on a 6% (w/v) polyacrylamide gel under nondenaturing conditions in TBE buffer (89 mM Tris-borate, pH 8.3, and 1 mM EDTA) to remove excess radioactive nucleotide. After autoradiography, the DNA was excised and eluted overnight in 400 μl of 10 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA. This suspension was filtered through a Millipore 0.22-μm filter, and the DNA was precipitated with 1 ml of ice-cold ethanol. After washing with 70% ethanol and

vacuum-drying of the precipitate, the 3′-end-labeled DNA was resuspended in 10 mM Tris and 10 mM NaCl, pH 7.0.

Sequencing of topo1-Mediated DNA Cleavage Sites. Each reaction mixture contained 200 cpm of 3′-end ^{32}P-labeled DNA, the tested compound, at the indicated concentration in topo1 relaxation buffer. After a 10-min incubation, the reaction was initiated by the addition of 4 U of human topo1. Samples were incubated for 20 min at 37°C, and the reaction was terminated by adding SDS and proteinase K. The DNA was precipitated with ethanol and then dissolved in 5 μl of formamide-TBE loading buffer, denatured at 90°C for 4 min, and then chilled in ice for 4 min before loading onto the sequencing gel. DNA cleavage products were resolved by electrophoresis on an 8% polyacrylamide gel in TBE buffer containing 8 M urea. Gels were then soaked in 10% acetic acid, transferred to Whatman 3M paper (Whatman, Clifton, NJ), and dried under vacuum at 80°C. A 425E PhosphorImager (GE Healthcare) was used to collect signals from the storage screens exposed to dried gels overnight at room temperature. Baseline-corrected scans were analyzed by integrating all of the densities between two selected boundaries using ImageQuant version 3.3 software (GE Healthcare). Each resolved band was assigned to a particular band within the DNA fragment by comparison of its position relative to sequencing standards (G-track) generated by treatment of the DNA with dimethyl sulfate followed by piperidine-induced cleavage at the modified guanine residues.

Cells. A panel of 31 human tumor cell lines was used, provided by American Type Culture Collection (Manassas, VA) and are listed in Fig. 4. The P388CPT5 resistant murine leukemia cell line (Madelaine et al., 1993) was provided by Dr. Riou (Reims, France). A primary culture of porcine aortic endothelial cells (PAEC) was used to investigate the effect of the drugs on rapidly proliferating normal cells (doubling time of 17 h). The PAEC cells were collected and cultured as described previously (Fournet-Bourguignon et al., 2000). All of the cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 or Dulbecco's modified Eagle's medium media supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Immunoblot Assay of topo1-DNA Complexes in Cells. The in vivo topo1 link kit of TopoGEN was used. Here, 1×10^7 exponentially growing cells were exposed to the drug at 5 μM for 1 h at 37°C and then incubated in drug-free medium for further 3 h as indicated. Cells were pelleted by centrifugation and resuspended in 0.8 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% sarkosyl). The lysed cell mixture was then overlaid onto a CsCl density gradient containing four different density steps (0.8 ml of CsCl at 1.82, 1.72, 1.50, and 1.37 g/ml) and centrifuged at 13,000*g* for 15 h at 25°C. From the top of the gradient, 12 fractions of 330 μl were collected. The DNA content in fraction was estimated by absorbance measurement at 260 nm. For the immunoblot analysis, 50 μl of each fraction was diluted with 100 μl of 25 mM PBS, pH 6.5, and applied onto Hybond-C nitrocellulose membranes (Amersham) fitted on the vacuum slot-blot device (Life Science, Cergy-Pontoise, France). The membranes were washed with PBS and then soaked for 2 h in Tris-buffered saline/Tween 20 buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, and 1% bovine serum albumin) supplemented with 5% nonfat dried milk. The membranes were washed three times with Tris-buffered saline/Tween 20 buffer before 1-h incubation at room temperature with a rabbit polyclonal anti-topo1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed and incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Life Sciences) for 30 min. The Western blot chemiluminescence reagent from PerkinElmer Life and Analytical Sciences (Waltham, MA) was used for the detection, and bands were visualized by autoradiography. Scans were analyzed by integrating all of the densities using ImageQuant version 3.3 software. The amount of cleavable complexes were estimated by summing the densities of fractions 6 to 12. Control experiments were routinely performed to check the specificity of the anti topo1 antibody.

Standard Proliferation Assay. This assay has been described previously (Léonce et al., 1996). Adherent cells were seeded in 96-well microplates and incubated for 2 days. Tested compounds were added, and plates were incubated for four doubling times. At the end of this period, 15 μl of 5 mg/ml 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to each well, and the plates were incubated for 4 h at 37°C. The medium was aspirated, and formazan was solubilized by 100 μl of dimethyl sulfoxide. All of the measurements were performed in triplicate. Results are expressed as IC_{50} and as $\Delta = \text{Log}(IC_{50})$ individual cell line $-$ mean $\text{Log}(IC_{50})$ of all cell lines (Boyd et al., 1995).

Cell Cycle Analysis. HCT116 cells in exponential growth were exposed to the drugs for 24 h, washed with culture medium, harvested, fixed with 70% ethanol, washed, and incubated for 30 min in PBS containing 100 μg/ml RNase and 50 μg/ml propidium iodide (PI). For each sample, 1×10^4 cells were analyzed on a Epics XL/MCL flow cytometer (Beckman Coulter, Roissy, France). PI fluorescence was collected through a 630-nm bandpass filter.

Detection of Apoptosis by Annexin-V Labeling. HCT116 cells were exposed to the drugs for 96 h, rinsed, and labeled with annexin-V-FITC as described previously (Léonce et al., 2001). Cells were resuspended in 200 μl of binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$) containing 10 μl of annexin-V-FITC (BD Pharmingen, San Diego, CA) and 10 μg/ml PI. After 15 min at 20°C in the dark, 800 μl of ice-cold binding buffer was added. Samples were kept at 4°C before flow cytometry analysis. FITC and PI fluorescences were collected through 520 and 630 nm bandpass filters, respectively. Results are expressed as a percentage of annexin-V-positive cells. Caspase-dependent apoptosis was revealed by exposing HCT116 cells to the drugs in the presence of 50 μM Z-VAD-FMK (Sigma), an inhibitor of caspases 1- and 3-like proteases.

Histone H_2AX Phosphorylation. Histone H_2AX phosphorylation was measured by flow cytometry (Léonce et al., 2006). HCT116 cells were exposed to the drugs for 3 h, washed, and fixed by 70% ethanol at -20°C for at least 2 h. Samples were washed with PBS and incubated for 5 min in PBS containing 0.5% Triton X-100 at 0°C. Cells were washed and incubated for 2 h at room temperature with 0.5 μg of anti-phosphohistone H2AX (Ser139) murine monoclonal antibody (Upstate Biotechnology, Charlottesville, VA). Cells were washed and incubated for 1 h with 4 μg of FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed, incubated for 30 min with 100 μg/ml RNase and 10 μg/ml PI, and analyzed by flow cytometry. FITC and PI fluorescence were collected through 520 and 630 nm bandpass filters, respectively. Results are displayed as linear bivariate distribution of phosphorylated histone H2AX (γH2AX) level versus DNA content and expressed as percentages of γH2AX-positive cells.

Antitumor Activity. Female Swiss nude mice, homozygous for the nude gene (nu/nu), were purchased from Charles River Laboratories (Lyon, France). Mice were 6 to 8 weeks old and weighed 20 to 22 g at the start of the experiments, and they received proper care and maintenance in accordance with institutional guidelines. HCT116 tumor fragments of 2 to 3 mm^3 were grafted subcutaneously into the flank of nude mice. When the tumors had reached a mean volume of approximately 50 to 100 mm^3, 13 days after tumor graft, mice were distributed among experimental and control groups of seven mice. S38809 was administered intravenously at 12.5, 25, and 50 mg/kg/injection and TPT at 2 mg/kg/injection. The volume of each tumor was estimated from two-dimensional measurements performed with a slide caliper following the formula length (in millimeters) $\times$ width (square millimeters)/2. Tumor sizes were measured twice a week from day 13 to the end. Tumor volumes in treated mice were compared with those of control animals using Student's *t* test. A one-way analysis of variance analysis was carried out, followed by a Newman-Keuls test for pairwise comparisons. All significance thresholds were fixed at 5%.

Results

Stereoselectivity, Efficiency, and Site Selectivity of topo1 Poisoning by S38809. S36272 is a novel camptothecin derivative having a five-membered E-ring ketone. Its asymmetric C-7 carbon atom, which corresponds to the C-20 carbon atom of CPT, was resolved, leading to the isolation of the two corresponding enantiomers, S38809 (*S*) and S38769 (*R*) (Fig. 1). Two sets of experiments investigated their topo1 inhibitory properties. We first used a conventional DNA relaxation assay with a supercoiled plasmid DNA to measure the formation of single-strand breaks induced by topo1 in the presence of increasing concentrations of the compounds. Supercoiled DNA was fully relaxed by topo1 in the absence of compound (compare lanes DNA and topo1 in Fig. 2A). CPT, used as a positive control, strongly stabilized the cleavable complex as shown by the amplification of the intensity of the band corresponding to the nicked DNA form. The racemic compound S36272 also increased the intensity of the nicked form of DNA. It is interesting that only the enantiomer S38809 converted supercoiled DNA into nicked DNA, showing that the absolute configuration of the carbon 7 was a key determinant for topo1 poisoning (Fig. 2A). Either by visual inspection (Fig. 2A) or quantification of the band intensity (percentage of DNA form II; Fig. *2B*), the racemate S36272 was approximately 3-fold less potent than camptothecin, whereas the active enantiomer S38809 was approximately 1.5-fold less potent than camptothecin but was as potent as SN38 and more potent than topotecan.

Additional DNA cleavage experiments were then performed using a 117-base pair radiolabeled DNA. The cleavage products were analyzed on sequencing polyacrylamide gels to locate the position of cleavage with a nucleotide resolution. S38809 promoted the cleavage of DNA by topo1 in a concentration-dependent manner and at several specific sites identical with those detected with CPT, SN38, and topotecan (Fig. 2C). Four cleavage sites were identified at positions 26, 48, 81, and 107 that correspond to T ↓ G sequences, known as the preferred recognition sequences of CPT (Jaxel et al., 1991; Pommier et al., 1995). Band intensities were proportional to the efficacy of the inhibitor, and it can be seen that S38809 was as potent as topotecan but less potent than SN38. The two compounds S38809 and SN38 were equally potent when a supercoiled plasmid was used as a substrate (Fig. 2A), whereas SN38 seemed more efficient than S38809 when a linear DNA fragment was used. This probably reflected a distinct DNA structure-dependence for the two compounds.

All together, data from DNA relaxation and cleavage experiments demonstrated that this novel lactone-free CPT derivative incorporating a five-membered E-ring effectively stabilized topo1-DNA covalent complexes with a potency and sequence-selectivity similar to those of camptothecin.

Topo1 Is the Main Intracellular Target of S38809. An immunoblot assay was then used to identify the drug-stabilized topo1-DNA complexes in P388 leukemia cells. Cells were incubated with each drug for 1 h before fractionating cell extracts by centrifugation on a CsCl gradient followed by immunological detection of topo1. In the control samples, topo1 was found exclusively as a free protein in fractions 1 to 5 (Fig. 3A). In contrast, in samples prepared from cells exposed to S38809 or SN38, topo1 was found also in fractions 6

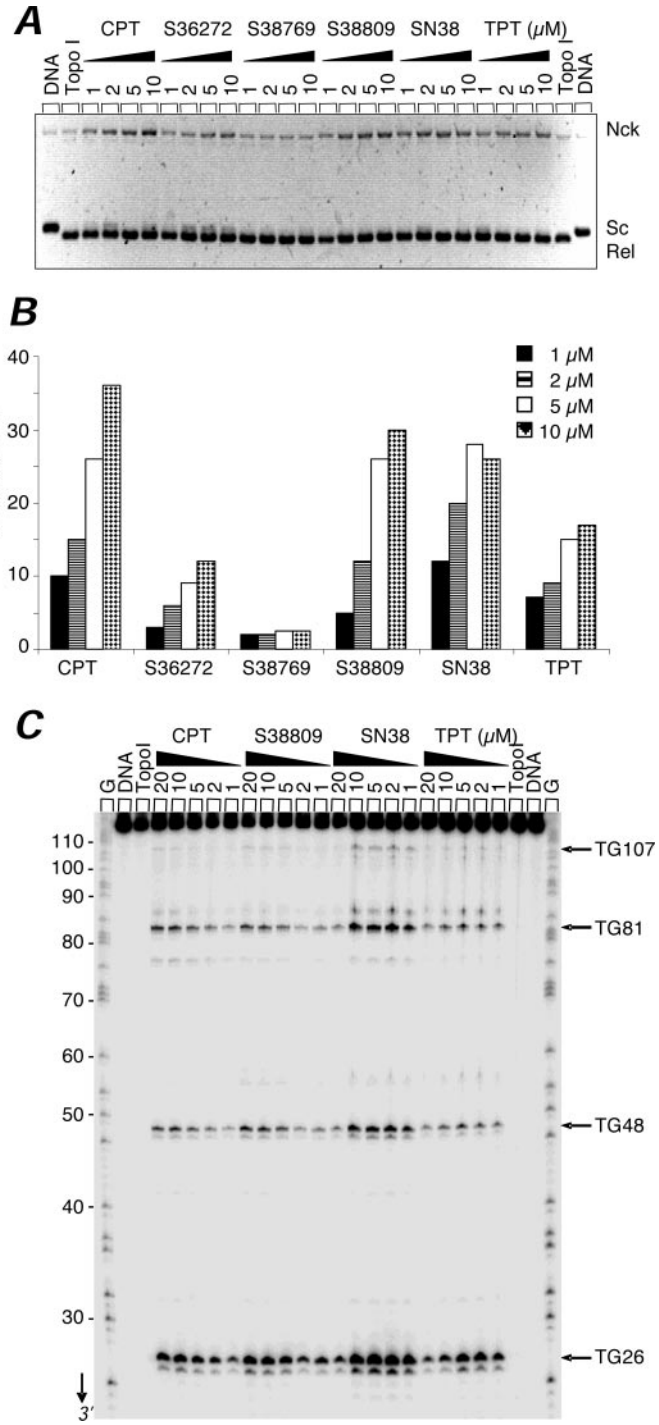

Fig. 2. Topo1 inhibition. A, effects of 1, 2, 5, and 10 μM CPT and derivatives on the relaxation of plasmid DNA by human topo1. Plasmid pLAZ (130 ng) was incubated with 4 U of topo1 in the absence (lane Topo1) or presence of drug at the indicated concentration (in micromoles). DNA samples were separated by electrophoresis on an agarose gel containing 1 μg/ml ethidium bromide. Nck, nicked; Rel, relaxed; Sc, supercoiled. B, nicked band density represented as histograms for each compound at 1, 2, 5, and 10 μM. C, sequencing of topo1-mediated DNA cleavage sites. The 3′-end labeled DNA fragment was incubated in the absence (lane TopoI) or presence of the compounds (from 1 to 20 μM). Topo1 cleavage reactions were analyzed on 8% denaturing polyacrylamide gels. Numbers on the left of the gel represent the nucleotide positions determined with reference to the guanine tracks labeled "G." The four cleavage sites are indicated by arrows.

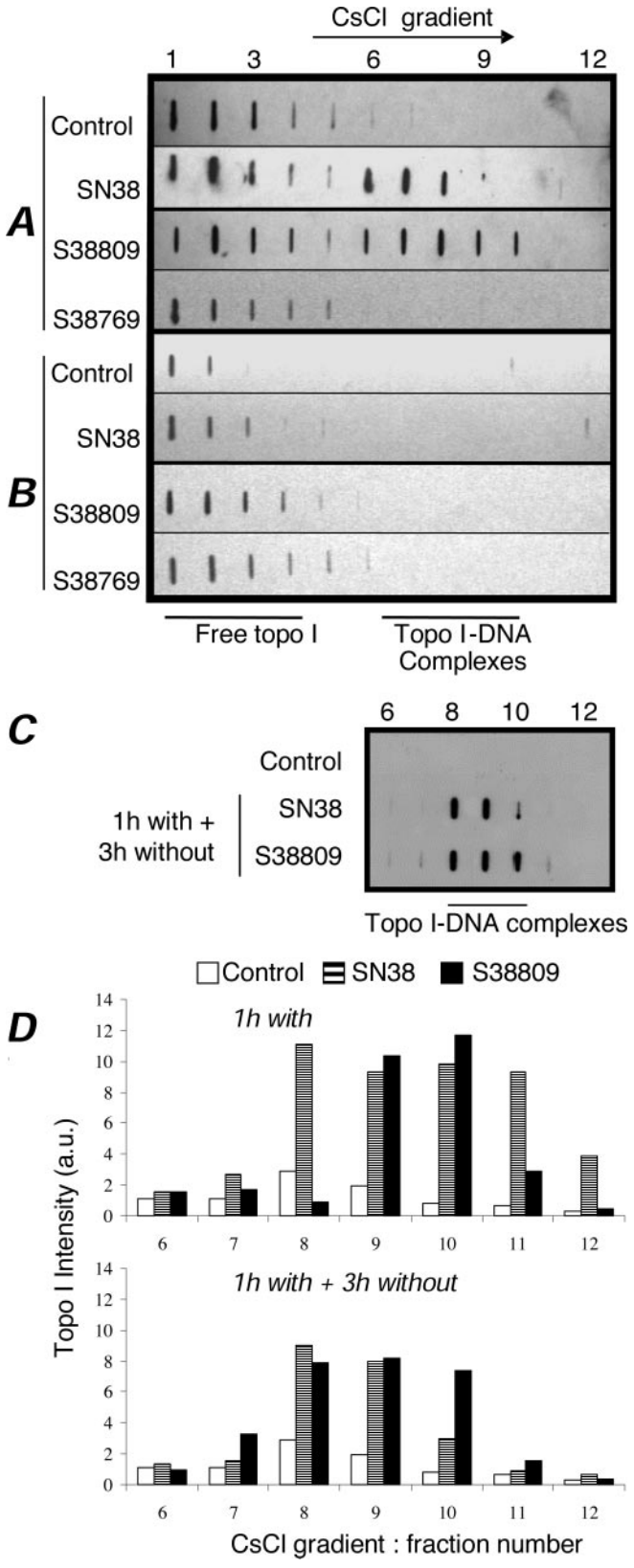

Fig. 3. Immunoblot analysis of topo1-DNA covalent complexes in intact cells. A, P388 cells were incubated with the indicated drug at 5 μM for 1 h at 37°C. Cell lysates were applied onto a CsCl gradient and centrifuged overnight. Twelve fractions were collected and analyzed by the slot blot method described under *Materials and Methods*. Fractions 1 to 4 contain free topo1, and fractions 6 to 10 contain covalent DNA-topo1 complexes. B, similar experiment performed with the CPT-resistant P388CPT5 cells. C, representative immunoblot analysis of lysates from HCT116 cells exposed first for 1 h to SN38 or S38809 and then incubated for 3 h in drug-free medium. D, bands density obtained in three separate experiments were scanned, averaged, and represented as histograms. Control (□), SN38 (▤), and S38809 (■).

to 10 that contain nucleic acids. The drug-trapped covalent complexes (fractions 6–10) were more abundant with S38809 than with SN38. In contrast, the enantiomer S38769, which did not inhibit topo1, did not induce complex stabilization. Similar experiments were performed with P388CPT5 cells, which express a mutated form of topo1 and are therefore CPT-resistant (Madelaine et al., 1993; Pilch et al., 2001). Samples exposed to SN38 or S38809 showed no sign of DNA-bound topo1 in fractions 6 to 10 (Fig. 3B). To investigate cleavable complexes stability after drug removal, HCT116 cells were exposed to the compounds either for 1 h or for 1 h and then incubated in compound-free culture medium during 3 h before fractionating and immunoblotting cell extracts. In cells incubated after drug removal in drug-free medium, the topo1-DNA complexes were more abundant in cells pre-exposed to S38809 than SN38 (Fig. 3*C*). Primary data from three independent experiments were pooled, and the results are presented as a bar graph (Fig. 3D). Despite that S38809 stabilized approximately 2-fold less cleavable complexes than SN38 after 1 h of exposure (Fig. 3D, top), the S38809-stabilized complexes remained stable after 3 h in drug-free medium, whereas those induced by SN38 decreased by 2-fold (Fig. 3D, bottom). These data suggest that the complexes induced by S38809 were more stable than those induced by SN38.

In summary, these results were consistent with the enzymatic data presented above and clearly establish that topo1 was the main intracellular target of S38809. The lower rate of complex dissociation suggests a prolonged action of S38809 at the cellular level with respect to CPT derivatives.

S38809 Potently Inhibits Tumor Cell Proliferation with a G_2/M Arrest. The cytotoxicity of S38809 was studied on a panel of 34 cell lines, including 31 human tumor cell lines, P388 and P388CPT5 subline, and normal PAECs. S38809 proved to be a potent cytotoxic agent against the 31 human tumor cell lines with a mean IC_{50} value of 5.4 nM versus 11.6 nM for topotecan and 3.3 nM for SN38. The pattern of sensitivity of the three drugs was similar, with colon, leukemia, and ovary cell lines being relatively more sensitive (Fig. 4). It is interesting that the rapidly proliferating normal PAEC cells were more resistant than the average panel. The P388CPT5 cells, which displayed a mutated topo1, were more than 100-fold resistant to S38809, SN38, and topotecan than the parental P388 cells.

To investigate the effect of S38809 on the cell cycle, HCT116 cells were exposed for 24 h to increasing concentrations of S38809 or SN38, and DNA content was measured by flow cytometry. The two drugs induced in a dose-dependent manner a similar accumulation of cells in the G_2/M phases of the cell cycle, with 73 to 75% of cells in the G_2/M phases at 10 nM (Fig. 5A). Topotecan induced a similar effect but at a higher concentration of 50 nM (data not shown).

H2AX Phosphorylation. The phosphorylation of histone H2AX on Ser139 by the ataxia telangiectasia-mutated and ataxia telangiectasia-mutated and Rad3-related kinases is an early event observed after the generation of DSBs into

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DNA by ionizing radiation or antitumor drugs (Rogakou et al., 1998; Banath et al., 2003; Furuta et al., 2003). The high number of phosphorylated residues covering each DSB confers to this detection a high sensitivity, allowing the detection of few DSBs in cells. To investigate whether S38809 could induce H2AX phosphorylation, the expression of γH2AX was measured by flow cytometry with a specific monoclonal antibody. A short duration of exposure was used to avoid apoptosis-mediated DNA fragmentation. The biparametric analysis of γH2AX and DNA content showed that the expression of γH2AX induced by these drugs was restricted to cells in S phase for a 3-h duration of exposure (Fig. 5B). The three derivatives induced a dose-dependent increase in γH2AX, with S38809 being as potent as SN38 and 5-fold more potent than topotecan as shown in Fig. 5C.

S38809 Induces Caspase-Dependent Apoptosis. To investigate whether cells exposed to S38809 underwent apoptosis, HCT116 cells were exposed to S38809, topotecan, or SN38, labeled with annexin-V-FITC and PI, and then analyzed by flow cytometry. Figure 6A shows a typical biparametric histogram of HCT116 cells exposed for 96 h to 10 nM S38809. More than 60% of cells were detected as annexin-V-positive, and this effect was significantly reduced by the caspase inhibitor Z-VAD-FMK, suggesting caspase-dependent apoptosis. The percentage of cells engaged into primary apoptosis (annexin-V-positive, PI-negative) was reported in Fig. 6B, which clearly shows that S38809 was as active as SN38 and more than topotecan. Similar experiments performed with different durations of exposure showed that annexin-V-positive cells became detectable starting from 48 h of exposure (data not shown). These data demonstrate that the arrest of cells in the G_2/M phases preceded apoptosis.

S38809 Shows Marked in Vivo Antitumor Activity. The antitumor activity of S38809 was investigated in

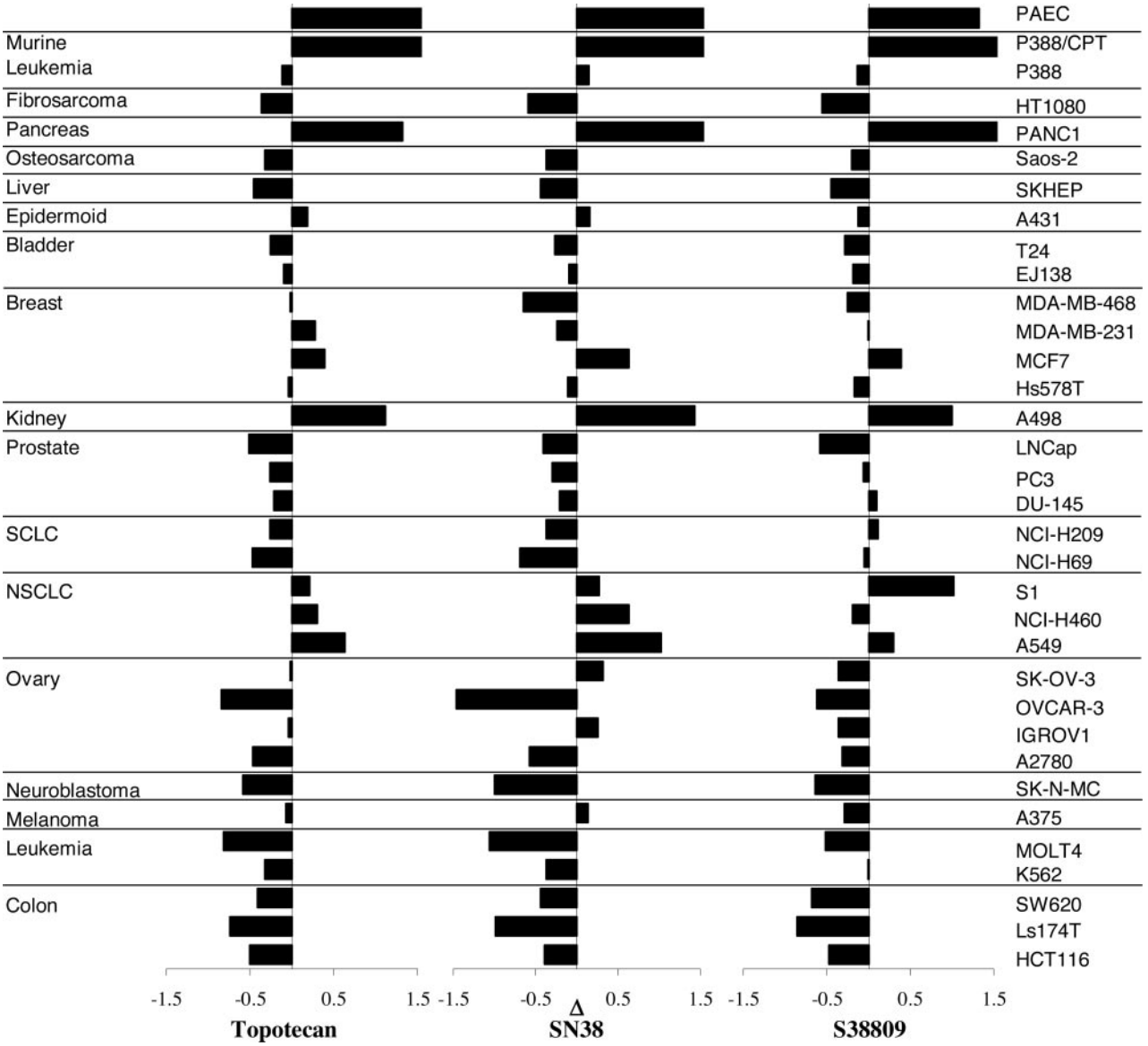

Fig. 4. Cytotoxicity in a panel of tumor cell lines. Cells were exposed to nine graded concentrations of the test drug for four doubling times, and viable cells were estimated by the 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay. Results are expressed as Mean Graph, with Δ = log(IC_{50} individual cell line) − mean of all the log(IC_{50}). The bar on the right indicates that the cell line is more resistant and on the left more sensitive than the average sensitivity of the panel.

HCT116 xenografts in nude mice. Preliminary experiments show that an intermittent schedule (once a week for 3 weeks) is the optimum schedule of administration of these nonlactone derivatives, in contrast to topotecan or irinotecan, which are more active in a repeated schedule, such as once a day for 5 days for several cycles (Houghton et al., 1995). The maximal tolerated dose of S38809 is 50 mg/kg when following the intermittent schedule. It was thus administered at 50, 25, and 12.5 mg/kg, whereas topotecan was administered at its maximal tolerated dose of 2 mg/kg for two cycles (i.e., 10 administrations). At the optimal dose, S38809 inhibited the growth of the tumor by 95% on day 40, whereas topotecan was slightly less active, with an inhibition of 84% (Fig. 6*C*). All of the growth curves of the treated groups were significantly different from the control from day 20 ($p < 0.01$). In addition, the tumor growth delay induced by S38809 was almost 2-fold higher than that of topotecan, and the difference was statistically significant ($p < 0.01$). The weight losses induced by the two compounds at their maximal tolerated dose were similar (Fig. 6D).

Discussion

CPT derivatives target the protein-DNA interface of the DNA-topo1 cleavable complex. These molecules are composed of different domains that play a role in the interaction with DNA and/or topo1. For many years, the E-ring lactone of CPT was considered to be absolutely necessary to inhibit the activity of topo1 through stabilization of the enzyme-DNA covalent complex. From a molecular modeling point of view, the recent crystallographic model of the ternary complex topotecan/topo1/DNA (Staker et al., 2002) suggests that the lactone O-atom directly interacts with the Arg364 residue of the enzyme. Another model proposed by Pilch and coworkers (Kerrigan et al., 2001) also envisions a bidentate interaction between Arg364 residue of topo1 and O-CO lactone function of CPT. Therefore, according to these models, the removal of the O-atom may be considered as detrimental for the poisoning of topo1 and the ensuing cytotoxicity and antitumor activity. Early studies indicated that modification of the CPT lactone moiety produces analogs that were inactive against topo1 (Bailly, 2000). A key example is the replacement of the endocyclic lactone O atom of CPT with a sulfur atom that afforded a thiolactone analog that failed to stabilize the topo1-DNA cleavage binary complex (Hertberg et al., 1989). However, this lactone function was recognized earlier as the Achilles' heel of CPT derivatives because of its opening into an inactive form. Considerable effort had been made to increase the lactone stability, and few novel lactone-modified CPT analogs still active against topo1 were finally identified. These compounds can be grouped in three categories: 1) CPT analogs substituted at the 20-OH group, such as 20-Cl and 20-Br derivatives that are capable of stabilizing the covalent complex (Wang et al., 1999) or high molecular weight derivatives; 2) hCPT derivatives containing an extended seven-membered lactone ring, among which the difluoro derivative diflomotecan seems to be a promising drug candidate (Bailly, 2003); and 3) the five-membered E-ring compounds without a lactone, studied in this work (Hautefaye et al., 2003). The key discovery that CPT derivatives lacking the lactone moiety still function as a topo1 poison is important. First, all CPT analogs currently in development, including diflomotecan, were designed to increase the stability of the lactone ring, but the hydrolysis can still occur. In the present case, S38809 cannot be hydrolyzed, thus overcoming one of the major drawbacks of CPT. This property of these five-membered E-ring ketone derivatives confers a potential therapeutic advantage over topotecan and irinotecan and over many derivatives that are currently in clinical development. From a molecular point of view, our results demonstrate that the O-atom on the lactone E-ring can be removed, leading to derivatives that maintain high potency, both at the enzymatic and cellular levels.

It is striking that the stimulation of topo1-mediated cleavage of DNA by S38809 was strongly dependent on the configuration of the C-7 atom, which is equivalent to the C-20 atom of other camptothecin derivatives. The deletion of the endocyclic O-atom of the lactone clearly has no effect on the known stereoselectivity of CPT derivatives; only the *S* enantiomer inhibits topo1. The sites of DNA cleavage at T↓G-containing sequences were strictly identical for CPT and S38809, suggesting that the recognition of the enzyme-DNA complex at TG sites obeys the same recognition rules and that the postulated H-bond between the O-atom of CPT and Arg364 residue is not essential for drug recognition and cleavage. This is in contrast with hCPT, another E ring-

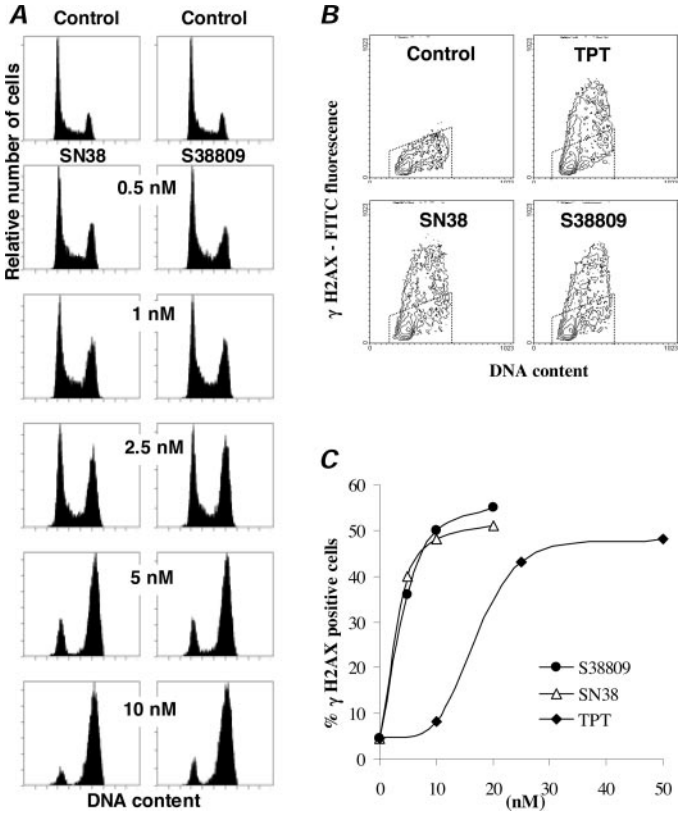

Fig. 5. G_2/M arrest and induction of histone H2AX phosphorylation. HCT116 cells were exposed for 3 h to the indicated concentrations of compounds, fixed, and labeled with either PI alone (A) or PI and an anti-phospho-histone H2AX monoclonal antibody (B and C) before flow cytometric analysis. A, results are displayed as monoparametric histograms of PI fluorescence (DNA content). B, typical biparametric histograms of γH2AX-FITC fluorescence and DNA content for HCT116 cells exposed to 20 nM S38809 or SN38 and 50 nM TPT for 3 h. C, γH2AX-positive cells were quantified and expressed as a percentage as a function of drug concentration.

modified derivative, that presents supplementary cleavage sites (Bailly et al., 1999).

Overall, in all of our in vitro experiments, S38809 proved slightly less potent than SN38 but as potent as camptothecin or topotecan at inhibiting topo1. The 10,11-methylendioxy group present on S38809 is known to increase the potency of camptothecin (Hsiang et al., 1989; Wall et al., 1993). On the basis of these data, S38809 might be less potent than 10,11-methylenedioxy camptothecin, suggesting that the replacement of a six-membered lactone ring by a five-membered ketone ring would lead to a moderate, but significant, decrease in potency.

At the cellular level, the drug was much less cytotoxic to the topo1-mutated P388CPT5 cells compared with the parental P388 cells, and the stabilization of topo1-DNA covalent complexes was observed only in the parental cells. This leaves little doubt that S38809 exerts its cytotoxic action via topo1 inhibition. S38809 was strongly cytotoxic for a panel of human tumor cells, with a mean IC_{50} value of 5.4 nM. It is interesting that topotecan, SN38, and S38809 showed a similar pattern of cytotoxicity. The overall cytotoxic potential of S38809 was comparable with that of SN38 and was slightly superior to topotecan. This trend was seen in vivo because S38809 seemed to be more active than topotecan at inhibiting the growth of HCT116 tumor xenografts.

The cleavable complexes stabilized by S38809 seemed to be more persistent than those stabilized by topotecan or SN38 after removal of the molecules. This property is consistent with the high chemical stability of these nonlactone derivatives. It is interesting that the optimal schedule of administration of S38809 to tumor-bearing mice was once a week for three weeks, in contrast to a daily administration for topotecan or irinotecan. This suggests that, even in vivo, the ternary complexes formed with S38809 might be more stable than those formed with lactone-containing CPT derivatives.

Topo1 poisons are highly potent cytotoxic agents, despite that they induce a reversible DNA lesion. To explain this apparent inconsistency, it was proposed that the ternary complex can be further converted into a highly toxic DSB that results from the collision with the DNA replication fork (Strumberg et al., 2000). Using histone H2AX phosphorylation as a marker, the induction of DSBs by CPT was shown to be restricted to *S* phase cells (Furuta et al., 2003), supporting the replication fork collision model. We observed a strong induction of γH2AX restricted to *S* phase cells at cytotoxic concentrations of S38809, topotecan, or SN38. In terms of potency, S38809 was as potent as SN38 and more potent than topotecan. These results suggest that, similarly to CPT and derivatives, S38809 induces DNA replication-mediated DSB that precedes cell cycle arrest in the G_2/M phases followed by apoptotic death.

In conclusion, S38809, a first representative of novel five-membered E ring ketone derivatives of CPT that lack the lactone function is a potent inhibitor of topo1 and a potent cytotoxic agent that is active in vivo. Taken together, our data show that the cytotoxicity of S38809 depends mainly on

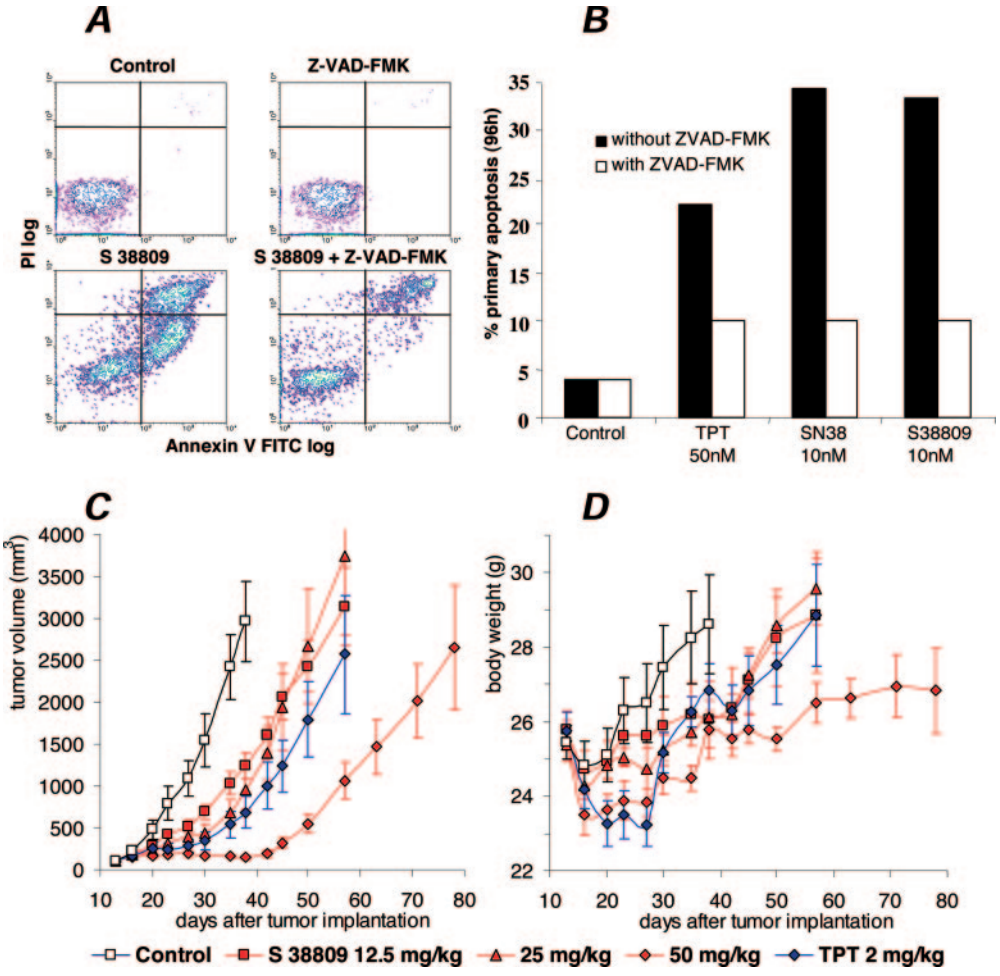

Fig. 6. Drug-induced apoptosis and in vivo antitumor activity. A, typical biparametric histograms of annexin-V-FITC and PI fluorescence of HCT116 cells exposed to 10 nM S38809 for 96 h with or without 50 μM Z-VAD-FMK and then analyzed by flow cytometry. B, primary apoptosis expressed as a percentage of annexin-V-positive PI-negative cells. HCT116 cells were exposed to the indicated concentrations of the three compounds for 96 h with (■) or without (□) 50 μM Z-VAD-FMK. C, nude mice bearing HCT116 tumors were dosed intravenously 13 days after tumor implantation. S38809 was administered on days 13, 20, and 27 at 12.5 mg/kg (red ■), 25 mg/kg (red ▲), and 50 mg/kg (red ◆); TPT was administered at 2 mg/kg (◆) on days 13 to 17 and 20 to 25; vehicle, □. Tumor sizes were measured twice a week from day 13 to the end of the experiment (mean ± S.E.M., $n = 7$). D, mean body weight ± S.E.M. of the control and treated groups.

the prolonged stabilization of the cleavable complex in whole cells and the subsequent cellular responses that lead to apoptosis. In cellular assays, S38809 seems to be at least as potent as SN38 and more potent than topotecan. These findings open a new route for improving the pharmacological properties of CPT derivatives and argue well for the development of novel series of lactone-free CPT as anticancer agents in the clinic.

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