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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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**Running title:** Topo1 poisoning by lactone-free camptothecins.

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**Non-standard abbreviations :**

AMV: Avian myeloblastosis virus

ATM: Ataxia telangiectasia mutated

CPT: Camptothecin

CPT-11: Irinotecan

DSB: Double-strand break

HCPT: Homocamptothecin

PAEC: Primary aortic endothelial cell

SN38: Irinotecan active metabolite

Topo 1: Topoisomerase 1

TPT: Topotecan

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**Abstract**

The E-ring lactone is the Achille's heel of camptothecin derivatives: although it is considered as 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$^{\downarrow}$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, as well as 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, 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.

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## Introduction

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) (Pommier, 2006 ; Wall et al., 1966 ; Hsiang et al., 1985). Amongst these, topotecan (TPT) and irinotecan (CPT-11) 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 (Redinbo et al., 1998 ; Fan et al., 1998 ; Kerrigan et al., 2001). For this reason, the lactone E-ring was considered as 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 as 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 analogues with a stabilized lactone E-ring.

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The structural modifications of CPT skeleton aimed at reinforcing the lactone ring and/or at reducing the binding of the carboxylate form to serum albumin can be grouped in two categories) 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) 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 were detrimental to the inhibition of topo1. Similarly, E-ring modified CPT analogues 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), novel family of potent CPT analogues with a more stable 7-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 ; Soepenberg et al., 2002).

If topo1 inhibition is preserved with a larger 7-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 5-membered E-ring ketone. The synthesis and preliminary biological evaluation of different A-ring substituted congeners were recently reported (Hautefaye et al., 2003) and the methylenedioxy derivative

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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). As 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.

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## Materials and Methods

**Drugs**. CPT was purchased from Sigma Chemicals (St Louis, MO), SN38 from Sanofi Aventis (Paris, France) and TPT from SmithKline Beecham (Brentford, United Kingdom). The racemate S36272 was synthesized as described (20). 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/minute). The drugs were dissolved in DMSO at 5 mM, (except TPT in water), and kept at -20°C.

**DNA relaxation.** Experiments were performed as previously described (Bailly, 2001). Plasmid pLAZ DNA (130 ng) was incubated with 4 units of human topo1 (TopoGen, Colombus, OH) at 37°C for 45 minutes in relaxation buffer (10 mM Tris pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1% BSA, 0.1 mM spermidine, 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 *E. coli* by NaOH-SDS lysis and purified by banding in CsCl-EB gradients. The 117-bp DNA fragment was prepared by 3'-[$^{32}$P]-end labeling at the *Eco*RI site of the *Pvu*II and *Eco*RI double digest of the plasmid pBS using α-[$^{32}$P]-dATP (Amersham Biosciences, Piscataway, NJ) and avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Neuilly/Seine, France). The labeled digestion products were separated on a 6% (w/v) polyacrylamide gel under non-denaturing conditions in TBE buffer (89 mM Tris-borate pH 8.3, 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, 1 mM EDTA. This

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suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with 1 mL of cold ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the 3'-end-labeled DNA was resuspended in 10 mM Tris, 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-minute incubation, the reaction was initiated by addition of 4 units of human topo1. Samples were incubated for 20 minutes 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 minutes then chilled in ice for 4 minutes prior to loading onto the sequencing gel. DNA cleavage products were resolved by electrophoresis on a 8% polyacrylamide gel in TBE buffer containing 8 M urea. Gels were then soaked in 10% acetic acid, transferred to Whatman 3MM paper, and dried under vacuum at 80°C. A Molecular Dynamics 425E PhosphorImager was used to collect signals from the storage screens exposed to dried gels overnight at room temperature. Base line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. 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 dimethylsulfate 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 (Rockville Pike, MD) and are listed in Fig. 4. The P388CPT5 resistant murine leukemia cell line (Madelaine et al., 1993), 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 17h).The PAEC cells were

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collected and cultured as previously described (Fournet-Bourguignon et al., 2000). All the cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$ in RPMI 1640 or DMEM media supplemented with 10% fetal calf serum (FCS), 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. 1 x $10^7$ exponentially growing cells were exposed to the drug at 5 µM for 1 hour at 37°C, and then incubated in drug-free medium for further 3 hours 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, 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 x g for 15 hours 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 hours in TBST buffer (20 mM Tris HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20, 1% BSA) supplemented with 5% nonfat dried milk. The membranes were washed three times with TBST buffer prior to 1-hour incubation at room temperature with a rabbit polyclonal anti-topo1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed, incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham LifeSciences) for 30 minutes. The western blot chemiluminescence reagent from NEN (Boston, MA) was used for the detection and bands were visualized by autoradiography. Scans were analyzed by integrating all the densities using ImageQuant version 3.3 software. The amount of cleavable

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complexes were estimated by summing the densities of fractions 6-12. Control experiments were routinely performed to check the specificity of the anti topo1 antibody.

**Standard proliferation assay.** This assay has been previously described (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 4 doubling times. At the end of this period, 15 µL of 5 mg/mL 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemicals) were added to each well and the plates were incubated for 4 hours at 37°C. The medium was aspirated and formazan solubilized by 100 µL of DMSO. All the measurements were performed in triplicate. Results are expressed as $IC_{50}$ and as Delta = Log ($IC_{50}$) individual cell line – mean 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 hours, washed with culture medium, harvested, fixed with 70% ethanol, washed and incubated for 30 minutes in PBS containing 100 µg/mL RNase and 50 µg/mL propidium iodide (PI). For each sample, 1 x $10^4$ 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 hours, rinsed, labeled with annexin-V-FITC as previously described (Léonce et al., 2001). Cells were resuspended in 200 µL of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 10 µL annexin-V-FITC (Pharmingen, San Diego, CA) and 10 µg/mL PI. After 15 minutes at 20°C in the dark, 800 µL of cold binding buffer were 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 percentage of annexin-V positive cells. Caspases-dependent apoptosis was revealed by exposing HCT116

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cells to the drugs in the presence of 50 μM Z-VAD-FMK (Sigma), an inhibitor of caspase 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 hours, washed, fixed by 70% ethanol at –20°C for at least two hours. Samples were washed with PBS, incubated for 5 minutes in PBS containing 0.5% triton X-100 at 0°C. Cells were washed, incubated for 2 hours at room temperature with 0.5 μg of anti-phosphohistone H2AX (Ser139) murine monoclonal antibody (05-636, Upstate Biotechnology, Charlottesville, VA). Cells were washed, incubated for 1 hour with 4 μg of FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Cells were washed, incubated for 30 minutes with 100 μg/mL RNase, 10 μg/mL PI, 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 (Lyon, France). Mice were 6-8 weeks old and weighed 20-22 g at the start of the experiments, they received proper care and maintenance in accordance with institutional guidelines. HCT116 tumor fragments of 2-3 $mm^3$ were grafted subcutaneously into the flank of nude mice. When the tumors had reached a mean volume of approximately 50-100 $mm^3$, 13 days after tumor graft, mice were distributed among experimental and control groups of seven mice. S38809 was administered i.v. 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 (mm) x width ($mm^2$)/2. Tumor sizes were measured twice a week from day 13 to the end.

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Tumor volumes in treated mice were compared with those of control animals using Student's *t*-test. A one-way ANOVA 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. 2*A*). CPT, used as a positive control, strongly stabilized the cleaveable 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. Interestingly, only the enantiomer S38809 converted supercoiled DNA into nicked DNA, showing that the absolute configuration of the carbon 7 is a key determinant for topo1 poisoning (Fig. 2*A*). Either by visual inspection (Fig. 2*A*) or quantification of the band intensity (% DNA form II, Fig. 2*B*), the racemate S36272 was approximately 3-fold less potent than camptothecin, whereas the active enantiomer S38809 was about 1.5-fold less potent than camptothecin, but as potent as SN38 and more potent than topotecan.

Additional DNA cleavage experiments were then performed using a 117-bp radiolabeled DNA. The cleavage products were analyzed on sequencing polyacrylamide gels to locate the

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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 to those detected with CPT, SN38 and topotecan (Fig. 2*C*). 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 (28, 29). 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 2*A*) whereas SN38 appeared more efficient than S38809 when a linear DNA fragment was used. This probably reflects a distinct DNA structure-dependence for the two compounds.

Altogether, data from DNA relaxation and cleavage experiments demonstrate that this novel lactone-free CPT derivative incorporating a 5-membered E-ring effectively stabilizes 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 hour prior to 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 1-5 (Fig. 3*A*). In contrast, in samples prepared from cells exposed to S38809 or SN38, topo1 was found also in fractions 6-10 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

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or S38809 showed no sign of DNA-bound topo1 in fractions 6-10 (Fig. 3*B*). To investigate cleavable complexes stability following drug removal, HCT116 cells were exposed to the compounds either for 1 hour, or for 1 hour and then incubated in compound-free culture medium during 3 hours prior to fractionating and immunoblotting cell extracts. In cells post-incubated 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 presented as a bar graph (Fig. 3*D*). Despite S38809 stabilized about 2-fold less cleavable complexes than SN38 after 1 hour of exposure (Fig. 3*D*, top), the S38809-stabilized complexes remained stable after 3 hours in drug-free medium, whereas those induced by SN38 decreased by 2-fold (Fig. 3*D*, 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 is 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 G2/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 primary aortic endothelial cells (PAEC). S38809 proved to be a potent cytotoxic agent against the 31 human tumor cell lines with a mean $IC_{50}$ of 5.4 nM, versus 11.6 nM for topotecan and 3.3 nM for SN38. The pattern of sensitivity of the 3 drugs was similar, with colon, leukemia, and ovary cell lines being relatively more sensitive (Fig. 4). Interestingly, 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.

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To investigate the effect of S38809 on the cell cycle, HCT116 cells were exposed for 24 hours to increasing concentrations of S38809 or SN38, and DNA content was measured by flow cytometry. The 2 drugs induced in a dose-dependent manner a similar accumulation of cells in the G2/M phases of the cell cycle, with 73-75 % of cells in the G2/M phases at 10 nM (Fig. 5*A*). 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 (ATM) and ATR (ATM and Rad3 related) kinases is an early event observed after the generation of double-strand breaks (DSBs) into 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 confer 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-hour duration of exposure (Fig. 5*B*). The three derivatives induced a dose-dependent increase in γH2AX, S38809 being as potent as SN38 and 5 fold more potent than topotecan as shown in Fig. 5*C*.

**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. Fig. 6*A* shows a typical bi-parametric histogram of HCT116

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cells exposed for 96 hours 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 ZVAD-FMK suggesting caspase-dependent apoptosis. The percentage of cells engaged into primary apoptosis (annexin-V positive, PI negative) was reported in Fig. 6*B*, that 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 hours of exposure (data not shown). These data demonstrate that the arrest of cells in the G2/M phases preceded apoptosis.

**S38809 shows marked *in vivo* antitumor activity.**

The antitumor activity of S38809 was investigated in HCT116 xenografts in nude mice. Preliminary experiments had shown that an intermittent schedule (once a week for three weeks) is the optimum schedule of administration of these non lactone derivatives, in contrast to topotecan or CPT-11, 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 was 50 mg/kg 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 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 two-fold higher than that of topotecan, the difference being statistically significant ($p<0.01$). The weight losses induced by the two compounds at their maximal tolerated dose were similar (Fig 6*D*).

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**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 co-workers (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: i) 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; ii) hCPT derivatives containing an extended 7-membered lactone ring, amongst which the difluoro derivative diflomotecan appears as a promising drug candidate (Bailly, 2003) and iii) the 5-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. Firstly, 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 5-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.

Strikingly, 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. Clearly, the deletion of the endocyclic O-atom of the lactone has no effect on the known stereoselectivity of CPT derivatives: only the S enantiomer does inhibit topo1. The sites of DNA cleavage at T$^{\downarrow}$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-modified derivative, that presents supplementary cleavage sites (Bailly et al., 1999).

Overall, in all 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 6-membered lactone ring by a 5-membered keton ring would lead to a moderate, but significant, decrease in potency.

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At the cellular level, the drug was much less cytotoxic to the topo1-mutated P388CPT5 cells compared to 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 $IC_{50}$ of 5.4 nM. Interestingly, topotecan, SN38 and S38809 showed a similar pattern of cytotoxicity. The overall cytotoxic potential of S38809 was comparable to that of SN38 and slightly superior to topotecan. This trend was seen *in vivo* since S38809 appeared to be more active than topotecan at inhibiting the growth of HCT116 tumor xenografts.

The cleavable complexes stabilized by S38809 appeared 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 non-lactone derivatives. Interestingly, 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 G2/M phases followed by apoptotic death.

In conclusion, S38809, a first representative of novel 5-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 the prolonged stabilization of the cleavable complex in whole cells and the subsequent cellular responses that lead to apoptosis. In cellular assays, S38809 appears 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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We thank Dr Emmanuel Canet for his continuous interest in this project, Stéphanie Giraudet, Laétitia Marini, Valérie Pérez for excellent technical assistance in cellular pharmacology, Gaëlle Lysiak, Géraldine David for the HCT116 xenograft studies and Roy Golsteyn for critical reading of this manuscript.

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**Footnotes**

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**Figure legends**

**Figure 1**. Structure of camptothecin and the non-lactone camptothecin series with enantiomers S38809 (S) and S38769 (R).

**Figure 2**. Topo1 inhibition.

**A**, Effects of 1, 2, 5 and 10 μM of CPT and derivatives on the relaxation of plasmid DNA by human topo1. Plasmid pLAZ (130 ng) was incubated with 4 units topo1 in the absence (lane Topo1) or presence of drug at the indicated concentration (μM). DNA samples were separated by electrophoresis on an agarose gel containing 1 μg/mL ethidium bromide. Nck, nicked; Rel, relaxed; Sc, supercoiled.

**B**, 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 pointed out by arrows.

**Figure 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 in Materials and Methods. Fractions 1-4 contains free topo1, and fractions 6-10 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 one hour to SN38 or S38809 and then incubated for three hours in drug-free medium.

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**D,** Bands density obtained in three separate experiments were scanned, averaged and represented as histograms. Control (white bars), SN38 (score bars), S38809 (black bars).

**Figure 4**. Cytotoxicity in a panel of tumor cell lines.

Cells were exposed to 9 graded concentrations of the test drug for 4 doubling times and viable cells were estimated by the MTT assay. Results are expressed as Mean Graph, with delta = 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.

**Figure 5**. G2/M arrest and induction of histone H2AX phosphorylation.

HCT116 cells were exposed for 3 hours 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,C) before flow cytometric analysis.

**A**, Results are displayed as monoparametric histograms of PI fluorescence (DNA content).

**B**, Typical bi-parametric histograms of γH2AX-FITC fluorescence and DNA content for HCT116 cells exposed to 20 nM S38809 or SN38, 50 nM TPT for 3 hours.

**C**, γH2AX positive cells were quantified and expressed as percentage as a function of drug concentration.

**Figure 6**. Drug-induced apoptosis and *in vivo* antitumor activity.

**A**, Typical bi-parametric histograms of annexin-V-FITC and PI fluorescence of HCT116 cells exposed to 10 nM S38809 for 96 hours with or without 50 μM ZVAD-FMK and then analysed by flow cytotmetry.

**B**, Primary apoptosis expressed as % annexin V positive-PI negative cells. HCT116 cells were exposed to the indicated concentrations of the three compounds for 96 hours with (black bars) or without (white bars) 50 μM ZVAD-FMK.

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**C,** Nude mice bearing HCT116 tumors were dosed i.v. 13 days following tumor implantation. S38809 was administered on days 13, 20, 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 13-17 and 20-25; 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.

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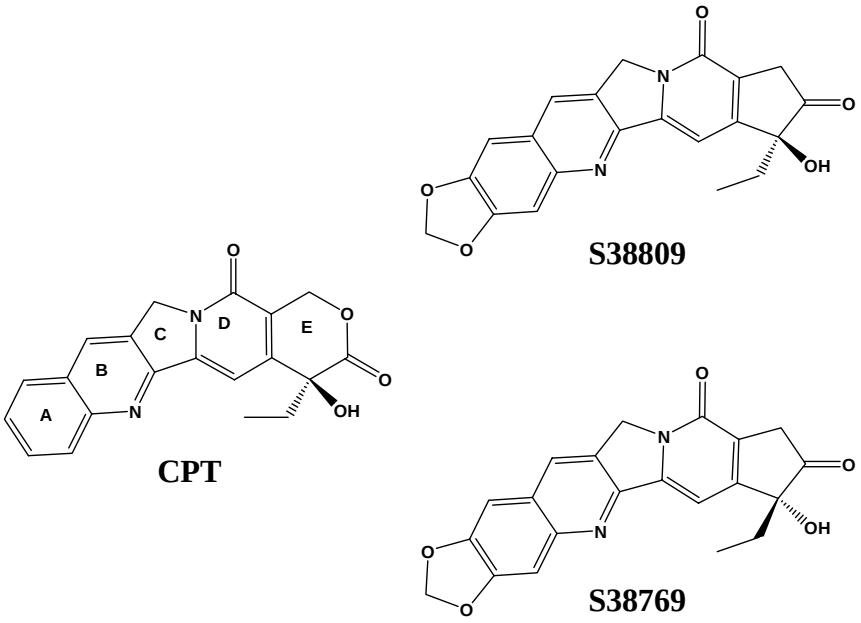
# Figure 1



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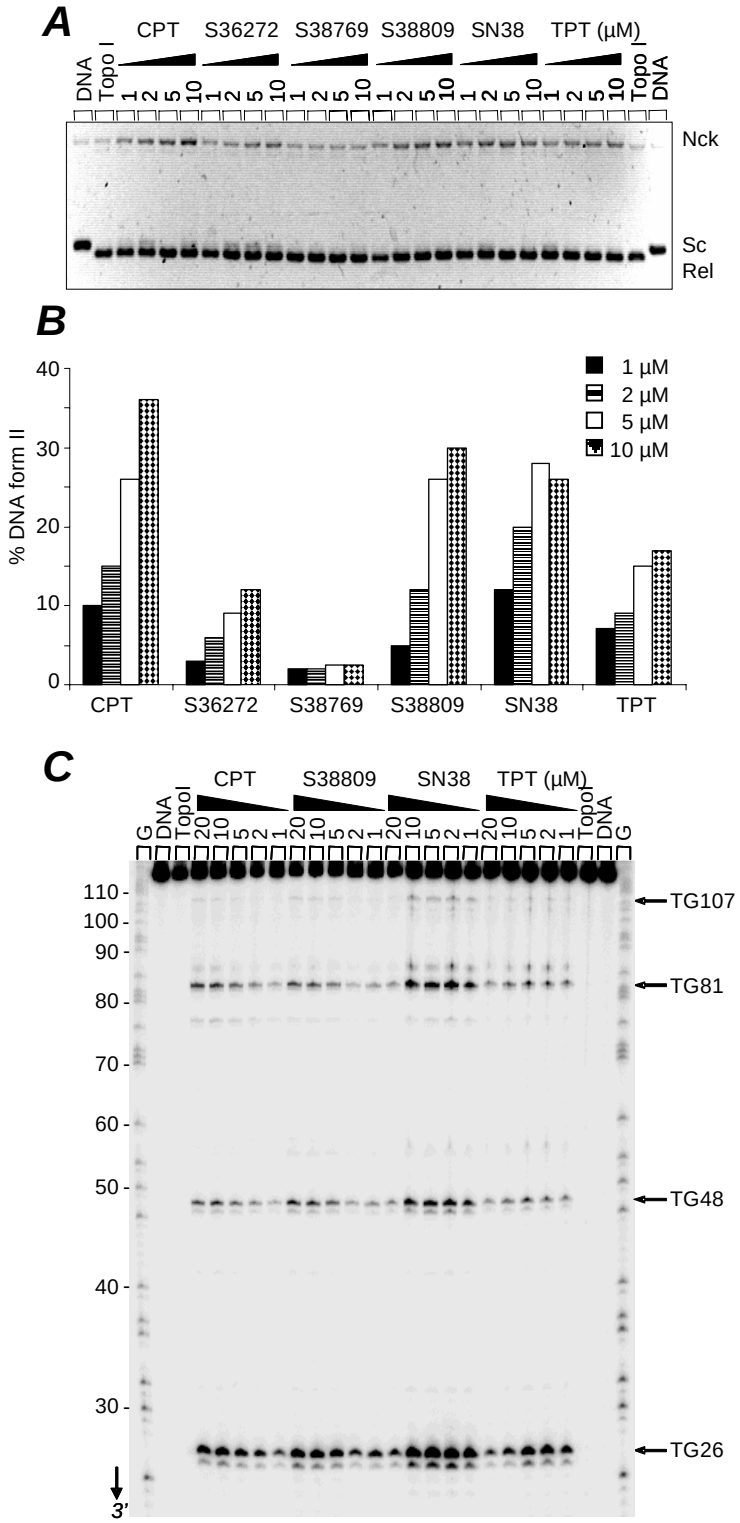
# Figure 2



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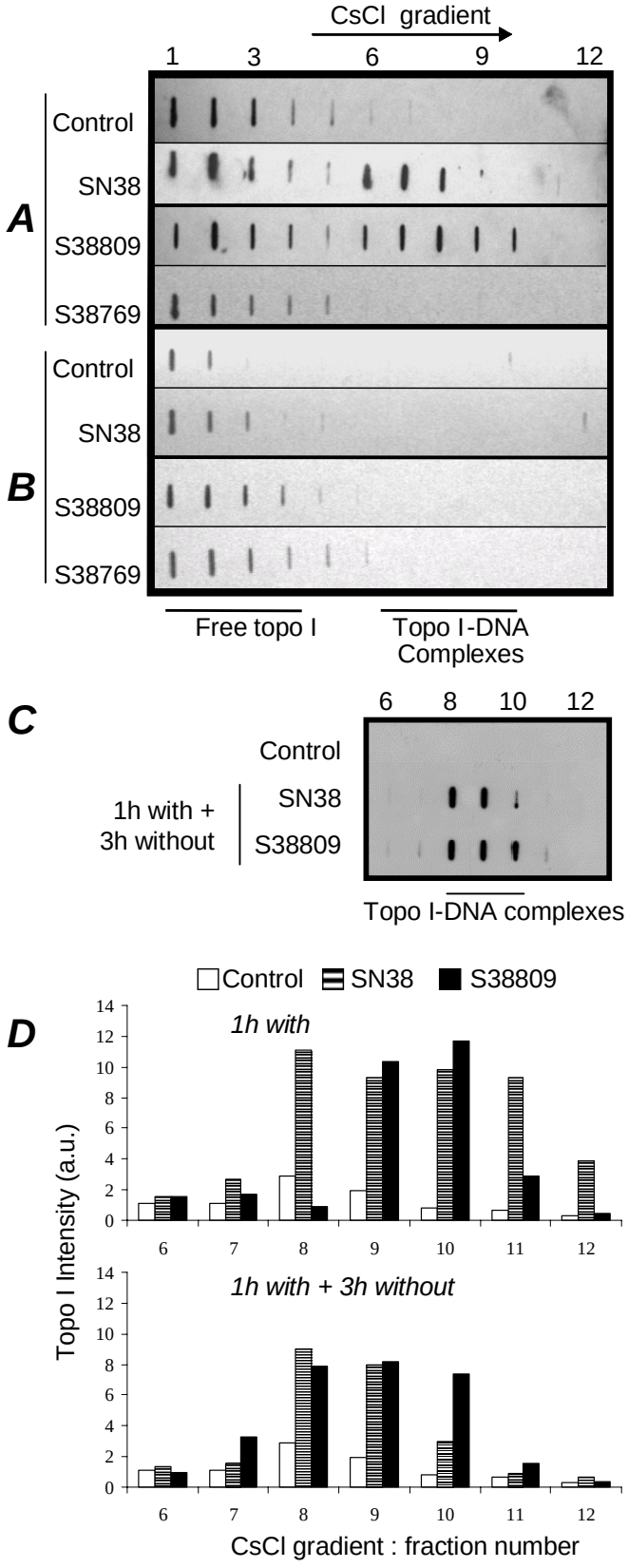
# Figure 3



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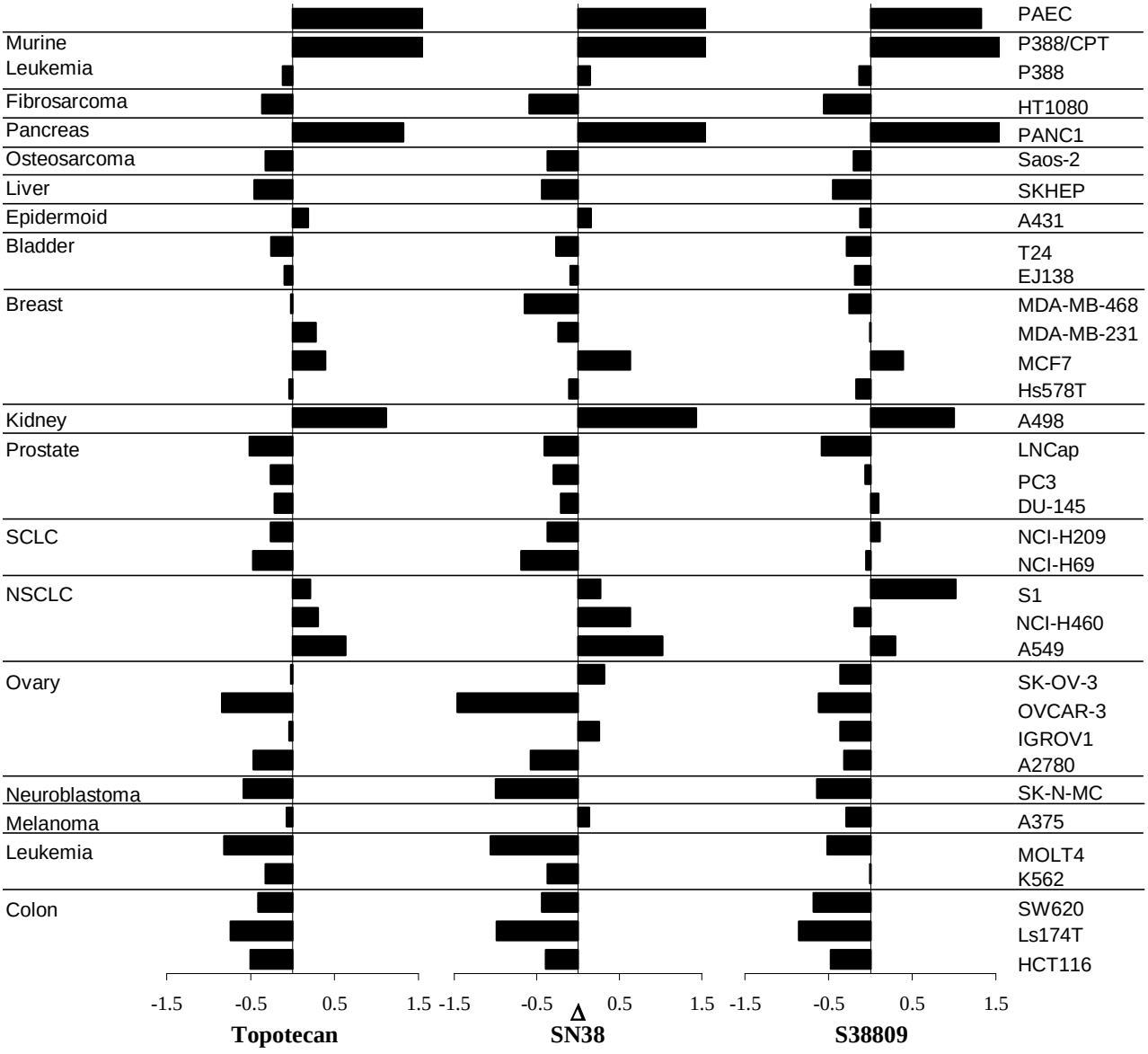
# Figure 4



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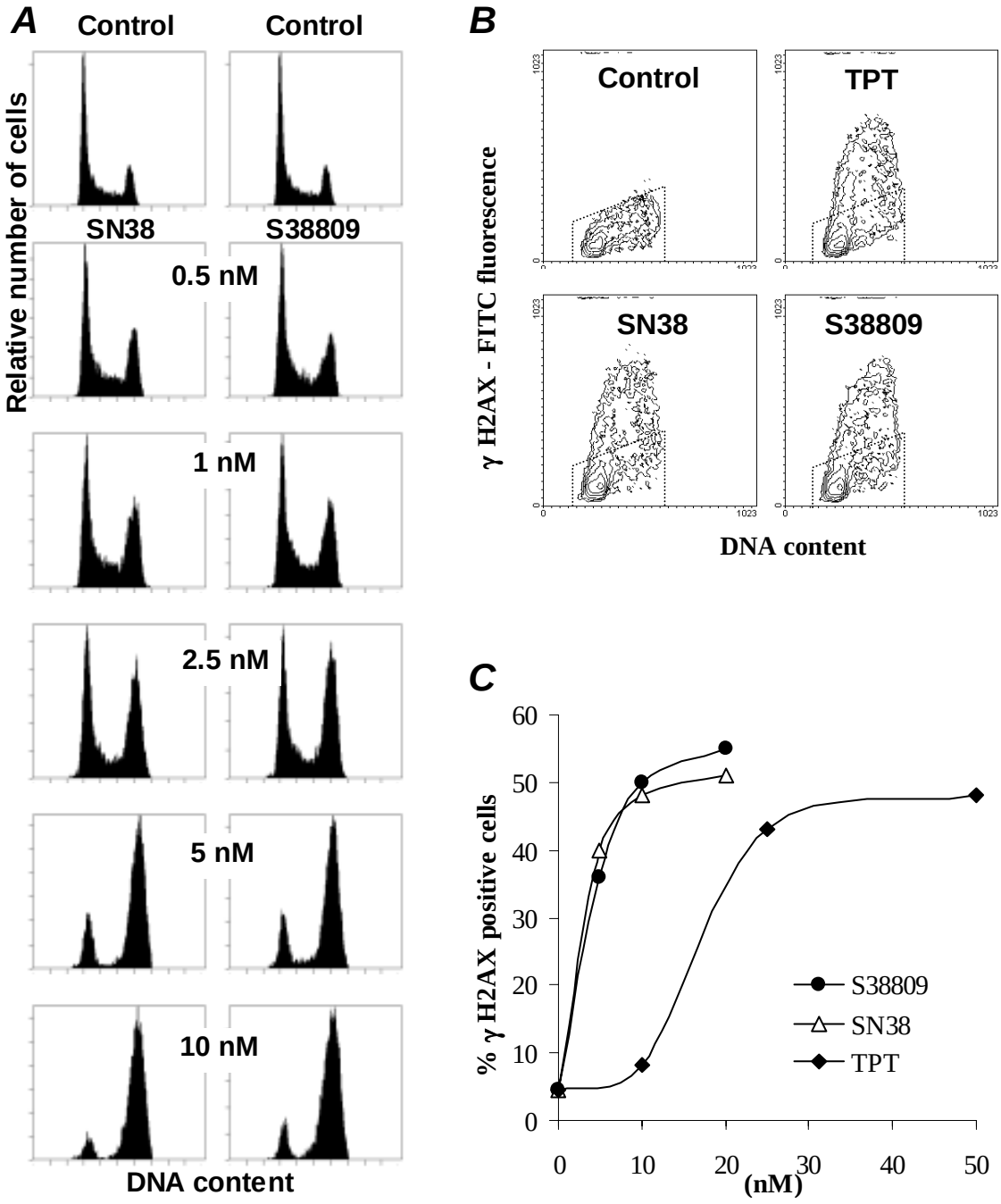
# Figure 5



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# Figure 6