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## Preclinical report

# The homocamptothecin BN 80915 is a highly potent orally active topoisomerase I poison

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**BN 80915, a lead compound of the homocamptothecin (hCPT) family, has entered clinical trials. BN 80915 is a difluoro-hCPT where the six-membered $\alpha$-hydroxylactone ring of camptothecin (CPT) is replaced by a seven-membered $\beta$-hydroxylactone ring. Preclinical data reported here show that in spite of the modification to the crucial E-ring of CPTs, BN 80915 retains topoisomerase I poisoning activity as shown in living HT29 cells as well as in cell-free assays, where BN 80915 always performs better than SN-38 or TPT. In antiproliferative assays BN 80915 is also very potent as evidenced by $IC_{50}$s values consistently lower than those of SN38 in sensitive cell lines as well as in their related multidrug-resistant lines overexpressing P-glycoprotein or multidrug resistance-associated protein. Furthermore, in human plasma, in contrast to CPT analogs, the hydrolysis of BN 80915 is slow, leading to improved plasma stability, and irreversible, thus avoiding toxicity related to the accumulation of active principle during excretion in the urinary tract. These findings may account for the good *in vivo* efficacy observed in PC3 xenograft experiments where BN 80915 administered orally at very low doses doubled the tumor growth delay in comparison to CPT-11 administered i.p. Altogether, these results strongly support further development of BN 80915. [© 2001 Lippincott Williams & Wilkins.]**

*Key words*: Cell proliferation, homocamptothecin, MDR, topoisomerase I inhibitor, xenograft.

## Introduction

Topoisomerase (Topo) I is a nuclear enzyme involved in vital cellular processes such as replication and transcription[1] through the regulation of DNA topology. Its mechanism of action involves transient DNA single-strand breaks with the reversible formation of cleavable complexes that can be stabilized by various drugs. The accumulation of such complexes can lead, ultimately, to cell death.[2] Camptothecin (CPT; Figure 1), a natural product extracted from the Chinese tree *Camptotheca acuminata*, was shown to have antitumor activity[3] via the selective inhibition of Topo I. It was used as a template in the search for efficacious analogs and several compounds are reported to be in preclinical development,[4] while others have been clinically evaluated.[5] Two compounds, topotecan (TPT; Hycamtin®) and irinotecan (CPT-11; Campto®) have been approved for clinical use for second-line treatment in ovarian and colorectal cancer, respectively.[6,7] The anticancer activity of CPT analogs in patients is, however, less impressive than that observed in human tumor xenograft models, confirming earlier findings that these compounds have major limitations due to the half-life of the active lactone form and other pharmacokinetic factors.[8] Thus, much effort has been invested in designing CPT analogs with more favorable pharmacokinetics, improved stability of the lactone ring and lesser toxicity. Other solutions to the problem may come from oral administration[9] or liposomal formulation of the drugs.[10–12].

Homocamptothecin (hCPT) is a new CPT derivative bearing a seven-membered $\beta$-hydroxy lactone ring. All other CPT-derived Topo I inhibitors under clinical development, or launched, have in common the six-membered $\alpha$-hydroxylactone ring, previously considered to be an absolute requirement for good antitumor activity.[13] We recently reported that this change in the crucial lactone ring conserved Topo I poisoning activity[14] as well as antitumor activity in cellular assays and in animal models.[15] Furthermore the E-ring modification provides a less-reactive lactone with enhanced stability which may contribute to the high efficacy observed *in vivo*. hCPT was thus adopted as a

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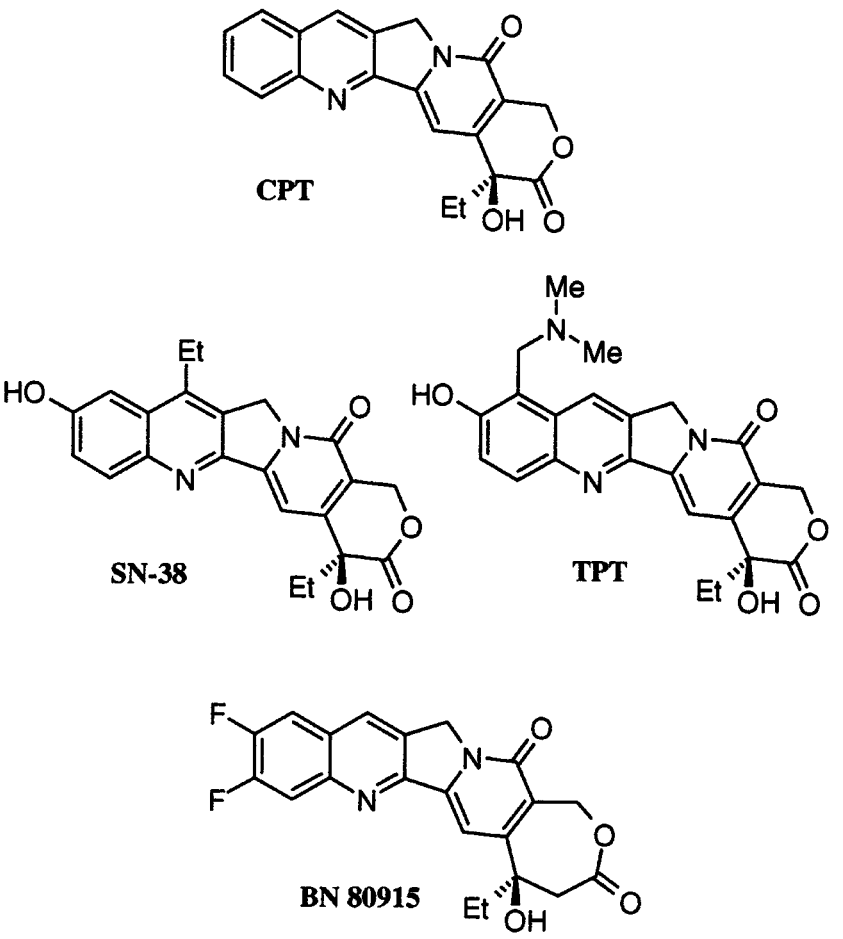


**Figure 1.** Structure of CPT, SN-38, TPT and BN 80915.

new template for an analog generation programme[16] which led to the difluorinated hCPT BN 80915 (Figure 1).

Oral and i.v. phase I clinical trials of BN 80915 were initiated in early 1999.

We present here preclinical data, which led to the selection of BN 80915 for clinical trials. We also discuss the differences between BN 80915 and classical CPTs, and whether these differences may lead to better treatment of refractory tumors.

## Material and methods

### Drugs

BN 80915, SN-38 and TPT were prepared according to published procedures.[16–18] The drugs were dissolved at $10^{-2}$ M in the appropriate solvent, water for TPT or *N*,*N*-dimethylacetamide (DMA) for SN-38 and BN 80915, and further diluted in the appropriate assay medium to the desired final concentration.

### DNA relaxation assay

Supercoiled pKMp27 plasmid DNA (500 ng) was incubated with 6 U human Topo I (TopoGen, Colombus, OH) at 37°C for 45 min in relaxation buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EDTA) in the presence of the drug at concentrations ranging from 1 to 100 $\mu$M. Reactions were terminated by adding SDS (0.25%) and proteinase K (250 $\mu$g/ml). DNA samples were then added to the electrophoresis dye mixture (3 $\mu$l) and electrophoresed in an ethidium bromide (EtBr)-containing agarose gel, at room temperature for 3 h.

### Purification and radiolabeling of DNA restriction fragment

Plasmid pBS (Stratagene, La Jolla, CA) was isolated from *Escherichia coli* by a standard SDS–NaOH lysis procedure and purified by banding in CsCl–EtBr

gradients. EtBr was removed by several isopropanol extractions followed by exhaustive dialysis against Tris–EDTA buffered solution. The purified plasmid was then precipitated and resuspended in appropriate buffered medium prior to digestion by the restriction enzymes. The 117 bp DNA fragment was prepared by 3′-$^{32}$P-end-labeling of the *Eco*RI–*Pvu*II double digest of the plasmid pBS, using [α-$^{32}$P]dATP (3000 Ci/mMol; Amersham, Orsay, France) and AMV reverse transcriptase. The digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE buffered solution (89 mM Tris–borate, pH 8.3 and 1 mM EDTA). After autoradiography, the DNA band was excised, crushed and soaked in water overnight at 37°C. This suspension was filtered through a 0.22 μm filter (Waters-Millipore, St Quentin, France) and DNA was precipitated with ethanol. After washing with 70% ethanol and vacuum drying, the labeled DNA was resuspended in 10 mM Tris buffer adjusted to pH 7.0 and containing 10 mM NaCl.

### Sequencing of Topo I-mediated DNA cleavage sites

Each reaction mixture contained 2 μl of 3′-$^{32}$P-end-labeled DNA (about 1 μM), 5 μl of water, 2 μl of 10 × Topo I buffer and 10 μl of drug solution at the desired concentration (1–100 μM). After 10 min incubation to ensure equilibration, the reaction was initiated by addition of 2 μl (20 U) calf thymus Topo I. Samples were incubated for 45 min at 37°C prior to adding SDS (0.25%) and proteinase K (250 μg/ml) in order to dissociate the drug–DNA–Topo I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 μl of formamide–TBE loading buffer, denatured at 90°C for 4 min, and then chilled in ice for 4 min prior to loading on to the sequencing gel. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis [about 2.5 h at 60 W, 1600 V in TBE buffer, BRL (Cergy Pontoise, France) sequencer model S2], gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper and dried under vacuum at 80°C. A Molecular Dynamics 425E PhosphorImager (Amersham Pharmacia Biotech, Saclay, France) was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Baseline-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 bond within the DNA fragment by comparison of its position relative to sequencing standards generated by treatment of DNA with SDS followed by piperidine-induced cleavage at the modified guanine residues.

### *In vivo* complex of topoisomerase (ICT) bioassay

The ICT bioassay was used to detect the formation of covalent DNA–Topo I complexes in living cells.[19] A commercially available *in vivo* Topo I-link kit (TopoGen) was used and its accompanying protocol was followed with few minor modifications. Exponentially growing HT29 cells ($1.5 \times 10^7$ cells/sample) in 10 ml serum-free DMEM were treated with BN 80915 (1 μM) or SN-38 (10 μM) for 60 min at 37°C. After removing the culture medium, the cells were washed with PBS and lysed by addition of 1.5 ml of 1% sarkosyl, pre-equilibrated at 37°C in TE buffer (10 mM Tris–HCl, pH 7.5 and 1 mM EDTA). Each lysate was overlaid onto a CsCl gradient of four different densities (1.37, 1.5, 1.72 and 1.82 g/ml). Gradient tubes were centrifuged (125 000 *g*) for 15 h at 25°C and fractions (0.35 ml) were collected from the top of the gradient. The double-strand (ds) DNA content of each fraction was quantified using PicoGreen[20] dsDNA fluorescence enhancement kit (Molecular Probes, Eugene, OR) on a Victor multitask-counter (EGG Wallac, Evry, France) operating at 485 nm excitation and 530 nm emission.

### Immunoblotting analysis

Aliquots of each fraction (50 μl) were incubated with 10 U DNase I (Boehringer Mannheim, Meylan, France) for 1 h at 37°C, then diluted with 100 μl sodium phosphate buffer (25 mM, pH 6.5) and applied with a dot-blot vacuum manifold (BioRad, Ivry-sur-Seine, France) onto Hybond nitrocellulose membranes (Amersham, Orsay, France) pre-equilibrated for 30 min in 25 mM sodium phosphate buffer. Nitrocellulose membranes were washed 3 times (10 min per wash) with TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20), followed by an overnight incubation in TBST containing 5% non-fat dried milk (BioRad). The membranes were washed 3 times in TBST and incubated overnight at 4°C, under gentle agitation, with Topo I antibody (TopoGen) at 1/5000 dilution in TBST. After three successive washes, the membranes were incubated with a goat anti-rabbit antibody conjugated to horseradish peroxydase (Amersham) at 1/5000 dilution in TBST, for 1 h at room temperature under gentle agitation. After four successive washes the presence of Topo I was revealed according to the enhanced chemoluminescence procedure (Amersham).

## Cell culture

HT29 (colon), A549 (lung), MCF7 (breast), SKOV3 (ovary), DU145 and PC3 (prostate), and K562 and HL60 (leukemia) cell lines were purchased from ATCC (Rockville, MD). Bladder cell line T24 and its resistant counterpart, T24anp, obtained by prolonged exposure to a cocktail of antitumor drugs consisting of adriamycin, navelbine and PE1001 (anp),[21] were a generous gift of Professor R Kiss (Université Libre de Bruxelles, Belgium). Resistant leukemia cell lines K562adr, HL60adr and HL60dnr, obtained by prolonged exposure to adriamycin (adr) or daunorubicin (dnr),[22] were a generous gift of Dr A-M Faussat (Hôpital Hôtel-Dieu, Paris, France). The MCF7mdr cell line, obtained by transfection of MCF7 cells with the mdr1 gene,[23] was a generous gift of Dr JC Kouyoumdjian (Hôpital Henri Mondor, Créteil, France). The resistance patterns of those cell lines were routinely confirmed in control experiments by Dr A-M Faussat: K562adr, HL60dnr, T24anp and MCF7mdr cell lines show classical multidrug resistance (MDR) phenotype with overexpression of P-glycoprotein (P-gp). HL60adr and A549 cell lines show also a MDR phenotype but with overexpression of the multidrug resistance-associated protein mrp.[24]

The cells were cultured at 37°C in a humidified atmosphere containing 5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM) at 4.5 g/l glucose supplemented by 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 $\mu$g/ml streptomycin and 2 mM glutamine (all from Life Sciences, Cergy-Pontoise, France).

## Cytotoxicity assays

The growth inhibition was evaluated by the WST colorimetric assay (Boehringer Mannheim, Meylan, France) as previously described.[15] Depending on the cell lines, 1000–4000 cells were seeded in 80 $\mu$l DMEM/well on a microtiter plate 24 h prior treatment with 20 $\mu$l of each drug over final concentrations ranging from $5.12 \times 10^{13}$ to $1 \times 10^{-6}$ M. After 72 h incubation with drugs, 10 $\mu$l of WST labeling reagent was added to each well for 2 h at 37°C. Quantification of viable cells was made by measuring the absorbance at 450 and 620 nm. Experiments were performed 3 times, each experiment representing eight determinations per tested concentration. For each drug, the data points included in the linear part of each experiment's sigmoid were retained in a linear regression analysis (linearity, deviations from linearity and difference between the experiments) to estimate the 50% inhibitory concentration ($IC_{50}$) and its 95% confidence limits.

## Plasma stability

The drugs were incubated at 37°C in capped polystyrene tubes containing 500 $\mu$l fractions of pooled human plasma (TSEF, Rungis, France) and monitored by HPLC following protein precipitation, as previously described.[15,25] The lactone and carboxylate forms of the drugs were detected at 380 nm for SN-38 and TPT, and 360 nm for BN 80915. Samples were eluted on a 5 $\mu$m Nucleosil $C_{18}$ column (Macherey-Nagel, Düren, Germany) at 30°C with a flow rate of 1 ml/min of mobile phase, consisting of a mixture of acetonitrile/75 mM ammonium acetate/1 M tetrabutylammonium dihydrogenophosphate (250/750/5) under isocratic conditions for SN-38, a gradient of 3% triethylammonium acetate/water (from 950/50 to 700/300) for TPT and a gradient of water/acetonitrile/sulfuric acid (from 800/200/0.4 to 300/700/0.4) for BN 80915.

## PC-3 xenografts

Tumors were established by s.c. injection of non-androgen-dependent human prostate adenocarcinoma cells ($5 \times 10^6$ cells per animal, on the left dorsal surface) in 4- to 6-week-old NCr *nu/nu* female athymic nude mice (NCI, Frederick, MD). Tumour volume ($mm^3$) was calculated as $(w^2 \times l)/2$, where $w$ is the width and $l$ is the length of the tumor as measured with calipers. The animals were monitored until the tumors reached an average size of approximately 100 $mm^3$. The range of tumor volumes were then tallied and the animals were randomized into groups of 10 so that the average tumor volumes were approximately the same for each group at the onset of the experiment. Dosing with drugs or vehicle began the following day (day 10 post-implant).

BN 80915 was formulated in DMA/Montanox 80/0.9% NaCl (3/2/95 v/v) and orally administered according to one of the following schedules: (i) once a day for 14 consecutive days: QD × 14 (0.06 mg/kg); (ii) twice a day for 14 consecutive days: BID × 14 (0.03 mg/kg); (iii) once a week for 3 weeks: QW × 3 (1 mg/kg); (iv) once a day for 4 days followed by a rest period of 3 days, this 4/3 cycle repeated twice more for a total of 12 administrations over the course of 3 weeks: 4on/3off/ × 3 (0.08 mg/kg). CPT-11 and TPT were used as clinically relevant benchmarks, administered i.p. at 100 mg/kg on a QW × 3 schedule and 3 mg/kg on a QD × 5 schedule respectively.[26] The mice were monitored individually until their tumor grew to a cut-off size of 1500 $mm^3$. The tumor growth delay (TGD, measured in days) was calculated as the difference in time required for the mean tumor size to

reach 50% of the cut-off size, between the treated group and the vehicle group. Animal care was in accordance with institutional guidelines.

## Results

### Effects of BN 80915 on human Topo I in cell-free systems

Topo I, in the absence of drug, covalently binds negatively supercoiled DNA and relaxes it via the nick of a single strand of the double helix. Those enzyme-linked DNA breaks are referred to as 'cleavable complexes'. In the presence of a Topo I poison, the percent of nicked DNA is increased in a dose-dependent manner corresponding to the stabilization of the cleavable complexes. BN 80915 (structure in Figure 1) was compared to TPT in such a DNA relaxation assay. Electrophoresis on an EtBr pre-treated gel of the reaction products enables the separation of the nicked and relaxed forms of DNA. Such a gel is reproduced in Figure 2, along with the accompanying graph obtained by densitometric analysis, showing the percent of nicked form of DNA as a function of drug concentration. The higher level of nicked DNA is indicative of the greater potency of BN 80915 in stabilizing Topo I–DNA complexes compared to TPT.

BN 80915 was compared to both SN-38 and TPT in DNA cleavage experiments in order to map the Topo I cleavage sites induced by the drugs. The *Eco*RI–*Pvu*II 117 bp double digest of pBS plasmid was uniquely end-labeled at the 3′-end at the *Eco*RI site and used as a substrate for the Topo I cleavage reactions. As previously reported, the drugs stabilize only a subset of sites cleaved by the Topo I enzyme.[14] As shown in Figure 3, separation of the cleavage products by electrophoresis on a sequencing polyacrylamide gel revealed six cleavage sites: three major sites corresponding to TG sequences, three minor sites corresponding to two CG and a single TA site. Cleavage at the TG sites correspond to highly consensual sequences for the three drugs as shown by the intense bands obtained. The ability to promote Topo I-mediated DNA cleavage at these TG sites is, nevertheless, more pronounced for BN 80915 than for SN-38 or TPT. The three remaining sites detected at both 10 and 20 $\mu$M for BN 80915 are weaker or undetected for TPT and SN-38.

### BN 80915 stabilizes DNA–Topo I complexes in living HT29 cells

The ability of BN 80915 to stabilize DNA–Topo I complexes (cleavable complexes) in living cells was investigated using the ICT bioassay. Colon adenocarcinoma HT29 cells were lysed and fractionated using a CsCl gradient. For each fraction, the DNA content was estimated by fluorometry and the presence of Topo I was revealed by an immunoblotting assay, as shown in Figure 4. In a control experiment with untreated cells, free Topo I is found at the top of the CsCl gradient (fractions 1–6) while DNA is found at the bottom of the gradient (fractions 15–17). When HT29 cells are treated for 1 h with 1 $\mu$M for BN 80915 or 10 $\mu$M for SN-38, the DNA-containing fractions (fractions 13–17) are found to be immunoreactive to the anti-Topo I antibody, corresponding to DNA-bound Topo I,

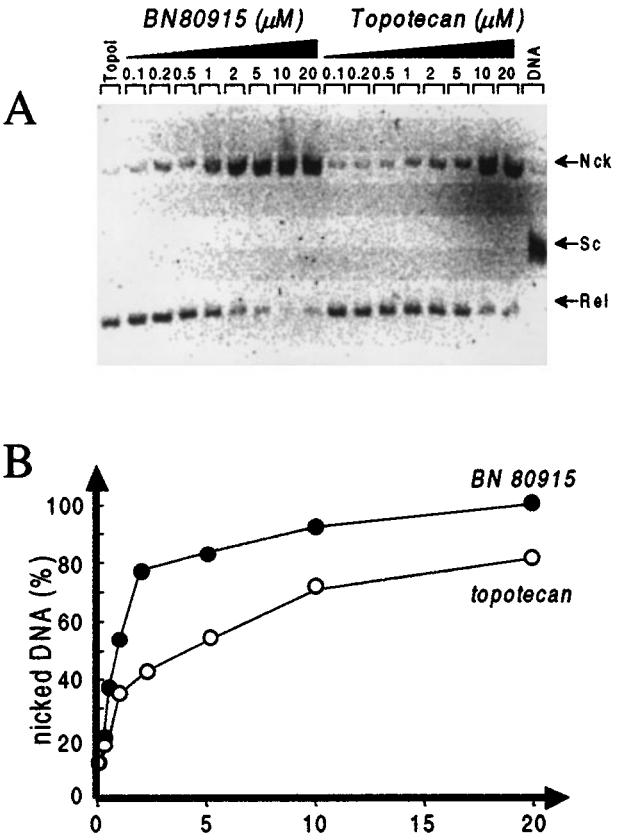


**Figure 2.** Effect of increasing concentrations of BN80915 and TPT on the relaxation of plasmid DNA by Topo I. Native supercoiled pKMp27 DNA (0.5 $\mu$g) (lane DNA) was incubated with 6 U Topo I in the absence (lane Topo I) or presence of drug at the indicated concentration ($\mu$M). DNA samples were separated by electrophoresis on an agarose gel (A) containing EtBr. Gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled. (B) Comparison of the Topo I-mediated cleavage efficiency of BN80915 (●) and TPT (○). The plots show the formation of nicked DNA as a function of the drug concentration. Data were compiled from quantitative analysis of three gels such as the one shown in (A) and must be considered as a set of average values.

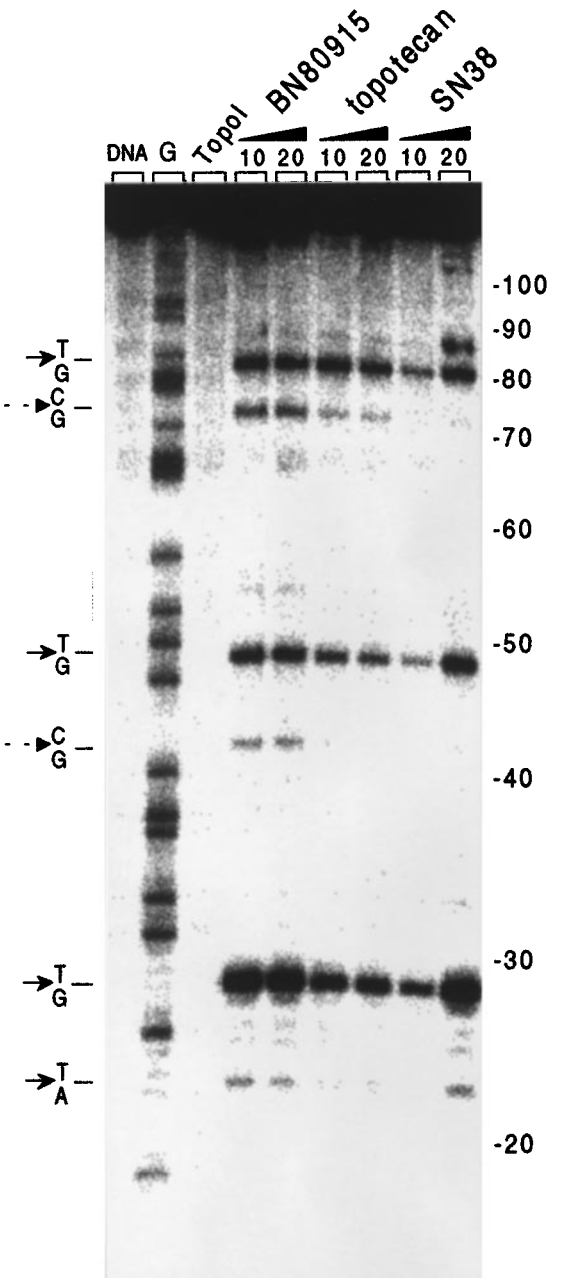


**Figure 3.** Phosphorimage comparing the susceptibility of pBS to cutting by human Topo I in the presence of SN-38, TPT and BN 80915. In each run, the 3′-end-labeled fragment was incubated in the absence (lane Topo I) or presence of the test drug at the indicated concentration ($\mu$M). Topo I cleavage reactions were analyzed on 8% denaturing polyacrylamide gels. Numbers at the right of the gels show the nucleotide positions, determined with reference to the guanine track (G). Short arrows point to the cleavage sites. Dotted arrows indicate hCPT-specific cleavage sites at sequences containing the AAC↓G element.

indicating a Topo I poisoning mechanism of action. Although the immunoassay is essentially qualitative, it is interesting to note that, in fractions 13 and 14 which have comparable DNA content for BN 80915 and SN-38, DNA-bound Topo I appears to be more abundant with 1 $\mu$M BN 80915 than with 10 $\mu$M SN-38. This suggests that BN 80915 stabilizes more cleavable complexes than SN-38. In a similar experiment, 1 $\mu$M SN-38 hardly stabilized any Topo I cleavable complexes (Data not shown).

## BN 80915 potently inhibits human tumor cell growth *in vitro*

The cytotoxic activities of the drugs were evaluated on nine human tumor cell lines corresponding to various tissues. Cultured cells were exposed to the drugs for 72 h and subjected to a WST assay to determine the 50% inhibitory concentrations ($IC_{50}$ values, given with 95% confidence limits). As shown in Table 1, BN 80915 exhibits a significantly higher antiproliferative activity than SN-38 on all the cell lines tested.

In order to evaluate the effects of the compounds on cells with the MDR phenotype, $IC_{50}$ determinations were performed on five resistant cellular strains: MCF7mdr, K562adr, HL60adr, HL60dnr and T24anp (Table 2). These cell lines were derived from their respective sensitive parent cell lines by selection or transfection (see Material and methods) and overexpress either P-gp or mrp, two efflux pumps known to reduce intracellular drug levels.[27,28] Here again BN 80915 shows higher cytotoxicity than SN-38 on the resistant cell lines. Table 2 also presents the ratio of $IC_{50}$ ($R_f$) between resistant and sensitive strains, for each pair of cell line. The $R_f$ values obtained for BN 80915 are always lower than those for SN-38, suggesting that BN 80915 could be more efficacious than CPT-11 for the treatment of drug-resistant-tumors.

## Enhanced plasma stability of BN 80915

The stability of BN 80915, compared to classical CPT analogs SN-38 and TPT, was studied upon incubation in human plasma at 37°C. The best-fit curves of experimental points obtained by HPLC monitoring of the lactone forms of the drugs, are reported in Figure 5. Exponential decays to an equilibrium of less than 20% lactone are observed for TPT and SN-38 as expected for CPT analogs bearing a six-membered $\alpha$-hydroxylactone ring. In contrast, a linear decay is observed for BN 80915, indicating that the lactone-ring hydrolysis is irreversible. The reaction is slow with about 80% lactone remaining after 1 h of incubation compared to 35% for SN-38 and TPT. After 3 h of incubation 35% of BN 80915 lactone is still present.

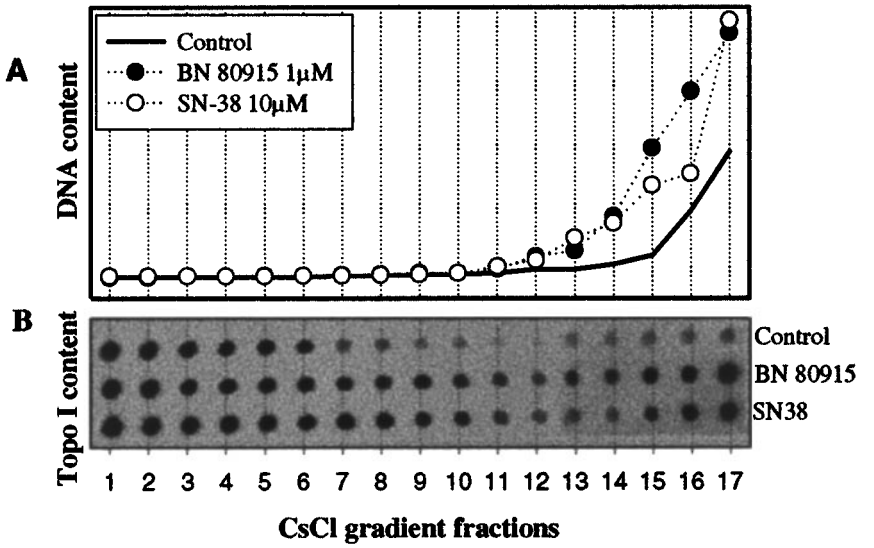


**Figure 4.** Detection of DNA–Topo I complexes in HT29 cells treated with the vehicle (—), 1 μM BN 80915 (●) or 10 μM SN-38 (○). After 1 h incubation, cells were lysed and fractionated using a CsCl gradient. For each fraction are presented the DNA content obtained by fluorometry (A) and the presence of Topo I as revealed on a dot-blot using an antibody specifically directed against Topo I (B).

**Table 1**. *In vitro* cytotoxicity against human tumor cell lines

| Cell line | | $IC_{50}$ (nM) [95% confidence limits] | | | |
|---|---|---|---|---|---|
| Name | Tissue | BN 80915 | | SN-38 | |
| HT29 | colon | 13 | [8–23] | 110 | [80–140] |
| A549 | lung | 19 | [3–130] | 61 | [33–110] |
| T24 | bladder | 0.4 | [0.27–0.6] | 4.59 | [3.57–5.95] |
| MCF7 | breast | 32 | [14–72] | 320 | [88–1300] |
| SKOV3 | ovary | 7.2 | [4.6–11] | 39 | [24–65] |
| DU145 | prostate | 1.6 | [0.86–3.40] | 18 | [12–25] |
| PC3 | prostate | 43 | [23–80] | 130 | [84–190] |
| K562 | leukemia | 0.55 | [0.38–0.8] | 9.2 | [6.2–14] |
| HL60 | leukemia | 0.28 | [0.11–0.7] | 4.67 | [2.74–8.28] |

**Table 2**. Cytotoxicity [$IC_{50}$ (nM)] against couples of resistant compared with sensitive cell lines

| Cell line pairs (resistant/sensitive) | BN 80915 | | | SN-38 | | |
|---|---|---|---|---|---|---|
| | Resistant | Sensitive | $R_f$ | Resistant | Sensitive | $R_f$ |
| T24anp/T24 | 0.372 | 0.4 | 0.93 | 10 | 4.6 | 2.17 |
| MCF7mdr/MCF7 | 460 | 32 | 14.4 | $>10^4$ | 320 | >31 |
| K562adr/K562 | 1.69 | 0.55 | 3 | 35 | 9.2 | 3.8 |
| HL60adr/HL60 | 0.09 | 0.28 | 0.32 | 7.3 | 4.67 | 1.56 |
| HL60dnr/HL60 | 1.14 | 0.28 | 4.07 | 32 | 4.67 | 6.85 |

$R_f$ is determined by the ratio $IC_{50R}/IC_{50S}$.

### BN 80915, orally administrated, potently inhibits tumour growth *in vivo*, in a prostate xenograft model

When implanted s.c. in nude mice, the non-androgen-dependent human prostate adenocarcinoma (PC-3) cells formed tumor-like colonies which, when untreated, grow to cut-off sizes of 1500 $mm^3$ in about 40 days. This *in vivo* model was used to evaluate the efficacy of orally administered BN 80915 in different schedules and to compare BN 80915 (p.o.) with CPT-11 and TPT ( i.p).

BN 80915 was tested in four different schedules: QD × 14, BID × 14, QW × 3 and 4on/3off × 3 (see Materials and methods) at doses approximating the MTD as shown in Figure 6(A). BN 80915 appears highly potent against PC-3 prostate tumor cell growth but, surprisingly, no schedule dependency was observed in this model. In view of this latter observation the QW × 3 protocol was chosen for BN 80915 in the benchmark comparison studies since this is close to the FDA-approved protocol for CPT-11 (weekly i.v. injections for at least 3 or 4 weeks).[29] Since most clinical data available with TPT are based on a once daily treatment for 5 days,[30] this schedule was used in the comparison studies. Reference compounds were administered i.p. according to usual practise in xenograft studies.[31]

Average tumor sizes were plotted on Figure 6(B) and TGD calculated as described in Material and methods. BN 80915 orally administered was clearly more efficacious than TPT and irinotecan in inhibiting tumor growth. Calculation of TGD results in 15 and 7 days for CPT-11 and TPT respectively, while BN 80915 gave a TGD of 27 days.

Moreover, when comparing the time–evolution curves of the tumor size for individual animals (Figure 7) a remarkably homogenous response was observed for BN 80915-treated animals in comparison to the CPT-11- and TPT-treated groups where much greater variability was observed.

## Discussion

BN 80915, a new anticancer agent, belongs to the hCPT family and acts as a Topo I poison. Its poisoning activity was evaluated in various *in vitro* assays and confirmed in living HT29 cells where it clearly stabilizes Topo I–DNA cleavable complexes. BN 80915 was found to be more potent than SN38 or TPT in relaxation as well as cleavage assays. In these experiments BN 80915 generates Topo I-mediated DNA breaks at additional sites. This is characteristic of hCPTs and consistent with a previous observation that changing the CPT lactone from a six- to a seven-membered ring generates new Topo I-mediated DNA cleavage sites, particularly at AAC↓G sequences that are not recognized by CPT[14] and which may lead to increased DNA damage and consequent cell death.

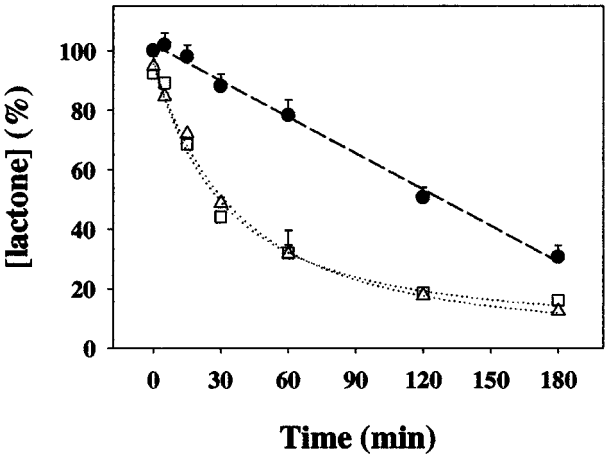


**Figure 5.** HPLC monitoring of the drugs (100 $\mu$M) in human plasma at 37°C and best-fit curves of the experimental points determined by least-squares regression. Stability of the lactone form for BN 80915 (●), SN-38 (□) and TPT (△).

The antiproliferative activities of BN 80915 and SN-38 were tested on a panel of human cancer cell lines. Estimated $IC_{50}$ values, always lower for BN 80915 than for SN-38, confirm its high potency in inhibiting cancer cell growth. Consistent with these results, studies on human colon cancers using a more clinically relevant model of organotypical culture of tumors directly obtained from surgical resection, showed that BN 80915 is significantly more active in inhibiting colon cancer cells proliferation than CPT, SN-38 and TPT.[32]

Interestingly, BN 80915 also outperformed SN-38 when tested on various resistant cell lines overexpressing P-gp (T24anp, HL60dnr, K562adr and MCF7mdr) or mrp (HL60adr and A549) (Table 2). This is of importance since the MDR phenomenon, known to limit the efficacy of chemotherapy of various human cancers by decreased accumulation or increased efflux of cytotoxic drugs, is often associated with overexpression of the MDR proteins P-gp and mrp.[33–35] These results suggest that BN 80915 may not only be a potent cytotoxic agent for a range of tumor types, but also could provide a good alternative to irinotecan for second-line chemotherapy.

Thus, the above results provide evidence that the hCPT BN 80915 is a potent Topo I poison which consistently outperformed the benchmarks in these *in vitro* assays.

In tumor cells, the antitumor activity of CPT analogs is due to the lactone form of the compounds. However, the CPT lactone rings rapidly and reversibly hydrolyze to an equilibrium with a ring-opened carboxylate form, biologically inactive, which at physiological pH represents about 80% of total compound. Thus, a major limitation to CPTs efficacy *in vivo* is related to the concentration of the lactone. The slower hydrolysis of BN 80915, combined with lower binding to serum albumin than CPT derivatives,[15] may lead to enhanced cellular drug delivery, improved pharmacokinetics[36,37] and enhanced effi-

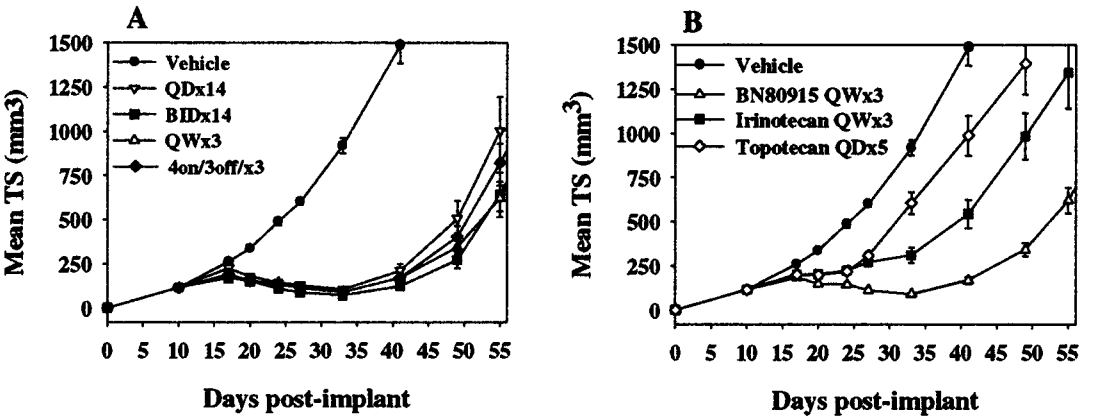


**Figure 6.** Evolution of tumor growth in the non-androgen-dependant human prostate adenocarcinoma PC-3 xenograft model: mean tumor volumes of 10 animals per group. (A) Four different schedules of orally administered BN80915: QD × 14, 0.06 mg/kg once a day for 14 consecutive days; BID × 14, 0.03 mg/kg twice a day for 14 consecutive days; QW × 3, 1 mg/kg once a week for 3 weeks; 4on/3off/ × 3, 0.08 mg/kg once a day for 4 days followed by a rest period of 3 days, this 4/3 cycle repeated twice more for a total of 12 administrations over the course of 3 weeks. (B) Comparison of tumor growth response to BN 80915 (QW × 3, p.o 1 mg/kg), CPT-11 (QW × 3, i.p. 100 mg/kg) and TPT (QD × 5, i.p. 3 mg/kg).

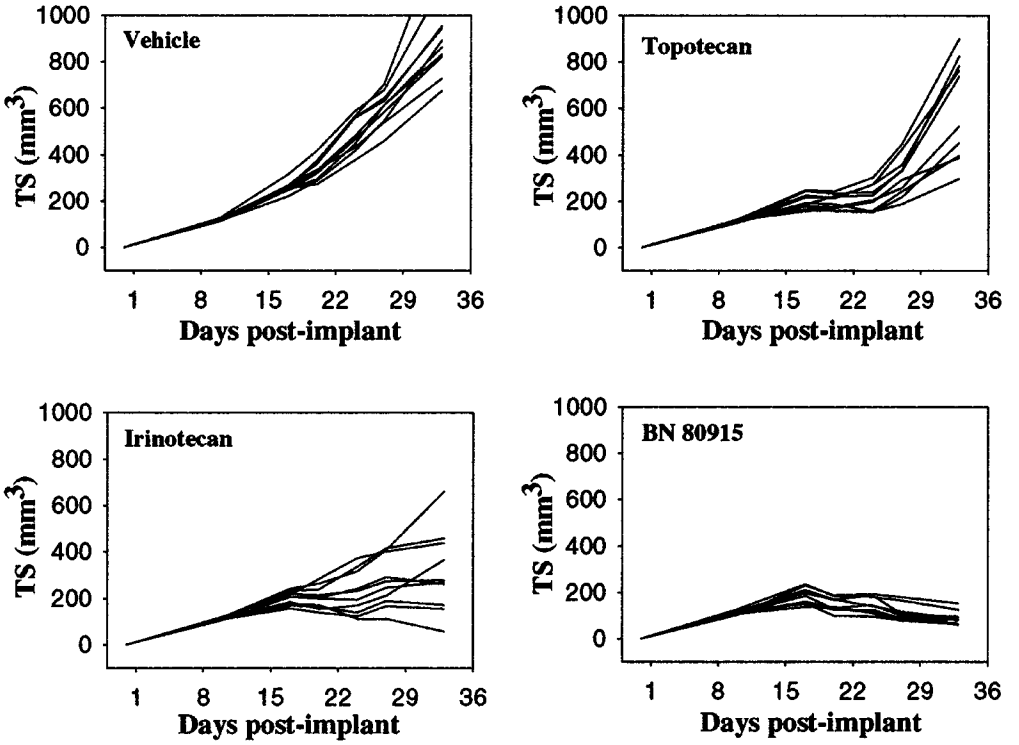


**Figure 7.** Comparison of intra-group variability (10 animals per group) of the tumor growth evolution in the non-androgen-dependant human prostate adenocarcinoma PC-3 xenograft model in response to treatment with BN 80915 (QW × 3, p.o 1 mg/kg), CPT-11 (QW × 3, i.p. 100 mg/kg) and TPT (QD × 5, i.p. 3 mg/kg). Each curve represents the tumor growth evolution of an individual nude mouse.

cacy *in vivo*. Another probable advantage for BN 80915 results from the irreversibility of the E-ring hydrolysis which will avoid accumulation of the active compound during excretion in the urinary tract and the resulting hemorraghic cystitis observed in several clinical trials.[9,38]

Prolonged, continuous, treatment schedules *in vivo* with CPTs tend to produce better results.[39,40]

Continuous i.v. infusions have been shown to be effective but the i.v. route is a source of discomfort and stress to patients,[41] whereas prolonged drug administration is facilitated by oral administration. Furthermore, in order to improve the stability, pharmacokinetic and toxicity profile of CPT analogs, many investigators have initiated clinical trials using the oral route of administration.[12,42] Further development of oral formulations of BN 80915 seems also attractive, hence *in vivo* xenograft studies were conducted by oral gavage.

Preliminary assays on athymic mice xenografted with PC-3 human prostate adenocarcinoma were performed using low oral doses of BN 80915 in different schedules. BN 80915 induced potent tumor regression in all cases. In the benchmark comparison studies (Figure 6B), BN 80915 administered once a week for 3 weeks (QW × 3) was shown to be more active than CPT-11 or TPT administrated QW × 3 and QD × 5, respectively. The results are remarkable in view of the doses employed and the difference in the route of administration: 1 mg/kg of BN 80915 p.o. is sufficient to give a TGD of 27 days compared to 15 days with CPT-11 at 100 mg/kg i.p. and 7 days with TPT at 3 mg/kg i.p. In addition, the tumor growth inhibitions observed, in this model, for individual animals (Figure 7) are homogenous in the BN 80915-treated group in contrast to CPT-11 or TPT-treated groups. This is of importance since several phase one trials of oral CPTs have reported substantial inter-patient variability in systemic exposure[12] which increase the difficulties in the dosage/toxicity relationships, and may result in insufficient antitumor activity in some patients and enhanced risk of toxicity in others. Hence low inter-individual variability for BN 80915 would be an obvious advantage, if such xenograft results could be extrapolated to man.

In conclusion, the hCPT BN 80915 is a new Topo I poison with unique features that lead to remarkable efficiency both *in vitro* and *in vivo*. BN 80915 consistently outperformed the clinically relevant benchmarks CPT-11 (or its active metabolite SN-38) or TPT in these preclinical studies. Thus, clinical trials have been initiated on the basis of this promising profile.

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