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BN80927: A Novel Homocamptothecin That Inhibits Proliferation of Human Tumor Cells *in Vitro* and *in Vivo*

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ABSTRACT

BN80927 belongs to a novel family of camptothecin analogs, the homocamptothecins, developed on the concept of topoisomerase I (Topo I) inhibition and characterized by a stable seven-membered β-hydroxylactone ring. Preclinical data reported here show that BN80927 retains Topo I poisoning activity in cell-free assay (DNA relaxation) as well as in living cells, in which *in vivo* complexes of topoisomerase experiments and quantification of DNA-protein-complexes stabilization, have confirmed the higher potency of BN80927 as compared with the Topo I inhibitor SN38. In addition, BN80927 inhibits Topo II-mediated DNA relaxation *in vitro* but without cleavable-complex stabilization, thus indicating catalytic inhibition. Moreover, a Topo I-altered cell line (KBSTP2), resistant to SN38, remains sensitive to BN80927, suggesting that a part of the antiproliferative effects of BN80927 are mediated by a Topo I-independent pathway. This hypothesis is also supported by *in vitro* data showing an antiproliferative activity of BN80927 on a model of resistance related to the noncycling state of cells (G_0-G_1 synchronized). In cell growth assays, BN80927 is a very potent antiproliferative agent as shown by IC_{50} values consistently lower than those of SN38 in tumor cell lines as well as in their related drug-resistant lines. BN80927 shows high efficiency *in vivo* in tumor xenograft studies using human androgen-independent prostate tumors PC3 and DU145. Altogether, these data strongly support the clinical development of BN80927.

INTRODUCTION

The preparation of camptothecin (CPT) analogs has led to the discovery of the anticancer drugs topotecan (TPT; Hycamtin; Refs. 1, 2) and irinotecan (CPT11; Campto; Refs. 2, 3), which are becoming the standards of treatment for ovarian and colon cancers, respectively, in addition to a large number of compounds undergoing clinical or preclinical evaluation. Exerting remarkable antiproliferative activity over a broad spectrum of experimental tumor types, CPT analogs (4) have been shown to target topoisomerase I (Topo I; Ref. 5). Their mechanism of action consists in the stabilization of normally transient DNA-Topo I complexes resulting in irreversible DNA damage (6). An innovative modification of the CPT lactone ring (7), changing the six-membered α-hydroxylactone ring to a seven-membered β-hydroxylactone ring led to the development of the homocamptothecin (hCPT) family. Surprisingly, this modification of the crucial E-ring of CPT conserves Topo I inhibition (8), and hCPTs show high activity *in vitro* and *in vivo* (9). In contrast to classical six-membered lactones, hCPTs undergo slow, linear, ring opening in plasma. The apparent lack of recyclization to the cytotoxic lactone at physiological pHs may also enable hCPTs to circumvent the urinary track toxicity encountered with classical CPTs possessing low aqueous solubility. In the course of an analog generation program, several promising molecules were identified (10–12) including BN80915 (13–16) and BN80927 (17–19), presently under evaluation for clinical applications (Fig. 1).

Here, we report the characterization of BN80927: plasma stability, mechanism of action, *in vitro* antiproliferative activities, and antitumor effects in animal models bearing androgen-independent prostate tumors.

MATERIALS AND METHODS

Drugs. BN80927, BN80915, SN38, and topotecan were prepared according to previously published procedures (19, 20). CPT was purchased from Janssen (Bischeim, France), and etoposide (VP-16) from Sigma (La Verpillière, France). The drugs were dissolved at 10^{-2} M in the appropriate solvent, water for topotecan and *N,N*-dimethylacetamide (Aldrich) for CPT, SN38, BN80915, and BN80927, and were further diluted with the appropriate assay medium.

Drug Stability in Plasma. To 500-μl fractions of pooled human or mouse plasma, preincubated at 37°C for 5 min, were added 5 μl of a 10^{-2} M drug solution, and the samples were incubated in capped vials (to prevent CO_2 loss), at 37°C. At defined times, three vials were opened, and the plasma proteins were precipitated by the addition of 2 ml of cold acetonitrile at −50°C. The resulting mixture was centrifuged (2000 × *g*) at 4°C for 5 min, and the supernatant was analyzed immediately to avoid further chemical transformation. Samples were run on a 5-μm Nucleosil C18 column (4.6 × 150 mm) at 22°C with a flow rate of 1 ml/min in an isocratic eluent composed of 1 M tetrabutylammonium dihydrogenophosphate/acetonitrile/75 mM, ammonium acetate (pH 6.9) at 5/250/750 (v/v/v). The eluted analytes were detected at 380 nm for SN38 and 360 nm for BN80927. The results are expressed using the best-fitting curves determined by least square linear regression.

DNA Relaxation and Decatenation Assays. Relaxation assays were performed as originally described by Jaxel *et al.* (21) and later modified by Lesueur-Ginot *et al.* (9). Supercoiled plasmid DNA pUC19 (300 ng; Pharmacia) was incubated with Topo I (Life Technologies, Inc.) or human Topo II (TopoGen Inc., Colombus, OH) in the presence of the drugs at defined concentrations (final range, 10–200 μM). *N,N*-Dimethylacetamide was diluted in the same way as the drugs, and used as negative control; SN38 and VP-16 were used as positive controls for Topo I and Topo II assays, respectively. Reactions were carried out for 15 min at 37°C and were stopped by a treatment with SDS/proteinase K (Boehringer Mannheim, Meylan, France) for 30 min at 37°C. Samples were run on electrophoresis in 1.2% agarose gels for 20 h in a phosphate buffer containing 2 mg/ml chloroquine. The gels were further stained with 2 mg/ml ethidium bromide. Each experiment was run in duplicate.

***In Vivo* Complexes of Topoisomerase Bioassay.** The *in vivo* complex(es) of topoisomerase (ICT) bioassay was used to detect the covalent DNA-Topo I complexes formed in living HT29 cells (22). A commercially available "*in vivo* Topo I-link kit" (TopoGen Inc.) was used with some modifications, described previously (13). Cells were treated with the drugs (BN80927, SN38, or VP-16) for 60 min at 37°C and were washed and lysed. Each lysate was overlaid onto a cesium chloride (CsCl) gradient of four different densities (1.37, 1.5, 1.72, and 1.82 g/ml). Gradient tubes were centrifuged, and fractions were collected. For each fraction, the DNA content was determined with a Pico-Green double-strand DNA fluorescence enhancement kit (Ref. 23; Molecular Probes, Eugene, OR); The Topo I or II content was determined by immunoblotting analysis, as described previously (13).

Formation of Covalent DNA-Protein-Complexes in Intact HT29 Cells. DNA-protein-complexes (DPCs) can be trapped within cells and quantified by the KCl-SDS coprecipitation assay (24). DNA and proteins of HT29 cells (5×10^5 cells) were radiolabeled for 18 h with, respectively, 0.6 μCi/ml [^{3}H]thymidine (Amersham) and 0.1 μCi/ml [^{14}C]leucine (Amersham). Cells were then exposed to the drugs at 37°C for various concentrations and times and were lysed in a SDS-containing solution; the lysates were precipitated as

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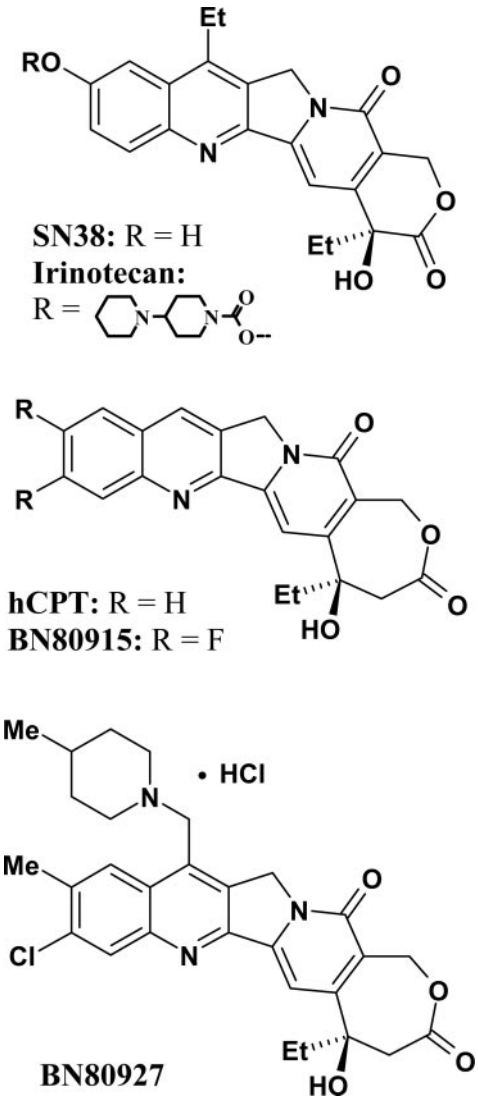

Fig. 1. Chemical structures. Chemical structure of the homocamptothecin (*hCPT*) BN80927 (*bottom*) together with the unsubstituted hCPT, the difluoro-hCPT BN80915 (diflomotecan; *middle*), and the camptothecin analog SN38 (*top*), active metabolite of irinotecan.

described previously (16). Data are expressed as the ratio of ^{3}H-DNA to ^{14}C-protein, with the protein being an internal standard for the number of cells used. All of the experiments were performed three times with each point in duplicate. For concentration-response experiments, HT29 cells were incubated for 1 h with concentrations ranging from 0 to 10 μM. For reversion experiments, HT29 cells were exposed to 500 nM BN80927 or 1000 nM SN38 for 1 h at 37°C. The drugs were then removed, and the cells were incubated in fresh medium from 0 to 240 min. Statistical analysis was performed by the Student *t* test (concentration-response experiments) or by a two-factor ANOVA without replication (reversion experiments).

Cell Culture. Fifteen human tumor cell lines were used. HT29 (colon), DU145 and PC3 (prostate), SKOV3 (ovary), A549 (lung), and MCF7 (breast) were purchased from American Type Culture Collection. T24 (bladder) sensitive and resistant cell lines (T24s and T24anp) were a generous gift from Professor R. Kiss (Université Libre de Bruxelles, Brussels, Belgium). K562 (leukemia)-sensitive and Adriamycin-resistant cell lines (K562s and K562adr) and HL60 (leukemia)-sensitive, HL60 daunorubicin-resistant and HL60 Adriamycin-resistant cell lines (HL60s, HL60dnr, and HL60adr) were a generous gift from Dr. Anne-Marie Faussat (Hôpital Hotel-Dieu, Paris, France). KB (epidermis) and the resistant KB-STP2 cell lines were kindly provided by Dr. Michihiko Kuwano (Kyushu University, Fukuoka, Japan). All of the cell lines were cultured in DMEM or Eagles MEM at 4.5 g/liter glucose supplemented with 10% heated inactivated FCS plus 2 mM glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (Life Technologies, Inc.), at 37°C in a humidified atmosphere containing 5% CO_2.

Multidrug-Resistance Positive Cell Lines. K562adr, HL60adr, HL60dnr, and T24anp cell lines were derived from their respective sensitive cell lines by prolonged exposure to adriamycine (adr), daunorubicin (dnr) or a cocktail of Adriamycin, navelbine, and PE1001 (anp). Those resistant cell lines show a classical multidrug resistance (MDR) phenotype with overexpression of P-glycoprotein for K562adr, HL60dnr, and T24anp cells and overexpression of the MDR-associated protein for HL60adr and A549 cells.

Topo I-Inhibitor Resistant Cell Line. The KB-STP2 resistant cell line was established by stepwise exposure of parental KB cells to the dual topoisomerase inhibitor saintopin and showed a 44-fold increase in resistance to saintopin relative to that of KB cells (25). Taniguchi *et al.* (25) suggested that the acquirement of drug resistance in KB-STP-2 cells is associated with the decrease in Topo I mRNA levels as a consequence of Topo I-inhibitor treatment, because KB/STP2 showed only a small decrease in sensitivity to Topo II inhibitor VP-16 but developed a marked cross-resistance to Topo I inhibitor SN38.

Antiproliferative Assays. Growth inhibition was evaluated by the WST-1 (water-soluble tetrazolium salt) colorimetric assay (Boehringer Mannheim), as described previously (9). Exponentially growing cells were seeded on a 96-well microtiter plate for 24 h before a 72-h treatment with drugs at final concentrations ranging from 5.12×10^{-13} to 1×10^{-6} M. For each drug, the data points that were 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. The effects of compounds on resistant cell lines were reported in terms of resistance factor(s) (*Rf*), defined as the ratio of the IC_{50} value obtained with the resistant cell line to the IC_{50} value obtained with the parent cell line. Antiproliferative experiments with the KB cell lines were performed by Oncodesign Biotechnology Inc. (Dijon, France) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay similarly to the WST-1 assay used above. IC_{50} values and *Rf* were estimated and reported as above.

Resting Cells: Synchronization of HT29 Cells in G_0-G_1 Phase of the Cell Cycle. HT29 cells were exposed for 48 h to 20 mM glucosamine (Sigma); this stress condition results in synchronization of HT29 cells in the G_0-G_1 phase of the cell cycle as described by Tomida and Tsuruo (26). The synchronization in G_0-G_1 phase of the cell cycle was confirmed by simultaneous flow-cytometric measurement of the expression of ki-67 (Ref. 27; specific nuclear antigen that reveals cells undergoing active division) and DNA staining with propidium iodide. Flow-cytometric analyses were carried out on a FACStar+ flow cytometer (Becton Dickinson, Le Pont de Claix, France).

Resting Cells: Determination of Cell Survival after Drug Treatment. After synchronization, cell cultures were incubated for 16 h in the presence of drugs at concentrations ranging from 0.64 to 400 nM. After medium removal and cell washes, fresh medium containing 10% FCS was added, and cells were restimulated to grow for an additional 72 h. Cell survival was quantified by determining the proportion of living cells, expressed as a percentage of the untreated population, using a Coulter counter (ZM Counter; Coultronics France S.A., Margency, France).

Schedule Dependency of BN80927 Antitumor Activity in Prostate Cancer Xenografted in Athymic Mice. Tumors were established by s.c. injection of non-androgen-dependent human prostate adenocarcinoma cells (5×10^6 cells per animal, on the left dorsal surface) in 4–6-week-old NCr nu/nu female athymic nude mice (National Cancer Institute, Frederick, MD). Mice were pair-matched, and treatments were initiated when tumors reached a mean group size of 104–128 mg for PC3 or 102–127 mg for DU145. Tumor volume (mm^3) was calculated as $(w^2 \times l)/2$, where w = width and l = length, as measured with calipers. BN80927 was formulated in 3% *N*,*N*-dimethylacetamide/1.8% Montanox 80/0.2% NaCl and was administered p.o. according to the schedules indicated in Fig. 7. The mice were monitored individually until day 50 postinjection. Mean day of survival (MDS) values were determined for all of the groups. Mice were weighed twice weekly and were examined for clinical signs. Animal care was in accordance with institutional guidelines.

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Comparison of BN80927, BN80915 and Irinotecan Activities in Androgen-Independent Prostate Tumors PC-3 and DU145. With the same protocols as described above, BN80927 p.o. at 5 mg/kg twice a day for 14 days, was compared with BN80915 p.o. at 0.03 mg/kg twice a day for 14 days. Both compounds were used at their respective maximum tolerated dose in this schedule. Irinotecan (28) was used as a clinically approved agent, administered i.p. at 100 mg/kg on a schedule of every week for 3 weeks.

RESULTS

BN80927 Is a Highly Stable hCPT Analog

Fig. 2*A* shows that more than 90% of BN80927 remains intact after 180 min of incubation in human plasma. Under the same conditions, the concentration of CPT analog SN38 decays exponentially to rapidly reach an equilibrium of about 20% lactone and 80% ring-opened compound. Lactone hydrolysis appears to be slightly faster in mouse plasma than in human plasma, but similar patterns are observed for each compound in both species. The BN80927 stability in human plasma was monitored for 24 h to estimate its *in vitro* plasma half-life. As shown on Fig. 2*B*, 50% of BN80927 was still in its lactone form after 21 h of incubation, in contrast to less than 30 min for SN38 (Fig. 2*A*).

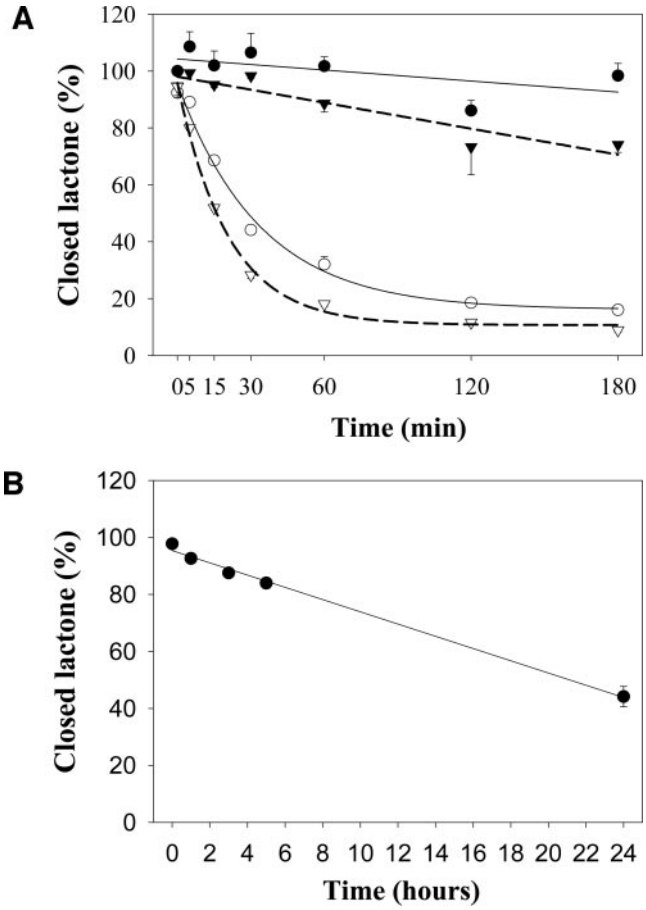

Fig. 2. Comparison study of lactone stability between BN80927 and SN38. High-performance liquid chromatography monitoring of the compounds in human plasma at 37°C over a 180-min period (*A*) for BN80927 in human (●) or mouse (▼) plasma and for SN38 in human (○) or mouse (▽) plasma. BN80927 lactone stability in human plasma over a 24-h period (*B*). Best-fit curves of the experimental points were determined by least square regression.

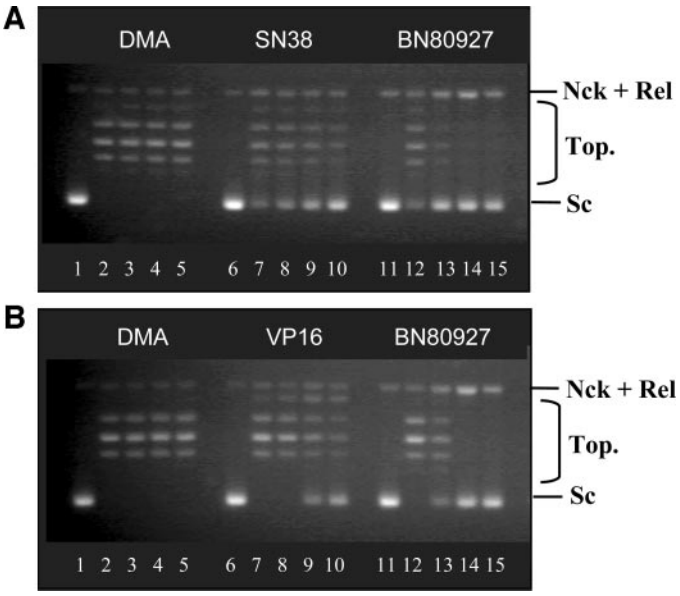

Fig. 3. Effect of increasing concentrations of BN80927 on plasmid DNA relaxation by topoisomerase I (Topo I) or Topo II; comparison with SN38 or with etoposide (VP-16). Native supercoiled pUC19-DNA (0.3 μg) was incubated with Topo I (*gel A*) or Topo II (*gel B*) in the presence of BN80927 (*Lanes 12–15*), or SN38 (*Lanes 7–10, gel A*) or VP-16 (*Lanes 7–10, gel B*) at 10, 50, 100, and 200 μM. *N,N*-dimethylacetamide (*DMA*; solvent diluted in the same way as the drugs) is used as negative control (*Lanes 2–5*). The first *Lane* of each group (*1, 6,* and *11*) correspond to the highest concentration of drug or solvent incubated in the presence of DNA but without enzyme, to see a possible direct effect of the drug on DNA. DNA samples were separated by electrophoresis on agarose gels. Gels were then stained in ethidium bromide and photographed under UV light. *Nck+Rel*, nicked DNA + fully relaxed DNA; *Top.*, topoisomers; *Sc*, supercoiled DNA.

BN80927 Inhibits Topo I- and Topo II-Mediated Relaxation of Supercoiled DNA

In the absence of topoisomerase enzyme (Fig. 3, *Lanes 1, 6,* and *11*), the compounds did not induce any DNA band changes in the gel. The ability of BN80927 to inhibit both Topo I- and Topo II-catalyzed relaxation of supercoiled DNA is illustrated in Fig. 3, *A* and *B*, respectively. The BN80927 concentrations leading to an inhibition of Topo I and Topo II are visualized from 10 μM (*Lane 12*) on Fig. 3*A* and 50 μM (*Lane 13*) on Fig. 3*B*, respectively. SN38 (Fig. 3*A*, *Lanes 7–10*) and VP-16 (Fig. 3*B*, *Lanes 7–10*) were used as positive controls for Topo I and Topo II assays, respectively.

BN80927 Promotes the Accumulation of Topo I-DNA Complexes in Living Cells

HT29 cells were lysed and fractionated on a CsCl gradient. For each fraction, Topo I or Topo II dot blots are shown in Fig. 4. (DNA-containing fractions, as measured by fluorometry, are *outlined in black*.) With control untreated cells, free enzymes (Topo I or Topo II) were found at the top of the CsCl gradient (*corresponding to the left part of the dot blot*), whereas DNA was found at the bottom of the gradient (*right part of the dot blot*). When HT29 cells were treated for 1 h with 10 μM BN80927 or SN38, the DNA-containing fractions were found to be immunoreactive to the anti-Topo I antibody, revealing the stabilization of covalent DNA-Topo I complexes (*right part of the dot blot*, Fig. 4*A*). Although the immunoassay is essentially qualitative, it is interesting to note that DNA-bound Topo I was more abundant with BN80927 than with SN38. This suggests that BN80927 stabilizes a greater number of cleavable complexes than does SN38. Even at the 1 μM dose, Topo I-DNA complexes were still detected with BN80927 but not with SN38 (data not shown).

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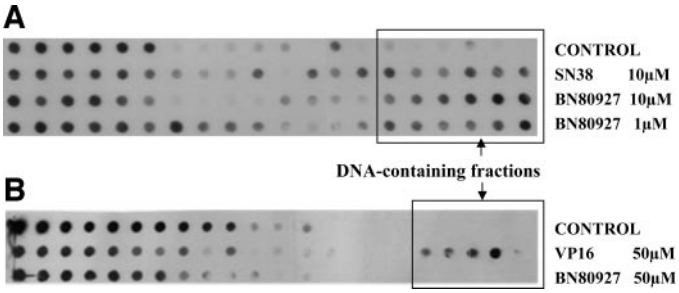

Fig. 4. Detection of DNA-topoisomerase I (DNA-Topo I) or DNA-Topo II cleavable complexes in BN80927-treated HT29 cells; comparison with SN38 or with etoposide (VP-16). Cells treated with BN80927 were compared with cells treated with the solvent alone (*CONTROL*), SN38, or VP-16, at indicated concentrations. After 1-h incubation, cells were lysed and fractionated using a CsCl gradient. For each fraction, DNA content was detected by fluorometry (data not shown), and the presence of Topo I or Topo II was revealed on a dot-blot using antibodies specifically directed against Topo I (*blot A*) or Topo II (*blot B*). *In the frames on the right side of the blots,* DNA-topoisomerase complexes (DNA-containing fractions).

BN80927 Does Not Promote the Accumulation of Topo II-DNA Complexes in Living Cells

When ICT experiments were repeated with Topo II antibodies on HT29 cells treated with 50 μM BN80927 or VP-16 (Fig. 4*B*), the immunoblot did not show any appreciable amount of BN80927 with stabilized DNA-Topo II-cleavable complexes, whereas such complexes were observed in the presence of VP-16.

DPCs Induced by BN80927 Are Formed More Rapidly and Are More Stable Than Those Induced by SN38

The ability of BN80927 and SN38 to induce DPCs in living HT29 cells was quantified by the KCl-SDS coprecipitation assay. In a first series of experiments, a concentration-dependent formation of DPCs after 1 h of drug exposure was observed. The results in Fig. 5*A* show that BN80927 induced significantly more DPCs than did SN38, with about 3-fold higher levels of concentration, at 0.02, 0.1, and 0.5 μM. With each drug, the amount of DPCs increased in a dose-dependent manner, then reached a plateau of about 10 times more DPCs than in the basal level of control cells. A concentration of >1000 nM SN38 was needed to reach this plateau, compared with ~500 nM for BN80927. These critical concentrations needed to reach the plateau were used in the DPCs reversion experiments. The BN80927-induced DPCs were found to be more stable than those induced by SN38 (Fig. 5*B*) because ~85% of BN80927-induced DPCs *versus* 45% of SN38-induced DPCs remained at 15 min and 70% *versus* 35%, respectively, remained at 30 min. The differences in remaining DPCs at 15 and 30 min were statistically significant ($P = 0.084$ and 0.032, respectively). After 1 h for BN80927 and 0.5 h for SN38, the DPC levels reached a plateau with, respectively, about 40–45% and 30–35% of DPCs that were not reversed (no statistical difference). Together, these experiments demonstrate the higher potency of BN80927 in inducing and stabilizing DPCs as compared with SN38.

BN80927 Is Strongly Active against Exponentially Growing Tumor Cells *in Vitro*

The antiproliferative activity of the drugs were determined on various human tumor cell lines from a diverse set of target organs, including leukemia and solid tumors (colon, prostate, ovary, lung, bladder, and breast cell lines). As shown in Table 1, BN80927 was an antiproliferative agent on all of the cell lines tested. On this set of cell lines, BN80927 (IC_{50} values, 2–81 nM) was consistently more potent than SN38 (IC_{50} values, 4–318 nM); however, the patterns of sensitivity were similar.

BN80927 Remains Highly Active against Several Types of Drug-Resistant Tumor Cells

The effects of BN80927 were investigated on three distinct types of drug-resistant cell lines including (*a*) cell lines expressing different multidrug-resistant pumps, (*b*) a tumor cell line with altered Topo I expression, and (*c*) nonproliferative tumor cells.

BN80927 Remains Highly Active against MDR Expressing Cells. Four MDR-expressing cell lines derived from their respective wild-type parent cell line by drug selection, K562adr, HL60adr, HL60dnr, and T24anp were used and were compared with their parental cells for their sensitivity to BN80927. As shown in Table 2, BN80927 performed better than the benchmarks on these resistant cell lines in terms of cytotoxic activity. Moreover, the *Rf* values were consistently lower for BN80927 than those of the resistance-inducing agents (adr, dnr). This shows that BN80927 overcomes MDR resistance, which suggests that BN80927 is not a substrate for these pumps.

BN80927 Remains Active against Cells Resistant to Topo I Inhibitors. Table 3 shows that, as expected, compared with the parental KB cell line, the variant KB/STP2 showed only a small decrease in sensitivity to Topo II-inhibitor VP-16 (*Rf*, 1.8) but pre-

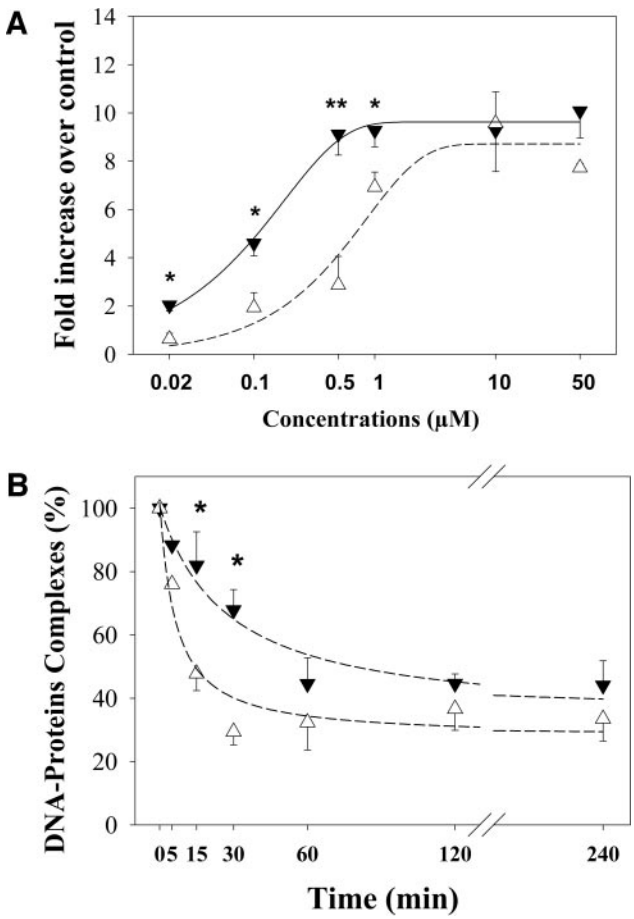

Fig. 5. DNA-protein complex (DPC) stabilization by BN80927 in HT29 cells. DNA and proteins were respectively radiolabeled with 0.6 μCi/ml [^{3}H]thymidine and 0.1 μCi/ml [^{14}C]leucine for 18 h. Cells were then exposed to drugs [BN80927 (▼) or SN38 (△] at various concentration and time, at 37°C. KCl-SDS coprecipitation of proteins, followed by determination of radioactivity, allowed a quantification of DPCs. Data are expressed by the ratio of ^{3}H-DNA to ^{14}C-protein. Statistical analyses were performed by the Student *t* test (dose-response experiments) or by a two-factor ANOVA without replication (reversion experiments). For dose-response experiments (*A*), HT29 cells were incubated for 1 h with concentrations of drugs ranging from 0 to 50 μM; *, $P < 0.05$; **, $P < 0.005$. For reversion experiments (*B*), HT29 cells were exposed to 0.5 μM BN80927 or 1 μM SN38 for 1 h at 37°C; *, $P < 0.1$. Drugs were then removed, and the cells were incubated in fresh medium for 0–240 min.

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Table 1 *Cytotoxicity of BN80927, SN38, and VP-16*[a] *in a panel of nine human tumor cell lines*

Tissue	Cell line	IC_{50}, nM [95% confidence interval]		
		BN80927	SN38	VP-16
Breast	MCF7	48 [20–120]	318 [88–1300]	100,000
Colon	HT29	21 [11–38]	106 [80–140]	4,500 [2,200–10,000]
Prostate	DU145	3 [1.6–6]	18 [12–25]	1,300 [410–4,000]
	PC3	81 [44–156]	126 [84–192]	> 10,000
Ovary	SKOV3	13 [10–16]	39 [24–65]	4,900 [1,400–23,000]
Bladder	T24	2.2 [1.2–4.2]	4.6 [3.6–6.0]	2,700 [1,200–5,500]
Leukemia	HL60	7.4 [1.8–30]	8.4 [1.1–64]	1,300 [80–8,000]
	K562	8.4 [5–14]	9.2 [6–14]	1,400 [640–3,140]
Lung	A549	17 [7.5–40]	61 [33–112]	> 10,000

[a] VP-16, etoposide.

Table 2 *Cytotoxicity against pairs of resistant and sensitive cell lines*

Cell line pairs (Resistance inducer)	Compounds	IC_{50}, nM		Rf[a] $IC_{50}R$:$C_{50}S$
		Resistant	Sensitive	
T24anp/T24 (Pgp)	BN80927	2.9	2.2	1
	SN38	10	4.6	2
	adr	310	20	16
	VP-16	2470	2640	1
K562adr/K562 (Pgp)	BN80927	18	8.4	2
	SN38	35	9.2	4
	adr	5030	5.3	100
	VP-16	14700	1430	10
HL60adr/HL60 (mrp)	BN80927	1.3	7.4	0.2
	SN38	10	8.4	1.2
	adr	3600	18	200
	VP-16	7400	1260	6
HL60dnr/HL60 (Pgp)	BN80927	32	7.4	4
	SN38	83	8.4	10
	dnr	1990	11	180
	VP-16	44000	1260	35

[a] *Rf*, resistance factor [defined as the ratio of the IC_{50} value obtained with the resistant cell line ($IC_{50}R$) to the one obtained with parent cell line ($IC_{50}S$)]; anp, Adriamycin, navelbine, and PE1001; Pgp, P-glycoprotein; adr, adriamycine; mrp, multidrug resistance-associated protein; dnr, daunorubicin.

sented a marked resistance to Topo I inhibitor SN38 (*Rf*, 9). BN80927 was highly potent on both sensitive and resistant KB cell lines with IC_{50} values lower than those obtained with SN38 and VP-16. It is noteworthy that the *Rf* value calculated for BN80927 (*Rf*, 2.6) is lower than that of SN38 (*Rf*, 9), suggesting an antiproliferative activity independent of Topo-I inhibition.

BN80927 Is Active on Resting Tumor Cells. Exponentially growing HT29 cells were exposed to 20 mM glucosamine for 2 days to achieve a selective accumulation of at least 90% of the cell population in G_0-G_1 phase of the cell cycle as confirmed by monitoring the expression of ki-67, a specific marker of cell proliferation (data not shown). The tested compounds were then added to the culture medium at various concentrations for 16 h. G_0-G_1 cell synchronization was confirmed just after compound and stress removal (data not shown); the cells were then cultured in drug-free medium for an additional 72 h. The sensitivity of G_0-G_1 HT29 cells to BN80927 is clearly shown in Fig. 6; the cell survival rate is concentration-dependent, which allows an estimation of the IC_{50} (10 nM). In comparison, G_0-G_1 HT29 cells exhibited only a limited sensitivity to BN80915 and high resistance to SN38 or CPT. VP-16 was also tested up to 10 μM without any inhibitory activity observed (data not shown).

BN80927, Orally Administrated, Inhibits Tumor Growth *in Vivo* in Two Human Androgen-Independent Prostate Xenograft Models

On the basis of the encouraging *in vitro* data, the antiproliferative activity of BN80927 was evaluated in human tumor models xenografted in animals. Using two human androgen-insensitive (hormone-refractory) prostate adenocarcinoma cell line, PC3 and DU145, we tested BN80927 in four different schedules: every day for 14 days, twice a day for 14 days, every week for 3 weeks, and 4-days-on/3-days-off for three cycles. BN80927 was highly potent in both models (average tumor sizes plotted on Fig. 7). In both models, the preferred schedule was twice a day for 14 days. In the PC3 study, calculation of the MDS for BN80927 on this twice-a-day-for-14-days schedule, resulted in a survival of 69.9 ± 2.8 days which translates to a 125% survival increase compared with matched control mice (MDS, 31 days). Similarly in the DU145 study, calculation of MDS for BN80927 in the twice-a-day-for-14-days schedule resulted in a survival of 66.1 ± 5.2 days, which translates to a 175% survival increase compared with matched control mice (MDS, 24 days). Moreover, no toxic death occurred after BN80927 treatment in either xenograft models, and the body weight loss never exceeded 10%. After treatment ended, the weight of all of the animals rebounded.

Comparison of BN80927 with Other Topo I Inhibitors in Two Human Androgen-Independent Prostate Xenograft Models

Antitumor activities of BN80927 have been compared with the clinically approved Topo I inhibitor irinotecan (prodrug of SN38) and to another hCPT, BN80915, presently evaluated in clinical trials. The three compounds have been tested at doses and regimens close to their maximum tolerated dosage (Fig. 8). In both models, irinotecan,

Table 3 *Cytotoxicity against topoisomerase I (Topo I) inhibitor-resistant cell line*

Cell line pairs	Compound	IC_{50}, nM		Rf[a] IC_{50R}:IC_{50S}
		Resistant	Sensitive	
KB-STP2/KB (T1 down-regulated)	BN80927	230	90	2.6
	SN38	3000	330	9
	VP-16	5600	3100	1.8

[a] *Rf*, resistance factor [defined as the ratio of the IC_{50} value obtained with the resistant cell line ($IC_{50}R$) to the one obtained with parent cell line ($IC_{50}S$)]; VP-16, etoposide.

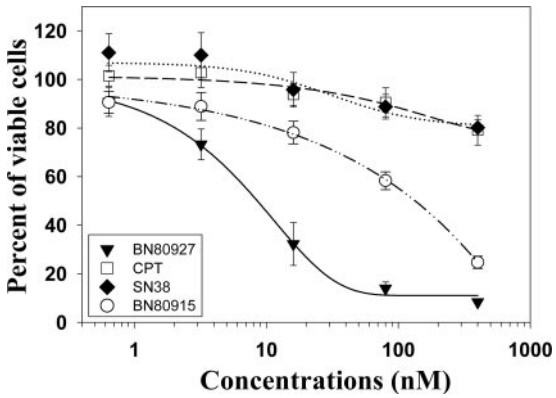

Fig. 6. Antiproliferative effects of BN80927 on human tumor resting cells *in vitro*; comparison with SN38 and camptothecin (*CPT*). Synchronized G_0-G_1 HT29 cells were exposed to the compounds for 16 h. Cell survival was determined 72 h after drug and stressor removal.

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BN80915, and BN80927 reduced the tumor growth rate. In both models, BN80915 and BN80927 compared well with irinotecan. In the PC3 model, BN80927 and BN80915 were equally efficacious, whereas in the DU145 model, BN80927 was more effective than BN80915. However, it is noteworthy that higher doses of BN80927 were required to achieve antitumor responses in comparison with BN80915.

DISCUSSION

The CPTs form a rapidly growing family of antitumor agents with high *in vitro* antiproliferative activities and impressive *in vivo* efficacy in preclinical tumor models. Among these compounds, the hCPT analogs represent an interesting group with improved stability due to the modified E-ring (29). The hCPTs were initially exemplified with BN80915 (diflomotecan; Refs. 13–16), a neutral molecule with two fluorine substituents at positions 10 and 11 in the A-ring (Fig. 1), that is currently undergoing Phase II clinical trials. The present paper presents the pharmacological characterization of a new hCPT, BN80927, which has a methyl group at position 10 and a chlorine at position 11 in the A ring, and a protonated 4-methyl-piperazinomethyl group at position 7 in the B ring (Refs. 17–19; Fig. 1).

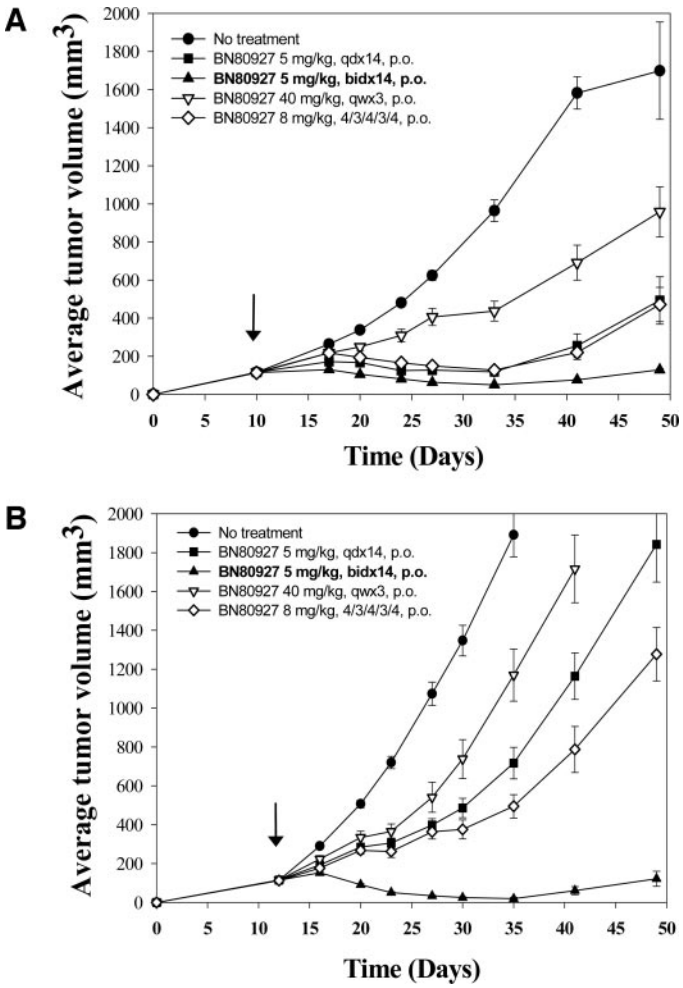

Fig. 7. Evolution of tumor growth in non-androgen-dependant human prostate adenocarcinoma PC-3 and DU145 xenograft models. BN80927 schedule dependency in PC3 (*A*) and DU145 (*B*) models. Four different schedules (numbered list *a–c*) of orally administered BN80927 were as follows: (*a*) *5 mg/kg, qd×14*, 5 mg/kg every day for 14 consecutive days; (*b*) *5 mg/kg, bid×14*, 5 mg/kg twice a day for 14 consecutive days; (*c*) *40 mg/kg, qwk×3*, 40 mg/kg once a week for 3 weeks; and (*d*) *8 mg/kg, 4/3/4/3/4*, 8 mg/kg once a day for 4 days followed by a rest period of 3 days (a 4/3 cycle) with the cycle repeated twice more for a total of 12 administrations over the course of 3 weeks. *Black arrows*, the start of treatments.

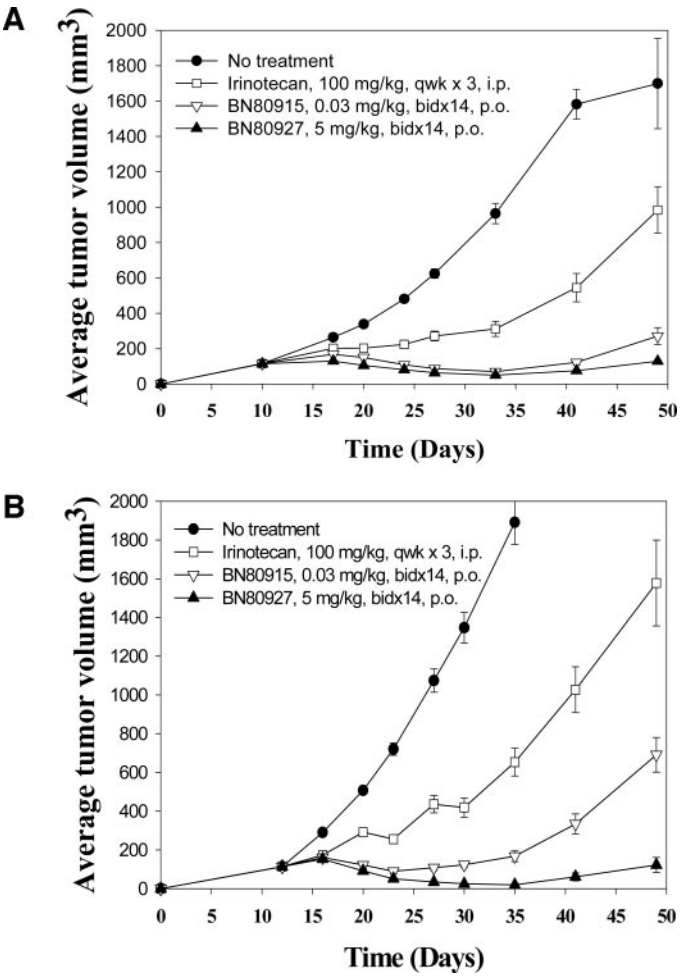

Fig. 8. Comparison of BN80927, BN80915, and irinotecan antitumor potency in non-androgen-dependent human prostate adenocarcinoma PC-3 and DU145 xenograft models. BN80927 and BN80915 were administered p.o. twice a day for 14 consecutive days (*bid×14*) at 5 and 0.03 mg/kg, respectively; irinotecan was administered i.p. at 100 mg/kg once a week for 3 weeks (*qwk×3*) in PC3 (*A*) and DU145 models (*B*).

As expected for a hCPT derivative (9, 13), BN80927 is very stable in plasma. The compound acts as a potent Topo I poison as expected for both CPT and hCPT analogs (12, 29, 30). BN80927 performs well in isolated enzyme assays but also on living cells. BN80927 promotes and stabilizes higher levels of protein-DNA complexes than does SN38 in quantitative DPC experiments as well as in ICT experiments in which BN80927 stabilized Topo I-DNA complexes (Figs. 4 and 5).

Moreover, an additional inhibitory activity, unexpected for a CPT analog, is observed with BN80927 on the Topo II-mediated enzymatic reactions on DNA, as revealed in relaxation and confirmed in decatenation assays (data not shown). This activity does not arise from an interaction with Topo II-DNA cleavable complexes as shown in whole cells (ICT experiments), and in this sense differs from the Topo II poisoning seen with the drug VP-16. Electrophoresis migration profiles do not support the hypothesis of BN80927 as an intercalating agent. This is reinforced by the absence of significant alteration of the denaturation temperature of double-strand DNA in the presence of BN80927.[3] However, it cannot be excluded that BN80927 may bind to specific DNA sequences or structures with biologically relevant affinity. Altogether, these results are in favor of a catalytic mechanism

[3] A. K. Larsen, personal communication.

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of action of BN80927 on Topo II, but additional experiments in whole cells (31) is rendered difficult by the important cytotoxicity due to Topo I poisoning. To better characterize the biological relevance of this Topo II inhibition, BN80927 was tested on a Topo I inhibitor-resistant cell line (KBSTP2; Ref. 25). Because BN80927 is highly potent on this Topo I inhibitor-resistant line, the antiproliferative effect seems to be due, in part, to a Topo I-independent pathway. Additional investigations are required to assess whether only Topo II inhibition is involved in the inhibition of KB-STP2 growth by BN80927 or whether additional pathways are involved.

As a dual topoisomerase inhibitor, BN80927 should be compared with other recently reported dual inhibitors of topoisomerase (32) having a dual poisoning mechanism of action, such as Tas-103 (33) or with a catalytic inhibition mode on either Topo I [lucanthone (34)] or both Topo I and II [such as F11782 epipodophyllotoxin derivative (35, 36)]. To date, to our knowledge, BN80927 seems to be the only dual inhibitor having both Topo I poisoning and Topo II catalytic inhibitory activities.

In *in vitro* cell-growth studies with proliferative human cancer cells, BN80927 appears to be consistently more potent than the topoisomerase inhibitors SN38 and VP-16. Compared with the other published hCPT, BN80915, over a large spectrum of cell lines (13, 37), BN80927 is slightly less potent.

It is well known that the clinical efficacy of anticancer agents, including some topoisomerase inhibitors, is limited by drug resistance mechanisms (38, 39). This resistance can be due to a decrease in the levels and/or activities of topoisomerase, to a decrease of intracellular drug accumulation (MDR phenotype; Ref. 40), or to the presence of nonproliferating tumor cells during the treatment period [for review, see Tomida *et al.* (26)]. We demonstrate that BN80927 remains an active antiproliferative agent in these three critical situations, and such data are particularly relevant clinically for the treatment of patients refractory to the standard drugs. However, if the expression of P-glycoprotein and MDR-associated protein in tumor biopsies are well documented, the importance of Topo I-inhibitor- or Topo-II-inhibitor-resistant phenotype is less documented. For solid tumors, the noncycling part of the tumor cell population is highly variable, depending on each tumor sample. These cells are refractory to numerous DNA-targeted agents that are active only on proliferating cells. In cultured cell lines, accumulation of cells in G_0-G_1 phase of the cell cycle has been reproduced by cell exposure to several stressor agents, including glucosamine (41, 42). In such an *in vitro* model of stress-induced G_0-G_1 cell cycle arrest, tumor cells remain sensitive to BN80927, whereas CPT, SN38, and VP-16 are devoid of activity. Only limited effects are observed with BN80915 under such conditions (Fig. 6). This difference represents an additional targeted cell population within a tumor composed of resting and dividing cells. This aspect should, however, be further studied over several consecutive cell cycles and also with the use of normal cells.

With the support of the *in vitro* data showing the antiproliferative activity of BN80927 in a wide spectrum of cell lines, BN80927 was evaluated in animal models and compared with BN80915. In two different androgen-independent prostate tumor models representing a large unmet clinical need, BN80927 has demonstrated strong antitumor activity with several dose administration regimens. The most efficient regimen by the oral route is a twice daily administration. However, despite close values of *in vitro* antiproliferative activities between the two tested hCPTs, the difference of dosing is large (>100). Only limited weight loss was observed, even at the highest doses, and was reversible at the end of the treatment.

In conclusion, BN80927 is a potent broad-spectrum, antiproliferative agent that retains activity against chemoresistant cell lines. The compound is a potent Topo I poison, but also acts as a catalytic inhibitor of Topo IIα and shows high activity on G_0-G_1 synchronized cells. These properties of BN80927 clearly differentiate it from previous CPT and hCPT analogs.

In addition BN80927 shows high plasma stability and demonstrates remarkable efficacy in the PC3 and DU145 prostate xenograft models.

The overall profile of BN80927 and, in particular, the mechanistic differences with respect to related molecules merit further study and clinical evaluation of the compound.

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BN80927: A Novel Homocamptothecin That Inhibits Proliferation of Human Tumor Cells in Vitro and in Vivo

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