ABSTRACT

BN 80915 is the lead compound from a novel class of E-ring modified camptothecin analogues, the homocamptothecins, which show potent antitumor activities in animal models. Here, we report that BN 80915 induces up to 2-fold more cleavable complexes between plasmid DNA and purified human topoisomerase I than SN-38 and camptothecin. BN 80915 also induces DNA-topoisomerase I complexes in living HT-29 colon carcinoma cells, as shown by the in vivo link assay. BN 80915 is an extremely potent inducer of DNA-protein complexes in these cells starting at a concentration of 5 nM in the media. BN 80915 is clearly more potent than SN-38, because at least 20 times more SN-38 is needed to induce comparable levels of cleavable complexes. Kinetic experiments show that BN 80915 induces cleavable complexes within minutes that remain stable for at least 6 h in the presence of drug. Whereas the majority of the complexes are reversed within 15 min after drug removal, a substantial fraction (30%) persists for at least 4 h, in contrast with SN-38-treated cells, where all complexes have disappeared by this time. BN 80915 shows strong antiproliferative effects toward HT-29 cells with an IC_{50} of 0.3 nM compared with 20 nM for SN-38 and 40 nM for topotecan. BN 80915 is also potent against other colon carcinoma cells as well as toward cells growing in three dimensions as multicellular spheroids. HL-60 cells expressing functional P-glycoprotein or multidrug resistance protein show no cross-resistance toward BN 80915. Taken together, our results show that BN 80915 is unusually potent toward human colon carcinoma cells because of the formation of high levels of stable, covalent DNA-topoisomerase complexes.

INTRODUCTION

DNA topoisomerase I is a multifunctional protein that is essential for the viability of dividing multicellular organisms. In addition to its classical DNA nicking-closing activities, which are needed for relaxation of supercoiled DNA, topoisomerase I can phosphorylate certain splicing factors. The enzyme is also involved in transcriptional regulation through its ability to associate with other proteins in the transcription factor IID- and, possibly, IIH-transcription complexes and is implicated in the recognition of DNA lesions (for recent review, see Ref. 1).

A crucial reaction intermediate in the relaxation of DNA is the formation of a transient DNA-topoisomerase I complex (the cleavable complex) where topoisomerase I is covalently linked to a 3’-end of DNA, thereby creating a single-stranded DNA break which allows DNA strand passage. The presence of topoisomerase I-targeted drugs such as the camptothecins prevents the relocation of nicked DNA, resulting in increased levels of covalent DNA-topoisomerase complexes (2, 3). Collision of the cleavable complexes with the replication fork or transcription machinery subsequently converts the protein-associated DNA strand breaks into frank DNA strand breaks, thereby triggering a series of reactions that eventually lead to cell death (4, 5).

It has been reported that topoisomerase I protein levels and catalytic activities are markedly higher in colorectal and prostate cancer and in immature neuroblastomas than in the corresponding normal tissues, providing a rationale for chemotherapy of these tumor types by topoisomerase I-directed compounds (6–8).

Two camptothecin derivatives, irinotecan (CPT-11; Campto) and topotecan (Hyacintin) are currently registered for second-line treatment of colorectal and ovarian cancers, respectively (9, 10). Other compounds, including 9-aminocamptothecin, 9-nitrocamptothecin, GG-211, and DX-8951f, have also been clinically evaluated (11), and many other potent camptothecin analogues are in preclinical development (12). The main flaw of these compounds is their intrinsic instability because of the presence of a highly reactive α-hydroxylactone, which, under physiological conditions, undergoes rapid hydrolysis to form an equilibrium with the biologically inactive carboxylate form. In the plasma, the equilibrium may be shifted even further toward the carboxylate form because of specific interactions with serum albumin (13). These interactions are species-specific and may explain the disappointingly low clinical efficacy of some of the camptothecin derivatives compared with the often spectacular results obtained in xenograft models (14).

To ensure sustained plasma levels of the active lactone form, drugs may be administered either as a prodrug or by continuous perfusion. Alternatively, the lactone ring may be chemically modified in a way rendering it more stable. However, previously reported modifications of the lactone ring failed to preserve biological activity, leading to general acceptance of an intact α-hydroxylactone as an indispensable structural feature for both topoisomerase I inhibition and antitumor activity (15–18). The preparation of a camptothecin homologue, homocamptothecin, wherein a seven-membered β-hydroxy lactone replaces the six-membered α-hydroxylactone of the parent compound, has been reported recently (19). This modified camptothecin shows greatly enhanced plasma stability and represents the only lactone ring modification known that conserves both the capacity to inhibit topoisomerase I and the antitumor activity (19). Furthermore, the ring opening of the homocamptothecins is irreversible, in contrast to the camptothecins, which can revert into the biologically active lactone form under acidic conditions. The modified lactone ring may therefore serve to avoid adverse effects such as hemorrhagic cystitis, which are associated with exposure to camptothecin and certain derivatives (19, 20).

The use of homocamptothecin as a template for the elaboration of new anticancer agents (20) resulted in the selection of the difluorinated analogue BN 80915 as a lead compound for additional development (see Fig. 1 for chemical structures). We now report that BN 80915 is unusually potent toward human colon carcinoma cells. This is associated with the induction of high levels of stable, covalent DNA-topoisomerase complexes. In addition, BN 80915 is not a substrate for P-glycoprotein and MDR2 protein, the two drug efflux
pumps most commonly associated with resistance of tumor cells to antineoplastic agents.

**MATERIALS AND METHODS**

**Chemicals.** Camptothecin was purchased from Janssen (Bisheim, France), whereas SN-38, topotecan, and BN 80915 were synthesized according to published procedures (19, 21). The drugs were dissolved in dimethylecetamide at 10 mM and stored as aliquots at −20°C. Ehtidium bromide and Hoechst 33342 were supplied by Sigma (St. Louis, MO), whereas netropsin was obtained from Serva (Heidelberg, Germany). [3 H]thymidine, [ 14 C]protein, with the protein being an internal standard for the exact number of cells used.

**DNA Substrates and Enzymes.** Supercoiled plasmid pBR322 DNA, EcoRI, and HindIII were purchased from Boehringer Mannheim (Mannheim, Germany) whereas Klenow fragment polymerase was obtained from United States Biochemical (Cleveland, OH). Human DNA topoisomerase I was expressed at high levels in a baculovirus system and purified by a two-step procedure consisting of ammonium sulfate precipitation and then nickel column chromatography (22).

**Melting Point.** The interaction of different compounds with DNA was determined by a classical DNA melting procedure (23, 24). Calf thymus DNA was incubated with different compounds in a buffer containing 5 mM Tris-HCl (pH 7.4) and 5 mM NaCl, and the absorbance at 260 nm was followed at increasing temperatures. The melting temperature (Tm) indicates the temperature at which one-half of the DNA is denatured, as determined by the increased absorbance at high levels in a baculovirus system and purified by a two-step procedure consisting of ammonium sulfate precipitation and then nickel column chromatography (22).

**Topoisomerase I-induced Cleavage of pBR322 Plasmid DNA.** pBR322 plasmid DNA was linearized with EcoRI and end-labeled at its 3’ ends with Klenow fragment and [α-32P]dATP as described previously (25). The end-labeled pBR322 was subjected to a second digestion with HindIII restriction enzyme, the fragments obtained were separated by agarose gel electrophoresis, and the larger fragment was used for the DNA cleavage assay. Reaction mixtures contained 20 mM Tris-HCl (pH 7.4), 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, −10°C (−50 ng) of end-labeled pBR322 DNA, and the indicated concentrations of drug. The reactions were initiated by the addition of DNA topoisomerase I (16 ng) and were allowed to proceed for 15 min at 37°C. Reactions were terminated by the addition of SDS and proteinase K (0.25% and 0.3 mg/ml final concentrations, respectively) and incubated for 1 h at 50°C. The samples were denatured by addition of NaOH (150 mM final concentration), loading buffer was added, and samples were electrophoresed in 1% agarose gels under denaturing conditions with TBE containing 0.1% SDS as the running buffer at 20 V/cm for 2–3 h. Gels were dried and autoradiographed with Hyperfilm MP (Amersham) for 1–2 days as described previously (26).

**Cells and Culture Media.** HT-29, HCT 116, and Caco-2 human colon carcinoma cell lines were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 mM glutamine. HL-60, HL60/Vinc, and HL60/AR human leukemia cell lines were kindly provided by Dr. Melvin Center (Kansas State University, Manhattan, KS). These cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 mM glutamine. All cells were grown at 37°C in a 5% CO2/95% air atmosphere.

**In Vivo Link Bioassay.** The in vivo link bioassay was used to detect the formation of covalent DNA-topoisomerase I complexes in living cells (27, 28). HT-29 colon carcinoma cells (2 × 105 cells/sample) were treated for 30 min with BN 80915 or, as a control, with growth media containing 0.1% DNA. The media was removed, and cells were lysed by direct addition of 1% sarkosyl (37°C) and overlaid onto a step CsCl gradient containing four different densities. To prepare the step gradients, a stock solution of CsCl (density, 1.86 g/cc) was diluted with TE buffer [10 mM Tris-HCl (pH 7.5)] and 1 mM EDTA] to give solutions of 1.37, 1.50, 1.72, and 1.82 g/ml. The densest solution (2 ml) was placed in a SW41 polyallomer tube and then 2 ml each of successively less dense CsCl solutions. Cells lysates were gently layered on top of the gradients, and the tubes were centrifuged in a Beckman SW41 rotor at 31,000 rpm for 18–24 h at 20°C. Fractions (0.4 ml) were collected from the top of the gradients, and DNA was located using fluorometry. Aliquots from each fraction (50 µl) were diluted with 100 µl of 25 mM sodium phosphate buffer (pH 6.5) and applied to Hybond C nitrocellulose membranes (Amersham) using a slot-blot vacuum manifold.

**Immunoblot Analysis.** Nitrocellulose membranes were equilibrated in TBST buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] for 30 min and then incubated in TBST containing 5% nonfat dried milk for 2 h. The filters were washed three times (10 min each time) in TBST and incubated overnight at 4°C with a rabbit antibody directed against human DNA topoisomerase I (TOPOGEN, Inc., Columbus, OH). The antibodies were diluted 1:3,000 in TBST before use. The filters were washed three times (10 min each time) in TBST, followed by a 2-h incubation with antirabbit antibodies coupled to horseradish peroxidase (Amersham). The antibodies were diluted 1:10,000 before use. The filters were rinsed three times (10 min each time) with TBST, and the presence of topoisomerase I was revealed according to the enhanced chemiluminescence procedure (Amersham Life Science).

**DNA-Protein Complex Formation in Living Cells.** To compare the capacity of the different camptothecin derivatives to stabilize covalent DNA-protein complexes (cleavable complexes) in living cells, the KCl-SDS coprecipitation assay was carried out as described (29). DNA and protein of HT-29 colon carcinoma cells were radiolabeled with 0.6 µCi/ml [3 H]thymidine and 0.2 µCi/ml [ 14 C]leucine, respectively, for 18 h. The cells were exposed to various drug concentrations for 1 h at 37°C and then lysed in a solution of 1.25% SDS, 5 mM KCl, 5 mM EDTA (pH 8), and 0.4 mg/ml salmon testes DNA (final concentrations). The lysates were passed through a 22-gauge needle five times, heated to 37°C for 15 min and precipitated with 65 mM KCl on ice for 15 min. The precipitates were washed three times in 10 mM Tris-HCl (pH 8), 100 mM KCl, 1 mM EDTA, and 0.1 mg/ml salmon testes DNA at 65°C before being dissolved in 0.5 ml water and mixed with 5 ml scintillation fluid for determination of radioactivity. Data are expressed as the ratio of [ 3 H]DNA: [ 14 C]protein, with the protein being an internal standard for the exact number of cells used.

**Growth Inhibition Assays.** Growth inhibition assays were carried out with exponentially growing cells as described previously (30). Adherent cell lines were plated at 5 × 104 cells/ml (HT-29) or 2.5 × 105 cells/ml (Caco-2 and HCT 116) in 4-ml drug-free media and incubated at 37°C for 24 h before drug treatment. One ml of various 5× drug concentrations (1× final concentration) was added, and the cells were incubated for 6, 24, or 72 h at 37°C. For 6- and 24-h drug exposures, the drug was withdrawn at the indicated times, the cells were washed once, 5 ml of fresh medium was added to each dish and additional incubation was carried out until a total of 72 h, when cells were trypsinized and counted on a Coulter Counter. HL-60 cell lines were seeded at 5 × 105 cells/ml with the indicated drug concentrations. After 24 h of incubation, the cells were rinsed once, resuspended in drug-free media and incubated for an additional 48 h before counting with a Coulter Counter. The liquid overlay system was used to generate spheroids from HT-29 cells as described (30, 31). Under these conditions, cells are unable to attach to the tissue plates, which are covered with 1% agarose, and grow as multicellular spheroids in three dimensions.
RESULTS

Interaction between BN 80915 and DNA. Recent results show that homocamptothecin is a more potent inducer of topoisomerase I-mediated DNA cleavage than camptothecin itself (20, 32). We therefore wished to compare the interaction of camptothecin- and homocamptothecin-derivatives with DNA. The ability of a compound to bind DNA can be estimated by its capacity to alter the thermal denaturation profile of DNA (23, 24). The average value of the midpoint thermal denaturation temperature for calf thymus DNA was 65.4°C (Table 1). The presence of the intercalator ethidium bromide or the minor groove binder Hoechst 33342 increases the thermal denaturation temperatures to 80°C and 84.5°C, respectively. In contrast, no significant alterations of the denaturation temperature are observed for camptothecin, the camptothecin derivative SN-38, or the homocamptothecin derivative BN 80915 (see Fig. 1 for chemical structures) under the same experimental conditions. These results show that there are no apparent differences in the way camptothecins and homocamptothecins interact with DNA. Furthermore, although we cannot exclude that BN 80915 might bind with reasonable affinity to specific DNA sequences or structures, it is clearly not a compound with much general affinity for DNA.

BN 80915 Is a Strong Inducer of Covalent DNA-Topoisomerase I Complexes in Vitro. There is general agreement that camptothecin and its clinically used derivatives act by stabilizing a covalent DNA-topoisomerase I complex called the cleavable complex. The ability of BN 80915 to stabilize the cleavable complex was evaluated by incubating purified human topoisomerase I and [32P] end-labeled linear plasmid DNA in the presence of drug followed by treatment with SDS and proteinase K. Cleavable complexes are revealed by the appearance of short DNA fragments when the samples are analyzed by gel electrophoresis under denaturing conditions. The results (Fig. 2) show that BN 80915 is a potent inducer of topoisomerase I-mediated DNA cleavage in vitro. The addition of increasing concentrations of BN 80915, SN-38, or camptothecin is accompanied by a dose-dependent increase in the level of cleavable complexes. The relative potency between the three compounds, as indicated by the remaining amount of intact DNA, is camptothecin, SN-38, and BN 80915 in increasing order. Scanning of autoradiographic images reveals that the difference between BN 80915 and SN-38 never exceeds 2-fold (data not shown). In addition, the topoisomerase I-mediated cleavage pattern induced by BN 80915 in pBR322 DNA is comparable with that observed for camptothecin and SN-38. In contrast, no detectable DNA cleavage is observed when BN 80915 was incubated with purified human DNA topoisomerase II or under similar conditions (results not shown).

BN 80915 Induces Covalent DNA-Topoisomerase I Complexes in Living Cells. The ability of BN 80915 to induce covalent DNA-topoisomerase I complexes in living HT-29 colon carcinoma cells was evaluated by the in vivo link assay (27, 28). Cells were lysed with the protein denaturant sarkosyl, which at the same time traps covalent DNA-protein complexes and dissociates noncovalent DNA-protein complexes, after which cesium chloride gradient ultracentrifugation was performed. Aliquots from the different fractions were applied to nitrocellulose membranes and the position of topoisomerase I identified by immunoblot analysis. In untreated control cells, most topoisomerase I sediments as free protein (Fig. 3). In clear contrast, part of the topoisomerase I sediments with the nucleic acids (as judged from the absorbance at 260 nm) in the presence of BN 80915 because of formation of covalent DNA-topoisomerase I complexes. These results show that BN 80915 targets topoisomerase I in living cells.

BN 80915 Is an Extremely Potent Inducer of Covalent DNA-Protein Complexes in HT-29 Human Colon Carcinoma Cells. The ability of BN 80915 and SN-38 to induce DNA-protein complexes in HT-29 human colon carcinoma cells was determined by the KCl/SDS link assay. Exponentially growing HT-29 cells (10⁷) were incubated in the absence (○) or presence (●) of 10 μM BN 80915 for 1 h and then lysed and CsCl gradient centrifugation. Thirty fractions were collected from the top and analyzed by immunoblotting with a topoisomerase I-directed antibody as described in “Materials and Methods.” Fractions 4–10 contained free topoisomerase I, whereas covalent DNA-topoisomerase I can be detected in fractions 19–21.

---

Table 1

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Tm (°C)</th>
</tr>
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<tbody>
<tr>
<td>DNA alone</td>
<td>65.4 ± 0.8</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>80.0 ± 0.8</td>
</tr>
<tr>
<td>Netropsin</td>
<td>72.7 ± 0.3</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>84.5 ± 0.1</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>65.3 ± 0.6</td>
</tr>
<tr>
<td>SN-38</td>
<td>65.4 ± 0.3</td>
</tr>
<tr>
<td>BN 80915</td>
<td>65.4 ± 0.7</td>
</tr>
</tbody>
</table>

* Calf thymus DNA was incubated in the absence or presence of the indicated compounds (10 μM) in a buffer containing 5 mM Tris-HCl (pH 7.4) and 5 mM NaCl.

Tm (melting temperature) indicates the temperature at which half of the DNA is denatured, as determined by the absorbance at 260 nm. Each value is the average of at least three independent experiments ± SD.
complexes remain stable for at least 6 h. For comparative studies, it was necessary to use 40 times higher concentrations of SN-38 than for BN-80915 to obtain similar levels of DNA-protein complexes. Interestingly, although the extracellular drug concentrations are much higher for SN-38 than for BN-80915, the levels of covalent DNA-protein complexes formed within the first 2 min are clearly more important for BN-80915 than for SN-38. Once formed, the total levels of both BN-80915- and SN-38-stabilized covalent DNA-protein complexes remained constant throughout the 6-h incubation period.

**Reversibility of BN 80915-induced DNA-Protein Complexes.**
To determine the reversibility of the drug-induced DNA-protein complexes, HT-29 cells were incubated with BN 80915 or SN-38 for 1 h, the drug was removed, and the disappearance of DNA-protein complexes was followed for the next 4 h. The results (Fig. 6) show both similarities and differences between the two drugs. In all cases, the reversion of cleavable complexes is rapid, because most of the DNA-protein complexes disappear within the first 15 min of drug removal. Furthermore, the reversion is dose-dependent for both compounds, with more extensive reversion at 500 nM than at 20 nM. However, important differences are observed with respect to the residual levels of cleavable complexes present in cells preincubated with 500 nM drug (29% of initial levels for BN 80915 compared with 4% of initial levels for SN-38, as determined after 4 h postincubation). In contrast, no clear differences between the two compounds are observed for cells exposed to 20 nM drug (69% of the initial levels for BN 80915 versus 65% for SN-38 after 4 h postincubation).

**BN 80915 Has Strong Growth-inhibitory Effects on HT-29 Cells.** Usually, the levels of drug-induced covalent DNA-protein complexes are directly correlated with cellular cytotoxicity (for recent review, see Ref. 33). Because 20–40 times more SN-38 than BN 80915 is required to induce comparable levels of cleavable complexes, we would also expect BN 80915 to be more cytotoxic than SN-38, assuming that DNA-protein complexes stabilized by BN 80915 and SN-38 are equally cytotoxic. The growth inhibitory effects of BN 80915 toward HT-29 colon carcinoma cells were determined after 6, 24, and 72 h of drug exposure. As expected, the results show that BN 80915 is at least 40 times more potent than SN-38 and topotecan for all exposure times tested. Even a 6-h exposure to BN 80915 has a strong antiproliferative effect on HT-29 cells with an IC_{50} of 1.5 nM (Table 2). An additional increase of the growth inhibitory effect is observed by increasing the drug exposure time to 24 h. In contrast, no additional increase is obtained by prolonging the drug exposure further.

---

**Fig. 4.** BN 80915 is an extremely potent inducer of DNA-protein complexes in HT-29 cells. Exponentially growing cells were incubated for 1 h in the presence of the indicated concentrations of BN 80915 ( ) or SN-38 (■), and the formation of covalent DNA-protein complexes as compared with untreated control cells was determined by the KCl/SDS coprecipitation assay. Each point is the average of at least three individual experiments, each done in duplicate. Bars, SD.

**Fig. 5.** The induction of DNA-protein complexes by BN 80915 is rapid and remains stable for at least 6 h. Exponentially growing cells were incubated for the indicated times in growth media containing 500 nM BN 80915 ( ) or 20 μM SN-38 (■), and the formation of covalent DNA-protein complexes as compared with untreated control cells was determined by the KCl/SDS coprecipitation assay. Each point is the average of at least two individual experiments, each done in duplicate. Bars, SD.

**Fig. 6.** Reversal of cleavable DNA-protein complexes in HT-29 human colon carcinoma cells. Exponentially growing cells were incubated for 1 h with 500 nM BN 80915 ( ), 20 μM BN 80915 ( ), 500 nM SN-38 ( ), or 20 μM SN-38 ( ). The drug was removed and the disappearance of DNA-protein complexes was followed during a 4-h period by the KCl/SDS coprecipitation assay. Each point is the average of two individual experiments, each done in duplicate. Bars, SD.
Table 2 BN 80915 has strong antiproliferative effects toward HT-29 human colon carcinoma cells for all exposure times tested

Cells were exposed to drug for 6 or 24 h and then to postincubation in drug-free medium for 72 h total. Alternatively, cells were incubated with drug throughout the entire 72-h incubation period.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (nM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>70 ± 20 40 ± 12 30 ± 12</td>
</tr>
<tr>
<td>SN-38</td>
<td>80 ± 20 20 ± 10 10 ± 0.5</td>
</tr>
<tr>
<td>BN 80915</td>
<td>1.5 ± 0.8 0.30 ± 0.05 0.27 ± 0.17</td>
</tr>
</tbody>
</table>

$^a$Concentration inhibiting the growth of drug-treated cells by 50% compared with untreated control cells. Each value is the average of at least three independent experiments, each done in duplicate ± SD.

Table 3 BN 80915 has strong antiproliferative effects towards different human colon carcinoma cells

Cells were exposed to the indicated compounds for 24 h and then to postincubation in drug-free media for an additional 48 h.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (nM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>40 ± 12 42 ± 13 22 ± 5</td>
</tr>
<tr>
<td>SN-38</td>
<td>20 ± 10 18 ± 9 4.1 ± 1.2</td>
</tr>
<tr>
<td>BN 80915</td>
<td>0.30 ± 0.05 0.83 ± 0.18 0.54 ± 0.14</td>
</tr>
</tbody>
</table>

$^a$Concentration inhibiting the growth of drug-treated cells by 50% compared with untreated controls. Each value is the average of at least three independent experiments, each done in duplicate ± SD.

BN 80915 Has Strong Antiproliferative Effects toward Different Human Colon Carcinoma Cell Lines. The growth inhibitory effects of BN 80915, SN-38, and topotecan toward different human colon adenocarcinoma cell lines were compared (Table 3). For all cell lines, BN 80915 was at least 10-fold more potent than SN-38 and 40-fold more potent than topotecan. Whereas the growth inhibitory dose of BN 80915 was similar toward all cell lines tested, topotecan and SN-38 were more toxic against HCT 116 cells than against HT-29 and Caco-2 cells.

BN 80915 Has Strong Growth Inhibitory Effect on HT-29 Cells Growing in Multicellular Spheroids. Although growth inhibition assays usually are carried out with cells growing as monolayer cultures in two dimensions, solid tumors grow in three dimensions. Therefore, the growth inhibitory effects of BN 80915, topotecan, and SN-38 were also determined for HT-29 cells growing in three dimensions as multicellular spheroids. The results (Fig. 7) show that BN 80915 inhibits the growth of HT-29 cells with an IC$_{50}$ around 2 nM after 72 h of continuous drug exposure. In comparison, SN-38 and topotecan showed IC$_{50}$ values of ~20 and 45 nM, respectively. Therefore, the potent ability of BN 80915 to induce cleavable complexes is accompanied by an important antiproliferative effect both toward cells growing in monolayer cultures and in cells growing in three dimensions as multicellular spheroids.

BN 80915 Is Not Recognized by the P-glycoprotein or the MDR Protein. Reduced drug accumulation and/or an altered intracellular drug distribution is a dominant feature of many drug-resistant tumors. HL-60/Vinc is a classical MDR cell line that overexpresses high levels of P-glycoprotein, whereas HL-60/AR expresses the MDR protein (34–37). None of these cell lines were cross-resistant toward BN 80915 (Table 4). In contrast, low levels of cross-resistance were observed for topotecan and SN-38 toward both HL-60/Vinc and HL-60/AR cells.

Table 4 HL-60 cells overexpressing functional P-glycoprotein (HL-60/Vinc) or MRP (HL-60/AR) show no cross-resistance toward BN 80915

Cells were exposed to the indicated compounds for 24 h and then to postincubation in drug-free media for an additional 48 h.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (nM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>25 ± 4 55 ± 7 46 ± 5</td>
</tr>
<tr>
<td>SN-38</td>
<td>5 ± 1.0 8.5 ± 1.5 10.5 ± 1.5</td>
</tr>
<tr>
<td>BN 80915</td>
<td>0.78 ± 0.1 0.78 ± 0.1 0.75 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$Concentration inhibiting the growth of drug-treated cells by 50% compared with untreated control cells. Each value is the average of at least three independent experiments, each done in duplicate ± SD.
of BN 80915 is present in the active lactone form after 30 min of incubation at 37°C compared with <50% for SN-38 (39). This observation is consistent with the findings of others, because different substitutions of the A-, B-, and E-rings of camptothecin have been reported to result in improved blood stability (40).

Another unexpected finding was the high stability of the cleavable complexes, which remained at a constant level throughout the 6-h incubation period. We might be dealing with a dynamic system, where there is constant exchange between the drug, the DNA, and topoisomerase I, which are all parts of the cleavable complex. Alternatively, the three partners initially involved in the cleavable complex might remain together throughout the entire incubation period. We favor the second possibility, because the concentration of the active lactone form of both BN 80915 and SN-38 decreases during the 6-h incubation period and therefore is unlikely to stabilize the same number of cleavable complexes at the beginning and at the end of the experiment. In agreement with this interpretation, it has been reported that interaction with certain sequences of double-stranded DNA results in a marked stabilization of the active lactone form of camptothecin drugs (41).

In order for the cleavable complex to persist for prolonged periods of time, not only the drug but also the topoisomerase must be stable. It has been shown previously that topoisomerase I engaged in cleavable complexes is targeted for proteasome degradation by ubiquitination (42, 43). Furthermore, recent results suggest the existence of a tyrosine-DNA phosphodiesterase that repairs topoisomerase I complexes (44). However, none of these pathways appear to be operating in HT-29 human colon carcinoma cells, indicating that these processes might be defective in at least some tumor cells, which may, at least in part, account for the selectivity of topoisomerase I-directed compounds toward such cells.

These results suggest that the relative importance of different pharmacodynamic parameters may be both drug- and tumor type-dependent. In HT-29 cells, the gradual conversion of the active lactone form of both BN 80915 and SN-38 into the inactive carboxylate form had no influence on the cellular levels of cleavable complexes, which remained stable throughout the 6-h incubation. In marked contrast, drug removal resulted in the rapid reversion of the drug-stabilized DNA-topoisomerase complexes in a manner that was different for the two drugs, inasmuch as the residual levels of the drug-stabilized cleavable complexes after drug removal were much higher for BN 80915 than for SN-38 (~30% compared with 4%, respectively). Therefore, for tumor cells such as HT-29, which appear to be partially defective for the ubiquitination/proteasome pathway, plasma clearance and cellular drug retention may be more important than the stability of the lactone ring in the plasma. For other tumor cells, such as KB human epidermoid carcinoma cells, FM3A mouse mammary carcinoma cells, and SJ-G5 human anaplastic astrocytoma cells, drug-stabilized cleavable complexes seem to be much more labile due to proteolysis of the topoisomerase I present in the cleavable complexes (42, 43, 45, 46). For such cells, lactone-ring stability becomes crucial because a more stable drug would remain active longer, thereby permitting the reformation of cleavable complexes during an extended period of time.

Whereas usually in vitro testing is carried out with cells growing in monolayer cultures, tumors grow in three dimensions. Importantly, BN 80915 also showed potent antiproliferative effects toward colon carcinoma cells growing in three dimensions. Another attractive feature of BN 80915 is the observation that this drug, in contrast with topotecan and SN-38, is not recognized by either the P-glycoprotein or the MDR protein. These membrane-associated drug efflux pumps are highly expressed in colonic tissues and are considered to be a leading cause of treatment failure in this tumor type (47–49).

In summary, the results presented here clearly show that BN 80915 is unusually potent toward colon human carcinoma cells. This is associated with formation of high levels of stable, covalent DNA-topoisomerase complexes. In addition, BN 80915 is not a substrate for the two major drug efflux pumps most commonly associated with cellular resistance of tumor cells to antineoplastic agents. These data should be of considerable theoretical and practical importance for the additional development of BN 80915 in experimental and clinical oncology.

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Unusual Potency of BN 80915, a Novel Fluorinated E-ring Modified Camptothecin, toward Human Colon Carcinoma Cells

Annette K. Larsen, Cristèele Gilbert, Ginette Chyzak, et al.


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