Homocamptothecin, an E-Ring-modified Camptothecin, Exerts More Potent Antiproliferative Activity than Other Topoisomerase I Inhibitors in Human Colon Cancers Obtained from Surgery and Maintained in Vitro under Histotypical Culture Conditions


ABSTRACT

Topoisomerase I (Topo I) is overexpressed in cancer colon tissues compared with normal colon tissues. Several anti-Topo I inhibitors are already successfully used in the clinic. We illustrate here the antiproliferative activity of a new class of Topo I inhibitors, i.e., E-ring-modified camptothecins with enhanced lactone stability (L. Lesueur-Ginot et al., Cancer Res., 59: 2939–2943, 1999). Forty-three human colon cancers were obtained from surgical resection and maintained under organotypical culture conditions for 48 h. Cell proliferation was assessed in these ex vivo tumor tissue cultures by tritiated thymidine autoradiography. As a validation of the methodology, we first analyzed in our model the antiproliferative activity of two clinically active topoisomerase II (Topo II) inhibitors, Adriamycin and etoposide, which are not active for colon cancers; and three Topo I inhibitors, camptothecin (CPT) and two clinically active compounds (especially for colon cancers), i.e., topotecan and the active metabolite of irinothecan, SN-38. We then compared the antiproliferative activity of CPT, topotecan, and SN-38 against those of two investigational E-ring-modified camptothecins, i.e., BN80245 and BN80915. Three concentrations (1, 10, and 100 nM) were studied for each compound. The results indicate that the three Topo I inhibitors used as references, i.e., CPT, irinothecan, and SN-38, were much more active than the two Topo II inhibitors, i.e., Adriamycin and etoposide, with SN-38 being the most efficient. The two investigational compounds BN80245 and BN80915 exerted higher antiproliferative activity than the three anti-Topo I reference compounds, with the highest activity observed for BN80915.

INTRODUCTION

Colon cancer affects ~5% of the population in the United States and most Western countries, with >155,000 new cases diagnosed in the United States each year (1). This disease thus constitutes a major public health problem and is associated with a high morbidity and mortality rate, with the 5-year relative survival rate having increased from 41% in the 1950s to only 54% in the 1980s (1). As reported by McCann (2), for years 5-fluorouracil, with or without oral leucovorin or levamisole, has been almost the only chemotherapy option for colon cancer patients. McCann (2) further reports that recent new insights into the biology of this cancer have spurred the development of new drugs, among which three classes of compounds (already approved for use in the United States) have emerged: the folate-based thymidylate synthetase inhibitors, the oral fluorinated pyrimidines, and the topoisomerase I inhibitors.

DNA Topo I is a nuclear enzyme that is very important for solving topological problems arising during DNA replication (3). The inhibition of Topo I activity leads to an accumulation of DNA strand breaks (cleavable complexes) and, ultimately, cell death (4). DNA Topo I represents the target of the camptothecins, a novel class of anticancer drugs active against refractory solid tumors (5). Giovanella et al. (3) showed that, on average,
Topo I levels are 14–16-fold higher (depending on clinical stages) in cancerous colon tissues than in normal colonic mucosa. These authors also observe that DNA Topo I is a principal target for the plant alkaloid, CPT, isolated from the Chinese tree *Camptotheca acuminata* (3). The antitumor activity of CPT has in fact been recognized for >20 years, but its use has been associated with severe and unpredictable toxicity (6). Two camptothecin derivatives, TPT (7, 8) and CPT-11 (9, 10), recently have been approved. With respect to CPT-11, SN-38, one of its metabolites, is responsible for antitumor activity (6, 10).

We have developed a new class of camptothecins, i.e., homocamptothecins, by modifying the crucial E-ring of camptothecin to afford a seven-membered homologous β-hydroxy-lactone derivative, BN80245 (11), whose structure is illustrated in Fig. 1. This compound is more stable than camptothecin and remains a potent inhibitor of both cell growth and Topo I (11). We then developed other homocamptothecins that combine enhanced plasma stability and potent Topo I-mediated activity (12). Like camptothecin, homocamptothecins carry an asymmetric tertiary alcohol and display stereoselective inhibition of Topo I (12). Many of the homocamptothecins that we prepared (among which is BN80915, tested in the present study) were better Topo I inhibitors than camptothecin. Various fluorinated homocamptothecins were found to have potent cytotoxic activity on human A427 non-small cell lung and PC-3 prostate cancer cell lines, and their cytotoxicity remained high for the human leukemic K562adr and breast MCF7mdr cell lines, which overexpress a functionally active P-glycoprotein (12). Fluorinated homocamptothecins were more efficacious in vivo than camptothecin for human colon HT-29 cancer xenograft (12).

The aim of the present work is to characterize the antiproliferative activity of two homocamptothecins, i.e., BN80245 and BN80915, in comparison with the antiproliferative effects obtained with three Topo I inhibitor reference compounds, i.e., CPT, TPT, and SN-38, and two Topo II inhibitor reference compounds, ADR and VP-16. Their antiproliferative activity was characterized for individual human colon cancers by means of an experimental model that uses tumors obtained from surgical resection and maintained under organotypical culture conditions for 48 h, with histological tritiated thymidine autoradiography to measure cell proliferation.

**MATERIALS AND METHODS**

**Tissue Samples.** Forty-three fresh and sterile human colon cancers (25 T₁N₀/M₀, 15 T₂N⁺M₀, and 3 T₃N⁺M₀) were obtained from surgical resections performed at the Center Hospitalier Universitaire Brugmann between January 1997 and February 1999.

**Ex Vivo Culture Techniques.** The 43 colon cancers were maintained under organotypical culture conditions in a manner identical to the methodology that we described recently for human brain (13) and prostate (14) tumors. Briefly, after their surgical removal the cancer specimens were rinsed twice in MEM and immediately cut in half. One half was used for histopathological diagnosis, and the other was immediately divided into pieces measuring ~2 mm². Between 40 and 130 2-mm² pieces were available for each tumor involved in this study. To overcome the problem of biological heterogeneity, each experiment included 10 randomly selected tumor pieces for each cancer. Thus, according to the biological materials available, the influence of between one and three compounds could be tested on any one tumor. We were therefore able to characterize the influence of each of the five compounds on a minimum of 10 and a maximum of 22 colon cancers (see Tables 1 and 2). Three concentrations (1, 10, and 100 nM) were studied for each of the seven compounds under study. The seven compounds included three Topo I inhibitors chosen as reference compounds, CPT, TPT, and SN-38 (the active metabolite of CPT-11; hCPT, homocamptothecin; BN 80915, fluorinated homocamptothecin under investigation; CPT. R¹ = R² = R³ = H; TPT. R¹ = H, R² = CH₂N(CH₃)₂; R³ = OH; SN-38. R¹ = C₂H₅, R² = H, R³ = OH; BN 80915. R = F). The tumor pieces were cultured for 48 h in Petri dishes (30 × 10 mm; Nunc), each containing the 10 tumor pieces immersed in 3 ml of culture medium. The culture medium corresponded to Eagle’s MEM supplemented with a mixture of 0.6 mg/ml glutamine, 200 units/ml penicillin, 200 units/ml streptomycin, and 0.1 mg/ml gentamicin (all from Gibco, Merelbeke, Belgium). The five compounds were dissolved in DMSO, with a final DMSO concentration of <0.1%. An equal volume of this solvent was added to the control cultures.

Cell proliferation in the *ex vivo* tumor tissue cultures was assessed by tritiated thymidine autoradiography. Details of this procedure are provided elsewhere (13, 14). Less than 1 h elapsed between the surgical removal of a tumor and the setting up of the *ex vivo* tumor tissue culture. The cutting of the tumor into 2-mm² pieces took an additional hour. Tritiated thymidine (1 μCi/ml MEM; specific activity, 44.0 Ci/mmol; code...
TRK.637; Amersham, Little Chalfont, United Kingdom) was added to the culture medium on four occasions (12, 24, 36, and 47 h after the addition of the compounds or the solvent). After 48 h of culture, the samples were fixed in ethanol-formalin-acetic acid (7 parts 96% ethanol, 2.5 parts 40% formalin, 0.5 parts glacial acetic acid, v/v). After paraffin embedding, 6-μm sections were prepared and dipped into K5 nuclear emulsion (Ilford, Sint Niklaas, Belgium), diluted with 1:3 (v/v) in double-distilled water, air dried, and stored at 4°C in a light-proof box for 3 weeks. After development with Dektol (Kodak, Vilvorde, Belgium) for 3 min and fixation (twice for 10 min each) in 25% sodium thiosulfate (Merck Sharp Dohme, Darmstadt, Germany), the autoradiographs were stained with H&E (BDH Chemicals, Poole, England), and mounted with DPX (BDH Chemicals).

The LI corresponds to the percentage of cell nuclei covered by 10 (background) silver grains each and represents the percentage of cells in the S phase of the cell cycle. Five sections were sliced per colon cancer case; each section included 10 different colon cancer pieces. The LI counts were made on 200 epithelial cells per tumor piece present in each section. Thus, a total of 2,000 epithelial cells were counted per colon tumor section, and a total of 10,000 epithelial cells per experimental condition. The fact that each section included 10 pieces enabled the microscope fields selected for the counts to be randomized. All of the samples were counted in a protocol-blinded fashion.

Routine H&E sections were prepared and analyzed for each of the tumor tissue specimens from which the ex vivo tissue culture samples were derived.

**Statistical Analysis.** The results are presented as the mean ± SE. The statistical comparisons of the data were carried out by means of the Fisher F (one-way ANOVA for more than two groups) or the Student t (for two groups) tests after a check of the equality of variance by means of the Levene test and of the normal distribution fitting of the data by means of the χ² test.

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Fig. 2 A, basal cell proliferation levels assessed by means of tritiated thymidine autoradiography on organotypical cultures of 43 human colon cancers obtained from surgical resection and maintained ex vivo for 48 h. Tritiated thymidine autoradiography enables the TLI (%) to be calculated. The TLI offers a good estimation of cell proliferation activity because it assesses the percentage of cells in the S phase of the cell cycle when the experiments were carried out. RU, relative units in percentages with respect to control (100%). B, TLI modifications occurring in a given colon cancer treated either with ADR or SN-38. TLI value modifications occurring in the range of +15% (i.e., a drug-induced increase of 15% of the TLI value above the TLI control value) or −15% (i.e., a drug-induced decrease of 15% of the TLI value below the TLI control value) were not associated with any statistical significance (P > 0.05). Drug-induced TLI modifications occurring in the −25 to −15% or +15 to +25% ranges were associated with P < 0.05. Drug-induced TLI modifications occurring in the −50 to −26% or +26 to +50% ranges were associated with P < 0.01. P < 0.001 when these modifications were above +50% or below −50% compared with control (CT). The data presented above correspond to 48 h of tumor sample incubation in the presence of the compound to be analyzed; tritiated thymidine was added into the culture medium on four occasions (12, 24, 36, and 47 h) after the addition of the compound.
Table 1  Number of colon cancers for which reference drugs at given concentrations modified the TLI values between 26 and 50% or >50% compared with control (see Fig. 2B)

The data presented are drug-induced tumor LI modifications. The "x/x" numbers represent the number of colon cancers whose TLI values were modified by >50% (+, increase, −, decrease) or between 26 and 50% (see Fig. 2B) compared with the TLI control value (the first "x" number) out of the total number of cases analyzed for this compound (the second "x" number). Drug-induced TLI modifications occurring in the −25 to −15% or +15 to +25% ranges were associated with P < 0.05. Drug-induced TLI modifications occurring in the −50 to −26% or +50 to +26% ranges were associated with P < 0.001. P was < 0.001 when these modifications were +50% or below −50% compared with control. The data presented above correspond to 48 h of tumor sample incubation in the presence of the compound to be analyzed. Tritiated thymidine was added into the culture media on four occasions (12, 24, 36, and 47 h) after the addition of the compound.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>10^{-7} M</th>
<th>10^{-8} M</th>
<th>10^{-9} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference drug</td>
<td>&lt;−50%</td>
<td>−50 to −26%</td>
<td>+26 to +50%</td>
</tr>
<tr>
<td>CPT</td>
<td>13/18</td>
<td>14/18</td>
<td>0/18</td>
</tr>
<tr>
<td>TPT</td>
<td>72/72</td>
<td>78%</td>
<td>0%</td>
</tr>
<tr>
<td>ADR</td>
<td>10/12</td>
<td>12/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>23%</td>
<td>46%</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>46%</td>
<td>62%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Table 2  Number of colon cancers for which investigational drugs at given concentrations modified the TLI values between 26 and 50% or >50% compared with control (see Fig. 2B)

The data presented are drug-induced tumor LI modifications. The "x/x" numbers represent the number of colon cancers whose TLI values were modified by >50% (+, increase, −, decrease) or between 26 and 50% (see Fig. 2B) compared with the TLI control value (the first "x" number) out of the total number of cases analyzed for this compound (the second "x" number). Drug-induced TLI modifications occurring in the −25 to −15% or +15 to +25% ranges were associated with P < 0.05. Drug-induced TLI modifications occurring in the −50 to −26% or +50 to +26% ranges were associated with P < 0.001. P was < 0.001 when these modifications were +50% or below −50% compared with control. The labeling period in the presence of tritiated thymidine was identical to the one described in the legend to Table 1.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>10^{-7} M</th>
<th>10^{-8} M</th>
<th>10^{-9} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigational drug</td>
<td>&lt;−50%</td>
<td>−50 to −26%</td>
<td>+26 to +50%</td>
</tr>
<tr>
<td>BN 80245a</td>
<td>7/10</td>
<td>8/10</td>
<td>0/10</td>
</tr>
<tr>
<td>BN 80915</td>
<td>70%</td>
<td>80%</td>
<td>0%</td>
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<td></td>
<td>12/12</td>
<td>12/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

a BN80245 and BN80915 are both homocamptothecins, i.e., E-ring-modified camptothecins, whose structures are illustrated in Fig. 1.

of goodness of fit. When these parametric conditions were not satisfied, the nonparametric Kruskal-Wallis (for more than two groups) or the Mann-Whitney (for two groups) tests were carried out. All statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK).

RESULTS

The marked biological heterogeneity encountered in the 43 colon cancers under study is illustrated in Fig. 2A. Indeed, the basal cell proliferation levels (assessed in the control condition) ranged from 2 to 52%.

The pattern of cell proliferation response in a colon cancer treated with either ADR or SN-38 is illustrated in Fig. 2B. We thus report in Table 1 the five reference drug-induced effects according to whether each concentration tested induced, compared with the control, either an increase or a decrease in cell proliferative activity in a 26–50% (P < 0.01) TLI range of modifications, both above and below a 50% cutoff value (P < 0.001), as detailed in the legend for Fig. 2B.

The data reported in Table 1 clearly indicate that the three Topo I inhibitors were very much more active than the two Topo II inhibitors. These data therefore represent a good validation of the model that we used to characterize the antiproliferative activity of the various Topo I inhibitors under study. Of the three Topo I inhibitors chosen as references, SN-38 appeared to be the most efficient. Neither TPT nor SN-38 increased cell proliferative activity at a 10 nM concentration, and SN-38 did so only marginally at a 10 nM concentration. In sharp contrast, in addition to their weak inhibitory effects even at the 100 nM concentration, the two Topo II inhibitors increased cell proliferation...
activity in a significant proportion of colon cancers, whatever the doses tested (Table 1).

Of the five Topo I inhibitors under study, i.e., the three reference inhibitors (Table 1) and BN80915 and BN80245 (Table 2), BN80915 was the most potent. Indeed, this compound induced a 50% reduction (at least) of proliferative activity in 100% (12 of 12) of the cases analyzed when administered at \(10^{-7}\) M in the culture medium. No other compound was able to induce such a dramatic antiproliferative effect. In the same vein, 75% (9 of 12) of the cases analyzed were associated with a 50% reduction of cell proliferative activity when BN80915 was tested at \(10^{-8}\) M. This high level of 75% of cases analyzed in which at least 50% of cell proliferative activity was reduced by BN80915 was never obtained with the other compounds under study (see Tables 1 and 2). At \(10^{-7}\) and \(10^{-8}\) M, no significant BN80915-induced increase of cell proliferation was observed (Table 2). At the lowest concentration tested, i.e., \(10^{-9}\) M, the BN80915 compound was still able to significantly reduce by at least 50% cell proliferative activity in 3 of 12 cases (25%), and only 1 case of 12 (8%) exhibited a BN80915-induced increase of cell proliferative activity. This is the lowest level of "proliferation burst" observed with the seven compounds under study (Tables 1 and 2).

**DISCUSSION**

Although new camptothecin derivatives are now available for clinical applications, the fact remains that a maximum of one-third of the patients treated with these compounds will actually obtain some objective benefit from the treatment (8, 11). New derivatives are therefore needed to improve the benefit of treatment offered by Topo I inhibitors. The homocamptothecins that we have developed for such a purpose (11, 12) contain a seven-membered \(\beta\)-hydroxylactone in place of the conventional six-membered \(\alpha\)-hydroxylactone ring found in camptothecin and its analogues TPT and CPT-11 (see Fig. 1). The homologation of the lactone E-ring reinforces the stability of the lactone and consequently considerably reduces the rate of conversion into the carboxylated form, which is inactive (15). We recently have shown that homocamptothecin is much more active than the parent compound against a variety of tumor cells in vitro and in xenograft in vivo models (16). For example, in two distinct in vivo models, using L1210 murine leukemia or human colon carcinoma HT29, homocamptothecin was found to be more efficacious than camptothecin (16). The results obtained in the present study fully confirm these experimental data in a clinically relevant model because they were obtained using human colon cancers obtained directly from surgical resections and maintained ex vivo (in vitro) for a short period, i.e., 48 h.

We recently showed (15) that the superior anti-Topo I activity of homocamptothecins compared with camptothecins may be related to a certain specificity of the drug-induced DNA cleavage by Topo I. Indeed, both camptothecin and homocamptothecin stimulate the cleavage by Topo I at T/G sites, but in addition, homocamptothecin induces cleavage at sites containing the sequence AAC/G (15). At low drug concentrations, the cleavage at the T/G sites and at the homocamptothecin-specific C/G sites is more pronounced and more stable with homocamptothecin than with camptothecin, a fact that could explain why at such low concentrations the antiproliferative activity mediated by homocamptothecins (BN80245 and BN80915) is more pronounced than that observed with camptothecins (CPT, CPT-11, and SN-38) as illustrated in Tables 1 and 2. In the same vein, higher levels of protein-DNA complexes were detected in HT29 colon carcinoma and P388 leukemia cells treated with homocamptothecins than those treated with camptothecin (15). Immunoblotting experiments revealed that endogenous Topo I was efficiently trapped on DNA by homocamptothecin in cells (15). These biochemical data thus show that the \(\beta\)-hydroxylactone ring of homocamptothecin plays an important and positive role in the poisoning of Topo I.

In addition to the fact that we bring strong experimental evidence that homocamptothecins are more potent inhibitors of cell proliferation in human colon cancers than the parent compound from which they derive, the present study also shows that we succeeded in setting up an experimental model that is able to predict which colon cancers will respond to a given Topo I inhibitor, and to what extent, and which will not. The model developed here is validated by the fact that Topo II inhibitors were found to be weak inhibitors of colon cancer cell proliferation compared with Topo I inhibitors, which mirrors the clinical situation (1). As detailed in the “Introduction,” human colon cancers exhibit higher amounts of DNA Topo I than normal colon tissues. With respect to DNA Topo II, colon tissues (whether normal, dysplastic, or neoplastic) also express significant amounts of Topo II (17), but with markedly smaller differences in terms of concentrations between normal and cancerous tissues (17) than is the case for Topo I expression (3). This absence of a marked difference in Topo II expression between normal and cancerous colonic mucosa could explain why Topo II inhibitors, including ADR and VP-16, are not included in colon cancer treatments despite the fact that they are actually clinically active for other tumor types (6).

The fact that Topo II inhibitors, such as ADR and VP-16, are able to stimulate cancer cell proliferation in the 0.1–10 nm range was demonstrated by Vichi and Tritton 10 years ago (18). Such drug-induced stimulation of human colon cancer cells was also observed in the present study with the camptothecin analogues as Topo I inhibitors, but was dramatically less with the homocamptothecins.

In conclusion, the data obtained in the present study clearly show that the investigational homocamptothecin BN80915 is significantly more active in inhibiting cell proliferation in human colon cancers than the parent camptothecins from which it derives.

**REFERENCES**


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