Intracellular Roles of SN-38, a Metabolite of the Camptothecin Derivative CPT-11, in the Antitumor Effect of CPT-11

Yasuyoshi Kawato,1 Masashi Aonuma, Yasuhide Hirota, Hiroshi Kuga, and Keiki Sato
Exploratory Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 16-13, Kitakasai 1-Chome, Edogawa-ku, Tokyo 114, Japan

ABSTRACT

It is known that 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), a semisynthesized derivative of camptothecin (CPT), has a potent antitumor activity in vivo, but 7-ethyl-10-hydroxycamptothecin (SN-38), a metabolite of CPT-11, shows much stronger cytotoxicity in vitro than CPT-11. In this study, we demonstrated that the relaxation of SV40 DNA plasmids by type I DNA topoisomerase prepared from P388 murine leukemia cells was inhibited by 50% by SN-38 at approximately 1 μM, although CPT-11 at 1 mM slightly inhibited the relaxation. SN-38 and CPT showed strong, time-dependent inhibitory activity against DNA synthesis of P388 cells. However, CPT-11 weakly inhibited DNA synthesis independently of time with coincident inhibition of the total thymidine uptake by the cells. By alkaline and neutral elution assays, it was demonstrated that SN-38 caused much more frequent DNA single-strand breaks in P388 cells than did CPT-11. The same content of SN-38 and a similar frequency of single-strand breaks were detected in the cells treated with SN-38 at 0.1 μM or with CPT-11 at 100 μM. Therefore, single-strand breaks by CPT-11 seem to be due to SN-38 produced from CPT-11 in cells. These results indicate that CPT-11 itself possesses a marginal antiproliferative effect but that SN-38 plays an essential role in the mechanism of action of CPT-11.

INTRODUCTION

CPT,1 first isolated from Camptotheca acuminata (1), demonstrated antitumor effects on experimental tumors (2). However, it showed severe toxicity not only in animal experiments (3) but also in clinical trials (4–6), and it is not utilized clinically as an anticancer drug.

A semisynthesized water-soluble derivative of CPT, CPT-11, has demonstrated potent antitumor activities against several murine tumors after i.v., p.o., or i.p. administration (7–9). CPT-11 is converted to SN-38 in mouse serum and tissue homogenate (10). SN-38 possesses much stronger growth-inhibitory activity against DNA synthesis of P388 cells than CPT-11 (11). SN-38 causes SSB, in order to estimate which compound has a dominant role in the antitumor effect after treatment with CPT-11.

Recent studies revealed that CPT inhibits type I DNA topoisomerase (topoisomerase I) through the formation of stable topoisomerase I-DNA cleavable complexes (11–13). The antitumor activity of CPT analogues correlates with the drug-induced accumulation of topoisomerase I-DNA cleavable complexes (14) and with inhibitory activity against DNA relaxation by topoisomerase I (15). The mutants of yeasts lacking this enzyme are resistant to CPT (16, 17). Furthermore, topoisomerase I prepared from CPT-resistant mammalian cells is markedly resistant to CPT or CPT-11, and/or the amount and total activity of this enzyme are reduced in comparison with those of the enzyme from wild-type cells (18–22). These observations indicate that inhibition of topoisomerase I is a principal mechanism in the cytotoxicity of CPT and its derivatives in eukaryotes including mammalian cells.

Topoisomerase I-DNA cleavable complexes stabilized by CPT appear to be responsible for SSB in cultured cells (12, 23, 24), and the production of these SSB in the S-phase may cause interference with or the arrest of the replication fork, resulting in cell death (25, 26). These mechanisms offer plausible reasons for such phenomena as the inhibition by CPT, in a time-dependent and S-phase-sensitive manner, of nucleic acid synthesis (2, 27–31) and induction by CPT of the degradation of DNA in an alkaline sucrose gradient (32–34). Therefore, inhibition of nucleic acid synthesis and induction of SSB are also important parameters for evaluating the antitumor activity of CPT derivatives.

In this study, we have examined both the inhibitory activities of SN-38 and CPT-11 against topoisomerase I and against nucleic acid synthesis and the abilities of these compounds to induce SSB, in order to estimate which compound has a dominant role in the antitumor effect after treatment with CPT-11.

MATERIALS AND METHODS

Tumor Cells. P388 mouse leukemia cells were cultivated and maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with sodium bicarbonate, 10% fetal bovine serum (Biocell Lab., CA), 20 μM 2-mercaptoethanol, and 60 μg/ml kanamycin sulfate at 37°C in a humidified atmosphere of 5% CO2 in air. For the preparation of topoisomerase I, P388 cells and Ehrlich mouse ascites tumor cells were maintained i.p. in DBA/2 mice and in ddY mice, respectively, purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Chemicals and Enzymes. CPT-11, SN-38, and CPT were supplied by Yakult Honsha Co., Ltd. (Tokyo, Japan). ADM was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). [methyl-3H]Thymidine, [methyl-3H]Thymidine, [6-3H]Thymidine, and [1-4,5-3H(N)]leucine were purchased from NEN Research Products (Boston, MA). SV40 DNA was purchased from Bethesda Research Laboratories (Bethesda, MD). Proteinase K was purchased from Sigma Chemical Co., (St. Louis, MO). Topoisomerase I was prepared from P388 cells or Ehrlich ascites tumor cells, essentially as described by Ishii et al. (35). Briefly, chromatin extracted from the cells was loaded onto a heparin-Sepharose CL-6B column (Pharmacia AB, Upsalla, Sweden), and crude topoisomerase I was eluted stepwise between 0.4 and 0.7 M NaCl in 10 mM Tris (pH 7.5), 0.5 mM EDTA, and 1 mM dithiothreitol. The active fraction was applied to a phenyl-Sepharose CL-4B column (Pharmacia) and topoisomerase I was eluted stepwise between 1 and 0.5 M (NH4)2SO4.

Topoisomerase I Assay. One unit (the minimum amount for full relaxation of 0.5 μg SV40 DNA under the conditions of this study) of topoisomerase I, 0.5 μl of the test compounds, and 0.5 μg SV40 DNA were added sequentially to the reaction buffer, which was composed of 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl2, 0.25 mM EDTA disodium salt, 0.25 mM dithiothreitol, 15 μg/ml bovine serum albumin, and 5% glycerol. Then, the reaction mixture (50 μl) was incubated for 10 min at 37°C, and the reaction was terminated by treatment with 7.5 μl of a solution consisting of 1% sodium dodecyl sulfate, 20 mM EDTA disodium salt, and 0.5 mg/ml proteinase K for an additional 30 min at 37°C. The samples were mixed with 5 μl of the loading buffer containing

---

Received 1/10/91; accepted 6/4/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed.

The abbreviations used are: CPT, camptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; SSB, DNA single-strand breaks; DSB, DNA double-strand breaks; ADM, Adriamycin; IC50, IC50, concentrations inducing 50% and 25% inhibition, respectively.
10 mM Na2HPO4, 31.3% sucrose, and 0.3% bromophenol blue. Relaxed (form I) DNA was separated from supercoiled (form II) DNA by electrophoresis on 0.8% agarose gel at 50 mA and 20 V for 17 h in the presence of 2 μg/ml chloroquine, 10 mM EDTA, 30 mM Na2HPO4, and 36 mM Tris-HCl (pH 7.8). After electrophoresis, the gel was stained with 0.05% ethidium bromide and photographed with UV light (302 nm). The amount of DNA was quantified using a densitometer (ACD-25D; ATTO Corp., Tokyo, Japan). The inhibition rate of topoisomerase I activity was calculated as

\[
\left( 1 - \frac{F_{\text{in}} - F_{\text{ex}}}{F_{\text{in}} - F_{\text{ex}}} \right) \times 100 \%
\]

where \(F_{\text{in}}\) and \(F_{\text{ex}}\) are the ratios of relaxed DNA to total DNA treated with topoisomerase I in the presence and absence of a test compound, respectively. \(F_{\text{ex}}\) is the proportion of relaxed DNA in untreated DNA. The IC50 of each test compound was estimated from the dose-response curve.

**Incorporations of Thymidine, Uridine, and Leucine.** P388 cells at 2 × 10⁶ cells/ml were cultured for 2 days and further incubated with the test compounds for a given period at 37°C. The cells were treated with [3H]thymidine (0.45 μCi/ml) for the last 15 min in the incubation period or with [3H]uridine (0.9 μCi/ml) or [3H]leucine (1.8 μCi/ml) for an additional 60 min. These cells were washed twice with ice-cold phosphate-buffered saline and were solubilized by treatment with 0.5 N NaOH for 10 min on ice. After addition of the same volume of 20% trichloroacetic acid to the solubilized cells on an ice bath, the sample was filtered through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, MA). The residue on the filter was washed twice with ice-cold 5% trichloroacetic acid. The radioactivities remaining on the filter (acid-insoluble fraction) and in the filtrate (acid-soluble fraction) were measured with a liquid scintillation counter (Liquid Scintillation System, LSC-700; Aloka Co., Ltd., Tokyo, Japan). The radioactivities of the acid-insoluble fraction and of both fractions were considered to be proportional to the syntheses of macromolecules and to the cellular uptakes of their precursors, respectively. The IC50 of each test compound for macromolecule synthesis was evaluated from the dose-response curve.

**DNA Strand Breaks.** SSB and DSB were measured by alkaline elution assay (36) and neutral elution assay (37), respectively. In both assays, the elution was carried out at a rate of 0.03 to 0.04 ml/min, and the fractions were collected at intervals of 90 min. The DNA strand break frequency was calculated as described by Zwelling et al. (38).

**Intracellular Content of SN-38.** An equal number of P388 cells (1 × 10⁷ cells) incubated with 100 μM CPT-11 or 0.1 μM SN-38 for 1 h at 37°C were washed once with ice-cold phosphate-buffered saline. Half of the cells were disrupted by alternately freezing in a dry ice-methanol bath and thawing in a water bath twice (within 3 min) and then were centrifuged (7000 × g; 5 min). To stop the formation of excessive SN-38 and CPT, the supernatant was mixed with an equal volume of 90% methanol, 0.2% phenylmethylsulfonyl fluoride, and 0.1 N HCl. The same procedure was performed on the other half without the freezing and thawing. SN-38 in the mixture was isolated by high-performance liquid chromatography using a TSK gel ODS 80TM column (Tosoh Corp., Tokyo, Japan) and an eluting solution composed of 0.001 N HCl and CH3CN:H2O (2:1, v/v) and was then quantified by fluorospectrometry at an excitation wavelength of 380 nm and an emission wavelength of 556 nm (Fluorescence Spectrophotometer F1000; Hitachi, Ltd., Tokyo, Japan). The intracellular content of SN-38 was determined as

\[
[\text{SN-38}]_{\text{RT}} - [\text{SN-38}]_{\text{C}}
\]

where \([\text{SN-38}]_{\text{RT}}\) and \([\text{SN-38}]_{\text{C}}\) are the amounts of SN-38 in cells processed with and without freezing and thawing, respectively.

**RESULTS**

Effects of CPT-11 and SN-38 on Activity of Topoisomerase I. SN-38 caused the strongest inhibition of the relaxation of SV40 DNA by topoisomerase I prepared from P388 cells, followed by CPT and then CPT-11 (Fig. 1). In this experiment, the respective IC50 of SN-38, CPT, and CPT-11 were 0.74, 2.3, and >1000 μM, as listed in Table 1. Each compound showed a similar relative activity against the enzyme prepared from Ehrlich tumor cells.

For comparison of the inhibitory activities of SN-38 and CPT-11, the IC50 value for topoisomerase I of P388 was calculated similarly to that of the IC50. This value of CPT-11 was 0.72 μM, whereas that of SN-38 was 0.20 μM (about 3600-fold stronger than CPT-11).

CPT-11 dose dependently shifted the position of relaxed DNA in the direction of nicked DNA, as shown in Fig. 1. SN-38 and CPT showed no effect on the position of relaxed DNA in the experiments described above.

Effects of CPT-11 and SN-38 on DNA, RNA, and Protein Syntheses and on Cellular Uptakes of Their Precursors. The dose-dependent effects of 2- or 3-h treatment of CPT-11, SN-38, or CPT on DNA, RNA, and protein syntheses are shown in Fig. 2. CPT-11, SN-38, and CPT dose-dependently inhibited DNA synthesis. The synthesis was decreased to 20% of the

![Fig. 1. Inhibition of topoisomerase I-induced relaxation of supercoiled DNA by CPT-11, SN-38, and CPT.](image-url)
INTRACELLULAR ROLES OF SN-38 IN EFFECT OF CPT-11

Fig. 2. Dependence of inhibition of DNA, RNA, and protein syntheses on concentrations of CPT-11 (A), SN-38 (B), and CPT (C). Respective syntheses were determined by the degrees of incorporation of [3H]thymidine (O), [3H]uridine (□), and [3H]leucine (■) into acid-insoluble fractions of P388 cells treated with each agent at 37°C for 2 (DNA synthesis) or 3 (RNA and protein syntheses) h.

Table 2: Inhibitory effects of SN-38, CPT, and CPT-11 on DNA and RNA synthesis in P388 cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC50 (μM)</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>0.077 (1.0)</td>
<td>1.3 (1.0)</td>
<td></td>
</tr>
<tr>
<td>CPT</td>
<td>0.18 (2.3)</td>
<td>2.4 (1.9)</td>
<td></td>
</tr>
<tr>
<td>CPT-11</td>
<td>19 (~250)</td>
<td>61 (~50)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, ratios of IC50 of respective agents to that of SN-38.

control at 100 μM of CPT-11. At a concentration of 1 μM, SN-38 and CPT reduced the synthesis to 12 and 21% of the control, respectively. The inhibitory effect of each compound on RNA synthesis was less than that on DNA synthesis. No inhibition was observed in protein synthesis after the treatment with any of the compounds. Respective IC50 values of CPT-11, SN-38, and CPT in DNA synthesis were 19, 0.077, and 0.18 μM, as listed in Table 2.

The time-dependent effects of each test compound on DNA synthesis and cellular thymidine uptake are illustrated in Fig. 3. The inhibitory effect of CPT-11 against DNA synthesis was time independent, although those of SN-38 and of CPT were time dependent. Moreover, the 20-min treatment with CPT-11 reduced the cellular thymidine uptake to 67 and 25% at 10 and 100 μM, respectively. This reduction also occurred in a time-independent manner. The cellular uptake was not inhibited by the 60-min treatment with 1 μM of SN-38 or CPT, whereas DNA synthesis was strongly inhibited. The cellular uridine uptake was also inhibited by CPT-11 but was not suppressed by the other compounds. The cellular leucine uptake was not affected by any of the compounds (data not shown).

DNA Strand Breaks by CPT-11 and SN-38. After a 1-h treatment, CPT-11 caused SSB much less frequently than SN-38 and ADM, as demonstrated in Fig. 4. The ability of CPT-11 above 400 μM to produce SSB was saturated at about 400 rad-equivalents (Fig. 4A). SSB frequencies induced by 0.1 μM SN-38 and by 100 μM CPT-11 were 74 ± 2 and 115 ± 22 rad-equivalents, respectively (Table 3). A similar frequency (118 rad-equivalents) was induced by 1 μM ADM. ADM also induced DSB at 2 μM, but no obvious DSB were detected after treatments with either SN-38 at 1 μM or CPT-11 at 1 mM (data not shown). The relationship between SSB frequency and DSB frequency is illustrated in Fig. 5, which reveals that few or no SSB arising from DSB are included in the apparent SSB induced by SN-38 or CPT-11 (38).

Relationship of SSB Frequency and Intracellular Content of SN-38. Table 3 compares the effects of the 1-h treatment with 100 μM CPT-11 and that with 0.1 μM SN-38 on the frequency of SSB and on the intracellular content of SN-38. No significant differences in either parameter were observed between these two treatments. These results indicate that SN-38 has a potency to induce SSB that is 1000 times stronger than that of CPT-11 and that most SSB induced by CPT-11 are due to SN-38.

DISCUSSION

CPT-11 shows potent antitumor activities in antitumor tests in vivo (7–9). SN-38, a metabolite of this compound, possesses a much higher cytotoxicity against tumor cells in vitro (10), although it seems to be less effective in vivo (7), in comparison with CPT-11. CPT, the mother compound of CPT-11, is known as a specific inhibitor of topoisomerase I (23). The inhibition of this enzyme is thought to be responsible for the cytotoxicity...
This result indicates that the inhibition of topoisomerase I by CPT-11 was 0.16 ± 0.01 μM (triplicate tubes), which is close to 0.23 μM. The concentration of SN-38 producing a 28% inhibition was estimated at 0.23 μM from the dose-response curve. When 1 mM CPT-11 is mainly attributable to the SN-38 derived from CPT-11. CPT-11 produces a 28% inhibition even at 1 mM, and the activity of CPT-11 was about 3600 times less than that of SN-38.

CPT-11 is mainly attributable to the SN-38 derived from CPT-11. The inhibitory activity of CPT-11 was about 3600 times less than that of SN-38. CPT-11 produced a 28% inhibition even at 1 mM, and the activity of CPT-11 was about 3600 times less than that of SN-38.

Therefore, it is believed that SN-38 plays a major role in the cytotoxicity of CPT-11 on the molecular level.

The inhibitory effects of CPT-11 on DNA synthesis and on RNA synthesis were approximately 250 and 50 times less than those of SN-38, respectively (Table 2). It is impossible to explain these results using the intracellular content of SN-38, because a concentration of CPT-11 1000 times higher than that of SN-38 was required to obtain a similar intracellular content of SN-38 (Table 3). Therefore, CPT-11 seems to have its own inhibitory effects on DNA and RNA synthesis at high concentrations (>10 μM). However, these effects have different characteristics from those of SN-38 and CPT. The inhibitory effects of CPT-11 were time independent, whereas those of the other compounds were time dependent. Moreover, CPT-11 induces coincident time-independent inhibitions of the cellular uptakes of thymidine and uridine, which were not induced by either SN-38 or CPT. When the cells were treated with SN-38 or CPT, the radioactivity of the acid-soluble fraction increased and compensated for the decrease in that of the acid-insoluble fraction, but it was unchanged after treatment with CPT-11. These results suggest that CPT-11 suppresses independently of time the regulatory mechanism(s) of the transport of nucleic acid precursors and that this suppression relates to the apparent inhibition of nucleic acid synthesis.

Even if CPT-11 possesses unexpected action(s) on nucleic acid synthesis, this compound itself appears to have a marginal antiproliferative effect, because the concentrations required to show such actions are much higher (>250 times) than those at which SN-38 affects topoisomerase I and DNA synthesis and the culture medium increased the concentration of SN-38 to less than 10 nM under the conditions under which SSB were tested. However, the treatment with CPT-11 at 100 μM induced a similar frequency of SSB and gave an intracellular content of SN-38 similar to those after treatment with SN-38 at 0.1 μM. These observations indicate that SN-38 was produced from CPT-11 in cells and that the SSB induced by CPT-11 were principally due to this SN-38.

The inhibitory effects of CPT-11 on DNA synthesis and on RNA synthesis were approximately 250 and 50 times less than those of SN-38, respectively (Table 2). It is impossible to explain these results using the intracellular content of SN-38, because a concentration of CPT-11 1000 times higher than that of SN-38 was required to obtain a similar intracellular content of SN-38 (Table 3). Therefore, CPT-11 seems to have its own inhibitory effects on DNA and RNA synthesis at high concentrations (>10 μM). However, these effects have different characteristics from those of SN-38 and CPT. The inhibitory effects of CPT-11 were time independent, whereas those of the other compounds were time dependent. Moreover, CPT-11 induced coincident time-independent inhibitions of the cellular uptakes of thymidine and uridine, which were not induced by either SN-38 or CPT. When the cells were treated with SN-38 or CPT, the radioactivity of the acid-soluble fraction increased and compensated for the decrease in that of the acid-insoluble fraction, but it was unchanged after treatment with CPT-11. These results suggest that CPT-11 suppresses independently of time the regulatory mechanism(s) of the transport of nucleic acid precursors and that this suppression relates to the apparent inhibition of nucleic acid synthesis.
induces DNA strand breaks. Furthermore, as stated above, it is believed that topoisomerase I inhibition and SSB after treatment with CPT-11 depend principally on SN-38. Therefore, at the intracellular level, it appears that SN-38 plays a dominant role in the antitumor effect caused by CPT-11.

REFERENCES


Intracellular Roles of SN-38, a Metabolite of the Camptothecin Derivative CPT-11, in the Antitumor Effect of CPT-11

Yasuyoshi Kawato, Masashi Aonuma, Yasuhide Hirota, et al.