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

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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-hydroxy-camptothecin (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, 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 (9). SN-38 possesses much stronger growth-inhibitory activity against tumor cells than CPT-11 in vitro and is thought to play an important role in the antitumor effect of CPT-11 in vivo (10).

Recent studies revealed that CPT inhibits type I DNA topoisomerase I (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, 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-4C]thymidine, [6-3H]uridine, and L-[4,5-3H(A')]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, Uppsala, 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...
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 mM, 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. 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 ( ), [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

DNA and RNA syntheses were measured at 37°C from the respective incorporations of [3H]thymidine and [3H]uridine into the acid-insoluble fractions, as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Agent</th>
<th>DNA IC₅₀ (μM)</th>
<th>RNA IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>0.077 (1.0)</td>
<td>1.3 (1.0)</td>
</tr>
<tr>
<td>CPT</td>
<td>0.38 (2.3)</td>
<td>2.4 (1.9)</td>
</tr>
<tr>
<td>CPT-11</td>
<td>19 (~250)</td>
<td>61 (~50)</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 of CPT-11, indicating that SN-38 is mainly attributable to the SN-38 derived from CPT-11. 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 syntheses 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.

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 of CPT (14–23, 25, 26). Therefore, it is important to evaluate the effects of CPT-11 and SN-38 on this enzyme. Andoh et al. (18) reported that the activity of topoisomerase I of a human lymphoblastic leukemia cell line was inhibited by SN-38 but not by CPT-11. In the present study, we have demonstrated that SN-38 has a stronger inhibitory effect on topoisomerase I of P388 cells than CPT, whereas CPT-11 only slightly inhibits the relaxation of DNA (Fig. 1). Using IC₅₀'s, 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 concentration of SN-38 producing a 28% inhibition was estimated at 0.23 μM from the dose-response curve. When 1 mM of CPT-11 was incubated under the same conditions as were used for the topoisomerase I assay, the concentration of SN-38 was 0.16 ± 0.01 μM (triplicate tubes), which is close to 0.23 μM. This result indicates that the inhibition of topoisomerase I by CPT-11 is mainly attributable to the SN-38 derived from CPT-11. Therefore, it is believed that SN-38 plays a major role in the cytotoxicity of CPT-11 on the molecular level.

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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
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.

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