Synergistic Effects of Topoisomerase I Inhibitor, 7-ethyl-10-hydroxycamptothecin, and Irradiation in a Cisplatin-resistant Human Small Cell Lung Cancer Cell Line

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ABSTRACT

7-ethyl-10-[4-(1-piperidyl)-1-piperidyl] carbonyloxy-camptothecin, a topoisomerase I (topo I) inhibitor, is one of the most active agents against lung cancer, and its radiosensitizing effect has been reported recently. We evaluated a combination in vitro effect of irradiation and 7-ethyl-10-hydroxy-CPT (SN-38), an active metabolite of 7-ethyl-10-[4- (1-piperidyl)-1-piperidyl] carbonyloxy-camptothecin, on a human small cell lung cancer cell line (SBC-3) and its cisplatin-resistant subline (SBC-3/CDDP). Growth-inhibitory effects of irradiation with or without SN-38 were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A modified isobologram method was used to evaluate the treatment interaction. The combination of irradiation and SN-38 showed a synergistic inhibitory effect on the growth of SBC-3/CDDP despite its cross-resistance to irradiation and SN-38. In contrast, the same combination showed only an additive effect on the growth of parental SBC-3 cells. There was no significant difference in topo I protein expression between these two cell lines. In SBC-3 cells, topo I catalytic activity was suppressed by 4 Gy of irradiation, without a decrease in nuclear topo I protein, whereas the exposure of SBC-3 cells to 1 μM SN-38 subsequent to irradiation showed no remarkable additional effects on both topo I activity and protein content. On the other hand, in SBC-3/CDDP cells, topo I activity was unchanged by irradiation, but the subsequent exposure to SN-38 gave rise to a decrease in topo I activity, which was accompanied by a significant decrease in the topo I protein content ($P = 0.02$). These observations may indicate that SN-38 induces sequestration of topo I onto DNA in radiation-treated SBC-3/CDDP cells and suggest that the synergistic effect of irradiation and SN-38 in SBC-3/CDDP cells was considered attributable to DNA repair-related enhanced recruitment of topo I onto the damaged DNA.

INTRODUCTION

Recently, combined modality treatment consisting of cisplatin (CDDP)-based chemotherapy and thoracic irradiation has been shown to produce survival benefit in patients with locally advanced non-small cell lung cancer (1). In SCLC,2 response rates and survival in patients with limited disease have been markedly improved by concurrent administration of the CDDP-containing chemotherapy and chest irradiation (2). However, despite a higher response rate and longer survival, relapses occur in the majority of patients treated with the CDDP-containing chemoradiotherapy. At the time of relapse, further radiation is difficult because of its toxicity, and second-line chemotherapy usually yields disappointing results (3).

The DNA topoisomerases have been considered as important therapeutic targets for cancer chemotherapy. Topo I inhibitors, such as CPT-11 and topotecan, a semisynthetic derivative of camptothecin, showed the strong anticancer activity against human tumor cells (4). CPT-11 was reported to be effective for treatment of patients with relapsed or refractory lung cancer after receiving the conventional CDDP-based chemotherapy (5). Furthermore, topotecan was reported to enhance potential lethal DNA damage induced by radiation (6). Recent clinical trials have implied the usefulness of topotecan or CPT-11 as a radiation sensitizer in the treatment of advanced localized non-small cell lung cancer and LD-SCLC (7–9).

In this study, we evaluated in vitro the synergistic effects of a combination of SN-38 (an active metabolite of CPT-11) and irradiation in a human SCLC cell line and its CDDP-resistant subline.

MATERIALS AND METHODS

Chemicals and Reagents. Drugs used in this study were as follows: adriamycin was obtained from Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan; cisplatin and etoposide were obtained from Bristol-Myers Squibb K. K., Tokyo, Japan; SN-38 (active metabolite of CPT-11) was from Yakult Honsha, Ltd., Tokyo, Japan; MTT was purchased from Sigma Chemical Co., St. Louis, MO.

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2 The abbreviations used are: SCLC, small cell lung cancer; topo I, topoisomerase I; CPT-11, 7-ethyl-10-[4-(1-piperidyl)-1-piperidyl] carbonyloxy-camptothecin; SN-38, 7-ethyl-10-hydroxy-CPT; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DSB, double-strand break.
Cell Line and Cultures. The human SCLC cell line SBC-3 (JCRB0818) was established from bone marrow aspirates of an untreated patient with SCLC in our laboratory (10). The adriamycin-resistant subline (SBC-3/ADM100), CDDP-resistant subline (SBC-3/CDDP), and etoposide-resistant subline (SBC-3/ETP) were established from SBC-3 by in vitro exposure to increasing concentrations of each drug (11–14). All cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.), penicillin-G (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% CO₂ in air.

Cytotoxicity Assay. Drug sensitivity was determined by an MTT assay (15) with a slight modification (16). Briefly, an adequate number of cells was plated in quadruplicate in 96-well, flat-bottomed microplates and incubated for 96 h in the media containing test agents. Ten μl of MTT solution (5 mg/ml in PBS) were added in each well, and the plates were incubated at 37°C for another 4 h. The absorbance at 560 nm was measured using Microplate Reader (Model 3550; Bio-Rad Laboratories, Richmond, CA). IC₅₀ was determined based on the dose-response curve obtained previously.

Radiosensitivity was also determined by an MTT assay, as described previously (17). An adequate number of cells were plated in quadruplicate in 96-well, flat-bottomed microplates, and then the cells were irradiated at a dose rate of 102 cGy/min for an appropriate time. The absorbance at 560 nm was measured using Microplate Reader (Model 3550; Bio-Rad Laboratories, Richmond, CA). IC₅₀ was determined based on the dose-response curve obtained previously.

Preparation of Nuclear Protein Extracts and Determination of Topo I Activity. Crude nuclear extracts were prepared as described previously (19). In brief, 5 × 10⁶ cells were incubated in 1 ml of PBS containing 10 mM MgCl₂ and 0.35% volume for volume Triton X-100. After 10 min of incubation on ice, nuclei were pelleted by centrifugation at 1.000 × g for 10 min. Nuclear protein was extracted by incubation with an extraction buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.35 M NaCl, 140 mM 2-mercaptoethanol, and 50 μg/ml BSA (Takara Shuzo, Kyoto, Japan). After 30 min of incubation of ice, the lysate was centrifuged at 12,000 × g for 5 min. The protein concentration was determined using the method of Bradford (20).

Topo I catalytic activity in nuclear extract was determined using a DNA unwinding assay kit (TopoGEN, Inc., Columbus, OH), according to the instructions of the manufacturer’s manual. In brief, 0.5 μg of super-coiled form pBR322 plasmid DNA was incubated in quadruplicate in 96-well, flat-bottomed microplates and then the cells were irradiated at a dose rate of 102 cGy/min for an appropriate time. The absorbance at 560 nm was measured using Microplate Reader (Model 3550; Bio-Rad Laboratories, Richmond, CA). IC₅₀ was determined based on the dose-response curve obtained previously.

Actual IC₅₀ values were obtained. When the experimentally observed IC₅₀ is plotted on the left side of the envelope, this combination is considered to show a supra-additive (synergistic) interaction. When the observed IC₅₀ is plotted within the envelope, this combination is regarded as additive, and when it is on the right of the envelope and within the dotted-line square, this combination is considered to be subadditive. When the observed IC₅₀ is plotted outside the square, this combination is considered to be protective.

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To evaluate the effect of a combination of irradiation and SN-38, the cells were incubated in media containing various concentrations of SN-38, followed by different doses of irradiation. After 96 h of incubation, an MTT assay was carried out. The combination effects were analyzed by a modified isobologram method (18). Briefly, three isoeffect curves (modes I, IIA, and IIB), which were made up on the basis of each growth inhibition curve, are drawn, and the total area enclosed by these three lines represents an “envelope of additivity” (Fig. 1). Actual IC₅₀ values were obtained. When the experimentally observed IC₅₀ is plotted on the left side of the envelope, this combination is considered to show a supra-additive (synergistic) interaction. When the observed IC₅₀ is plotted within the envelope, this combination is regarded as additive, and when it is on the right of the envelope and within the dotted-line square, this combination is considered to be subadditive. When the observed IC₅₀ is plotted outside the square, this combination is considered to be protective.

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unwinded by 15-min incubation at 37°C with adequately diluted nuclear protein extract and resolved on 1% agarose gel. Arrow, \( /H9262 \) separated on a 1% agarose gel, then visualized with 0.5% glycerol, and 0.1% solution containing 1% Sarkosyl, 0.025% bromphenol blue, 5% C, the reaction was terminated by adding 10\(^{-3}\)/H9262 37°/H9262 5% volume for volume glycerol. After 15 min of incubation at

Table 1 shows the IC\(_{50}\) values of adriamycin, cis-

was incubated with 1 \(\mu\)l of serially diluted crude nuclear extracts in reaction buffer comprised of 10 m\(\text{M}\) Tris-HCl (pH 7.5), 1 m\(\text{M}\) EDTA, 0.15 m\(\text{NaCl}\), 0.1% BSA, 0.1 m\(\text{M}\) Spermidine, and 5% volume for volume glycerol. After 15 min of incubation at 37°C, the reaction was terminated by adding 10 \(\mu\)l of dye solution containing 1% Sarkosyl, 0.025% bromphenol blue, 5% glycerol, and 0.1 \(\mu\)g of proteinase K. The reaction products were separated on a 1% agarose gel, then visualized with 0.5 \(\mu\)g/ml ethidium bromide, and photographed under UV light. To evaluate the inhibition of topo I activity by SN-38, 1 \(\mu\)M SN-38 was added to the reaction mixture.

**Immunoblot Analysis of Topo I Protein.** Nuclear protein extracts (50 \(\mu\)g) of each cell line were resolved on 7% SDS-polyacrylamide gel, electroblotted onto nitrocellulose membrane, and probed with antihuman topo I polyclonal antibody (1:300, Topogen, Inc.). The recognized proteins were visualized using an electro-chemiluminescence system (Amerham, Buckinghamshire, United Kingdom). Densities of the bands were quantified with a densitometer scanner (Molecular Dynamics, Sunnyvale, CA). To distinguish topo I protein band from nonspecific protein bands, we measured the density of both bands separately using NIH image ver. 1.61.

**RESULTS**

**Drug Sensitivity Profile of SBC-3 and Its Drug-resistant Sublines.** Table 1 shows the IC\(_{50}\) values of adriamycin, cis-platin, etoposide, SN-38, and irradiation for SBC-3 and its resistant sublines. SBC-3/CDDP cells were 10.4-fold resistant to CDDP compared with parental cells. SBC-3/ADM100 and SBC-3/ETP cells were 112.2- and 149.5-fold resistant to ADM and ETP, respectively, compared with parental cells. SBC-3/CDDP cells showed a cross-resistance to both SN-38 and irradiation. However, SBC-3/ADM100 and SBC-3/ETP cells showed only weak cross-resistance to SN-38 and collateral sensitivity to irradiation.

**Combination Effects of SN-38 and Irradiation by a Isobologram Analysis.** On the basis of growth inhibition curves of each treatment, three isoeffect curves (modes I, mode IIA, and IIB) were drawn (Fig. 1). In SBC-3/CDDP cells, the combination of irradiation and subsequent SN-38 showed a supra-additive effect. However, the same combination showed only additive effects in the other cell lines.

**Effects of Irradiation on the Topo I Catalytic Activity.** Fig. 2 shows sequential changes of the topo I catalytic activities in SBC-3 and SBC-3/CDDP cells after 4Gy of irradiation were given. In SBC-3 cells, topo I activity was immediately decreased after irradiation, whereas topo I activity in SBC-3/CDDP was unchanged after irradiation.

**Combination Effects of Irradiation and SN-38 on Topo I Catalytic Activity.** To evaluate the effect of subsequent incubation with SN-38 on topo I activity in irradiated cells, SBC-3 and SBC-3/CDDP cells were incubated with 1 \(\mu\)M SN-38.
Effects of the combination of radiation and SN-38 on topo I catalytic activity in SBC-3 and SBC-3/CDDP cells. Cells were incubated in the media with or without 1 μM SN-38 subsequent to 4 Gy of irradiation. After 15-min incubation at 37°C, nuclear protein was extracted from 5 × 10^6 cells as described in “Materials and Methods.” Supercoiled plasmid DNA (0.5 μg) was unwound by 15-min incubation at 37°C with serially diluted nuclear protein extract and resolved on 1% agarose gel. Amounts of nuclear protein used for assay were indicated at the top of each lane. For comparison, results of nuclear protein extracted from untreated cells and treated with SN-38 alone were included. Arrow, bands of supercoiled plasmid DNA. Bracket, bands of plasmid DNA unwound by topo I. Figures are representatives of multiple experiments.

Effects of the combination of radiation and SN-38 on topo I protein expression in SBC-3 and SBC-3/CDDP cells. Subsequent to 4 Gy of irradiation, SBC-3 and SBC-3/CDDP cells were incubated in the media with or without 1 μM SN-38. After 15-min incubation at 37°C, 50 μg of nuclear protein extracts were resolved on 7% SDS-polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with antihuman topo I polyclonal antibody. The bands of topo I were visualized using electro-chemiluminescence system. For comparison, results of nuclear protein extracted from untreated cells and treated with SN-38 alone were included. Arrow, M_r 102,000 topo I bands. Figures are representatives of multiple experiments.

for 15 min at 37°C after 4 Gy of irradiation (Fig. 3). Exposure to the same concentration and duration of SN-38 without irradiation did not affect topo I activities in both cell lines. In SBC-3 cells, the subsequent exposure to SN-38 gave no remarkable effects on topo I activity. However, in the SBC-3/CDDP cells, the subsequent exposure to SN-38 gave rise to a remarkable decrease in topo I activity.

**Combination Effects of Irradiation and SN-38 on Topo I Protein Expression.** To evaluate the effect of SN-38 on topo I protein expression in irradiated cells, SBC-3 and SBC-3/CDDP cells were incubated with 1 μM SN-38 for 15 min at 37°C after 4 Gy of irradiation (Fig. 4). Although the results of immunoblot analysis showed tendency of increased nuclear topo I contents in SBC-3/CDDP cells compared with SBC-3 cells, there was no statistically significant difference in the topo I content between these two cell lines.

In both cell lines, topo I protein levels were not altered by irradiation alone. In SBC-3 cells, an addition of SN-38 exposure after irradiation did not alter topo I protein levels. However, in the SBC-3/CDDP cells, the subsequent exposure to SN-38 resulted in a remarkable decrease in topo I protein levels (62.6%, P = 0.02). Table 2 shows the ratio of topo I protein:total nonspecific protein according to treatment. The ratio in SBC-3/CDDP cells receiving irradiation and SN 38 treatment was significantly decreased in comparison with that receiving no treatment (P = 0.02), whereas that receiving irradiation alone or SN-38 alone was not decreased.

**DISCUSSION**

A topo I inhibitor such as CPT-11 is an effective agent in cancer chemotherapy. Recently, CPT-11 has been reported to
produce high response rates for lung cancer when used in combination with cisplatin (21, 22). Furthermore, CPT-11 has been shown to increase ionizing radiation effects substantially in combination with cisplatin (21, 22). Furthermore, CPT-11 has produce high response rates for lung cancer when used in combination with cisplatin (21, 22). Furthermore, CPT-11 has been shown to increase ionizing radiation effects substantially in combination with cisplatin (21, 22). Furthermore, CPT-11 has

Boothman et al. (23, 25) reported that increases in topo I binding to DNA and DSB formation were associated with enhanced cytotoxicity in radiation-treated human melanoma cells which had been exposed to topo I modulators. However, augmentation of the radiation effect by topo I modulators was not accompanied by an increase in a protein level or activity of topo I, as might have been expected. Instead, the topo I activity diminished immediately after irradiation with no alteration in a topo I protein content (26). To provide an explanation for these findings, Boothman et al. hypothesized that: (a) down-regulation of topo I activity is a physiological cellular response to prevent its binding to single-stranded DNA nicks created by irradiation and facilitate the binding of the DNA repair complex to the single-stranded DNA nick; and (b) despite the cellular process to decrease topo I enzyme activity after irradiation, exposure of radiation-damaged cells to topo I modulators stimulates a topo I-mediated suicide pathway by affecting the topo I down-regulation and/or altering the active site of topo I, which results in irreversible conversion of single-strand breaks to unrepairable protein-linked DSBs. In our present study, a combination of irradiation and SN-38 showed a synergistic effect in CDDP-resistant SBC-3/CDDP cells but not in the parental cells. In SBC-3/CDDP cells, although irradiation itself did not suppress a topo I activity or topo I protein expression, subsequent exposure to SN-38 resulted in the decrease of both topo I activity and protein content. These decreases were considered to be relevant to the synergistic effect of SN-38 and irradiation in SBC-3/CDDP cells.

The possible explanation for the rapid decrease in topo I protein is sequestration of topo I onto the damaged DNA. According to our preliminary data, SBC-3/CDDP cells were considered to retain an increased ability to repair radiation-induced DNA damage compared with the parental cells. Furthermore, in the SBC-3/CDDP cells, the suppression of radiation-induced DNA repair by SN-38 was more remarkable than that in the SBC-3 cells (27). Although we did not examine a difference in cleaved complex formation induced by SN-38 in irradiated SBC-3/CDDP cells, it is conceivable that the formation of protein-DNA complexes was increased because of enhanced topo I recruitment, presumably related to increased ability to repair DNA damage. The cellular processing of radiation-induced DNA damage and SN-38-induced DNA DSBs produced at replication may share the common pathway. Although there have been few models which are able to explain how topoisomerases could influence DNA repair, it suggested that topoisomerase may play a role in DNA repair (28). It is also implied that DNA conformational changes mediated by topo I may have some contribution somewhat to DNA repair induced by irradiation. Another possible explanation for the decrease in topo I protein is proteasomal degradation of topo I protein. Recent studies have demonstrated that topo I-mediated DNA damage activates the ubiquitin/proteasome pathway, resulting in rapid degradation of topo I protein (29). In contrast to our results, this decrease of topo I by the proteasomal degradation is speculated to result in resistance to topo I inhibitors. Our experiments cannot eliminate the possibility of proteasomal degradation. However, because ubiquitination is supposed to occur on topo I trapped in the protein-DNA complex, it is highly indicated that the formation of protein-DNA complex is increased in SBC-3/CDDP cells treated with irradiation and SN-38. Our data suggest the potential advantage of topo I inhibitors in combination with radiation therapy for CDDP-resistant tumors. Accordingly, a mechanism of the synergistic effect of irradiation and SN-38 in SBC-3/CDDP cells is considered attributable to enhanced recruitment of topo I onto the DNA related to enhanced DNA-repair ability.

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Synergistic Effects of SN-38 with Irradiation


Synergistic Effects of Topoisomerase I Inhibitor, 7-ethyl-10-hydroxycamptothecin, and Irradiation in a Cisplatin-resistant Human Small Cell Lung Cancer Cell Line

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