**ABSTRACT**

Among the numerous clinical regimens used in combination chemotherapy, synergy is particularly marked in combinations containing cisplatin (CDDP). However, the clinical use of CDDP is sometimes limited due to its nephrotoxicity. Nedaplatin (NDP) is a second-generation platinum complex with reduced nephrotoxicity that may substitute for CDDP or even surpass it for use in combination with other drugs. We investigated the effects of combinations of NDP and other anticancer drugs on the growth of human small cell lung cancer cells (SBC-3) and non-small cell lung cancer cells (PC-14) using a three-dimensional analysis model. Among the combinations tested, the combination of NDP and irinotecan (CPT-11) showed the most marked synergistic interaction, and the synergism has also been observed against PC-14 cells. With regard to treatment schedule, a remarkable synergistic interaction was produced by concurrent exposure to NDP and CPT-11. On the other hand, sequential exposure to the two drugs led only to additivity. To analyze the interaction between the drugs, the effect of NDP on the 7-ethyl-1-hydroxy-CPT (the active form of CPT-11)-induced inhibitory effect on DNA topoisomerase I was examined. The topoisomerase I-inhibitory effect of 7-ethyl-1-hydroxy-CPT was enhanced 10-fold in the presence of NDP at microgram/milliliter concentrations. These biochemical interactions might be responsible for the synergistic interaction between NDP and CPT-11. These results suggest that the combination of NDP with CPT-11 may be clinically useful for the chemotherapy of lung cancer.

**INTRODUCTION**

Lung cancer is one of the three major causes of cancer-related deaths in Japan (1). For over 40 years, CDDP(1) has been the mainstay of systemic and regional chemotherapy, both for adjuvant chemotherapy and for the treatment of the advanced-stage disease (2). The response rates observed with the use of CDDP alone were relatively low (10–20%; Ref. 3). Various attempts have been made to improve the objective response rates to CDDP, including the use of CDDP in combination chemotherapy. Among the numerous CDDP-containing clinical regimens devised, synergy was found to be particularly marked in the case of the combination of VP-16 and CDDP (4–8). However, the clinical use of CDDP has sometimes been limited by its nephrotoxicity (9). Therefore, efforts have been made to obtain analogues of CDDP with lower toxicity and the same or greater antitumor potency. NDP was selected on the basis of its high activity against rodent solid tumors and human cancer cells (10–12), as well as its lack of renal toxicity (13). In clinical Phase II studies, NDP showed pronounced efficacy against lung, head and neck, testicular, and gynecological cancers (14, 15).

In the present study, we examined whether combination chemotherapy with NDP and VP-16 resulted in as marked a synergistic anticancer activity against lung cancers as that of the CDDP and VP-16 combination. We also selected CPT-11 as the best candidate drug for combination with NDP using a three-dimensional analysis model established by our group.

Because our final goal is to eventually develop the combination for use in a clinical setting, we attempted to identify the optimal treatment schedule that must be used to achieve the synergistic interaction and to determine the biochemical mechanism underlying the synergistic interaction between the two drugs.

**MATERIALS AND METHODS**

**Materials.** NDP and VDS were provided by Shionogi and Co., Ltd. (Osaka, Japan). CDDP and VP-16 were received...
as gifts from Bristol Myers Squibb Japan (Tokyo, Japan). ADM was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), and CPT-11 and SN-38 were obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). RPMI 1640 (Life Technologies, Inc.) and FCS were purchased from Nissui (Tokyo, Japan). Plasmid DNA pBR322 was purchased from Toyobo Co., Ltd. (Osaka, Japan).

**Cell Lines and Culture.** Human SCLC cell line SBC-3 and human NSCLC cell line PC-14 were used. They were kindly provided by Dr. I. Kimura (Okayama University, Okayama, Japan) and Dr. Y. Hayata (Tokyo Medical College, Tokyo, Japan), respectively. Both cell lines grew as very loosely adherent floating aggregates in small clumps. The cells were propagated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin in an incubator at 37°C and 100% humidity with 5% CO₂ and air, as described previously (16).

**Antiproliferative Activity.** We measured the inhibition of cell proliferation after drug treatments as the antitumor activity using a regrowth assay described previously (16). Briefly, cells were exposed to drugs alone or in combination for 6 days at 37°C in an atmosphere of 100% humidity with 5% CO₂; the cells were then pipetted six to eight times until almost all cells appeared as single cells and counted with a TOA Microcell counter (CC-108; TOA Medical Electronics Co., Kobe, Japan). For each drug, concentration-effect curves were drawn as plots of the fraction of surviving cells (unaffected cell fraction, fa) versus drug concentration.

The cell proliferation ratio of the treated:control cultures (T:C%) was calculated as follows: [(the number of treated cells on day 6)/(the number of control cells on day 6)] × 100%. The IC₅₀ was defined as the drug concentration required for a 50% reduction in the number of cells. Four or five independent experiments were carried out for each.

To check the effect of the drug treatment schedule on the effect of the combination, the cells were treated either by simultaneous exposure to the two drugs or by sequential exposure to NDP followed by CPT-11 (NDP→CPT-11) and *vice versa* (CPT-11→NDP) for 3 h. For the sequential exposure treatment, cells were exposed to the first drug for 3 h, washed in fresh medium once, and then immediately exposed to the second drug for 3 h. The treated cells were cultured in drug-free medium until evaluation.

**Assessment of the Effect of the Combination.** The theoretical basis of three-dimensional model analysis has been described in a previous study (17). Briefly, according to Chou and Talalay (18), the theoretical additive effect can be calculated from (fa)₀ and (fa)ₚ, which are the fractions affected by drug A and drug B, respectively. The calculated response surface (Sₐₐ) was obtained by integrating the theoretical additive effects with respect to (fa)₀ and (fa)ₚ given by Eq. A.

\[
(S_{A,B})_{cal} = \int_{a=0}^{1} \int_{b=0}^{1} \frac{(f_a)_0 + (f_a)_p + 2(f_a)_0(f_a)_p}{1 - (f_a)_0(f_a)_p} \]  \hspace{1cm} (A)

The observed response surface [(Sₐₐ)ₐₐ] was also obtained from (fa)₀,ₐ₋ₚ, which is the fraction affected by the drugs, by integrating with the concentrations of drug A and drug B. An observed response surface greater or lower than the calculated response surface is considered to reflect a greater or lesser effect than the additive combination effect, respectively. Furthermore, when the difference was statistically significant, the effect was defined as synergism or antagonism.

The 95% confidence limit values were obtained to multiply the variances Sₐ and Sₐₐ of drug A and drug B, respectively, and were integrated with respect to the drug concentrations in accordance with the following equation to obtain the 95% confidence limit surface, (Sₐₐ)ₚₚₚₚ.

\[
(S_{A,B})_{CL} = \int_{a=0}^{t} \int_{b=0}^{t} \frac{S_A(n_A - 1) + S_B(n_B - 1)}{(n_A + n_B)(n_A + n_B - 2)} \]  \hspace{1cm} (B)

where t is the t value (df = n – 1; α = 0.05), a is the concentration of drug A(k – l), and b is the concentration of drug B(m – n).

Dividing the raw combination effect surface by the 95% confidence limit surface, regions >1 or < -1 are defined as synergism or antagonism, respectively. The region between 1 and -1 represents additive effect.

**Preparation of Nuclear Extracts.** Crude nuclear extracts were prepared as described previously by Deffie et al. (19). In brief, cells were collected by centrifugation, washed twice with cold NB, resuspended in 1 ml of cold NB, and 9 ml of cold NB containing 0.35% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride were added. The cell suspension was kept on ice for 10 min and washed with Triton X-100-free cold NB, and the nuclear protein was eluted for 1 h at 4°C with cold NB containing 0.35 mM NaCl. A nuclear protein solution was obtained by centrifugation at 18,000 × g for 10 min, and its protein concentration was determined using the method of Bradford (20) with bovine plasma γ-globulin as the standard.

**DNA Topoisomerase I Activity.** The activity of DNA topoisomerase I was determined by measuring the relaxation of supercoiled *Escherichia coli* DNA (pBR322) essentially as described by Liu and Miller (21). For measurement of the total topoisomerase I activity in SBC-3 cells, the reaction mixtures used contained 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol, 50 mM Tris-HCl (pH 7.4), and 0.7, μg of pBR322, and crude nuclear extract. The reaction mixtures used for measuring the inhibition of DNA relaxation by topoisomerase I inhibitors comprised the specified amounts of the nuclear extract (1.0 μg/ml protein) and drug solution or the equivalent volume of water in addition to the above-mentioned components. The reaction mixtures were incubated at 37°C for 10 min, and the reactions were terminated by adding 45 μl of a dye solution containing 2.5% (w/v) SDS, 0.01% (w/v) bromphenol blue, and 50% (v/v) glycerol. The mixtures were applied to 0.7% agarose gel and electrophoresed for 4 h with a running buffer of Tris-acetate EDTA. The gel was stained with 0.5 μg/ml ethidium bromide and photographed under transillumination with 300 nm UV light.
RESULTS

Cytotoxicities of NDP and Other Drugs against SBC-3 and PC-14 Cells. Fig. 1 shows the dose-response curves for the growth of SBC-3 cells. NDP inhibited the proliferation of SBC-3 cells by 98%, 93%, 75%, 54%, 27%, 6%, and 2% at a concentration of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 μg/ml, respectively. Consequently, its IC50 value for growth inhibition of SBC-3 cells was 0.053 μg/ml, which is equivalent to 0.18 μM, which was almost the same as that of CDDP. The IC50 values of CPT-11, VP-16, ADM, and VDS for the growth inhibition of SBC-3 cells were 0.042, 0.025, 0.0027, and 0.00028 μg/ml, respectively.

On the other hand, the response of PC-14 cells to these drugs tended to be weaker than that of SBC-3 cells, as shown in Fig. 1. The IC50 values of NDP, CDDP, and CPT-11 for the growth inhibition of PC-14 cells were 0.61, 0.40, and 0.85 μg/ml, respectively.

We chose the one- or two-log dose range that included the IC50 values for the combination effect study (Table 1).

Effects of Combinations of NDP with Other Drugs in SBC-3 Cells. The drug interactions analyzed using the three-dimensional model are shown in Fig. 2, A–G, which illustrate the occurrence of complex drug-drug interactions with a concentration dependence. Fig. 2A shows the effects of the combination of CDDP with the topoisomerase II inhibitor VP-16 in SBC-3 cells, and the concentration dependence of the drug-drug interaction as well as the complexity of the interaction is demonstrated clearly. The region of approximately 0.05–0.25 μg/ml CDDP and 0.025–0.1 μg/ml VP-16, depicted in blue, represents a statistically significant synergic interaction. The effect of the combination at nadir concentrations of both drugs was observed to be less than additive. On the whole, these findings were consistent with those of a previous study (17).

We examined whether NDP can reasonably substitute for CDDP or even surpass it for use in combination with VP-16. The effects of the combination of NDP and VP-16 were superior to those of a combination of CDDP and VP-16 (Fig. 2B). The pattern of the concentration dependence of the drug-drug interaction was also similar to that for the combination of CDDP and VP-16. That is, the effect of the combination of NDP and VP-16 is biphasic and depends on the concentrations of both drugs.

To determine the role of the effect on topoisomerases as the cytotoxic mechanism of VP-16 on the synergistic interaction, we further investigated the effects of combinations of NDP + ADM, CPT-11, and other drugs. Fig. 2C shows the effect of the combination of NDP and the topoisomerase II inhibitor ADM (22). A synergistic interaction was observed within a limited dose range, such as 0.005 μg/ml NDP and 0.0005–0.001 μg/ml ADM. On the other hand, an additive effect was seen with NDP concentrations of more 0.01 μg/ml, regardless of the ADM concentration.

A remarkable synergistic interaction was observed between NDP and the topoisomerase I inhibitor CPT-11 at concentrations of approximately 0.005–0.05 and 0.005–0.1 μg/ml, respectively (Fig. 2D). Regions depicted in blue represent statistically significant synergic interactions. An additive effect, without any relation to the CPT-11 concentration, was noted at higher concentrations (>0.1 μg/ml) of NDP. A similar tendency was also observed with the CDDP and CPT-11 combination (Fig. 2E). In other words, a synergistic interaction was seen in combinations of low concentrations of both drugs, whereas an antagonistic interaction was noted at comparatively high concentrations.

The three-dimensional graph of the NDP and VDS combination showed that this combination has a less than additive effect (Fig. 2F). VDS is known to be a tubulin modulator but not a topoisomerase I or II inhibitor. A similar interaction was also observed in the NDP and CDDP combination (Fig. 2G). Both drugs are known to exert DNA damage but do not affect topoisomerases. These results suggest that a topoisomerase in-

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Table 1  IC50 values of anticancer drugs tested on human SCLC cell line SBC-3 and NSCLC cell line PC-14

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>Exposure time (h)</th>
<th>IC50 μg/ml</th>
<th>Dose range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBC-3</td>
<td>NDP</td>
<td>144</td>
<td>0.053 (0.18)</td>
<td>0.005–0.25</td>
</tr>
<tr>
<td></td>
<td>VDS</td>
<td>144</td>
<td>0.00028 (0.00033)</td>
<td>0.00001–0.0005</td>
</tr>
<tr>
<td></td>
<td>ADM</td>
<td>144</td>
<td>0.0027 (0.0048)</td>
<td>0.0001–0.005</td>
</tr>
<tr>
<td></td>
<td>CDDP</td>
<td>144</td>
<td>0.060 (0.20)</td>
<td>0.0025–0.1</td>
</tr>
<tr>
<td>PC-14</td>
<td>NDP</td>
<td>144</td>
<td>0.61 (2.07)</td>
<td>0.05–1.0</td>
</tr>
<tr>
<td></td>
<td>CPT-11</td>
<td>144</td>
<td>0.85 (1.26)</td>
<td>0.1–2.5</td>
</tr>
<tr>
<td></td>
<td>CDDP</td>
<td>144</td>
<td>0.40 (1.36)</td>
<td>0.05–2.5</td>
</tr>
</tbody>
</table>
Three-dimensional graphs representing the effects of combinations of NDP and other anticancer drugs on SBC-3 cells. The drug combinations tested are CDDP and VP-16 (A), NDP and VP-16 (B), NDP and ADM (C), NDP and CPT-11 (D), CDDP and CPT-11 (E), NDP and VDS (F), and NDP and CDDP (G). Cells were exposed to the drugs alone or in combination at various concentrations for 6 days, and then the cell proliferation ratio of the treated:control cultures was calculated. The effects of the combination were evaluated as described in “Materials and Methods.”
Synergistic Effect between NDP and CPT-11

Effects of Combinations of CPT-11 and NDP or CDDP in PC-14 Cells. Fig. 3, A and B, presents the three-dimensional graphs of the combinations of CPT-11 and NDP or CDDP, respectively, in NSCLC PC-14 cells. A remarkable synergistic interaction was also observed even when NDP and CPT-11 were combined at concentrations of <0.5 and <1.0 μg/ml, respectively. In the combination of CDDP and CPT-11, the drug interaction pattern was biphasic and depended on the drug concentrations. A synergistic interaction was observed at lower drug concentrations, whereas an antagonistic effect was observed at higher concentrations.

These results indicate that a synergistic interaction is a common phenomenon, irrespective of the tumor cell line examined, in combinations of CDDP or its analogues and topoisomerase I inhibitors.

Schedule-dependent Synergism between NDP and CPT-11. The synergy between these drugs seems to be affected by the drug treatment schedule because of their differing mechanisms. We sought to explore the influence of the drug treatment schedule on the effect of the combination to identify the most suitable schedule. In this experiment, 3 h was chosen as the exposure period because both NDP and CPT-11 disappeared within 3 h after administration to patients as short i.v. infusions. Table 1 shows the IC_{50} values of NDP and CPT-11 when SBC-3 cells were treated with the two drugs for 3 h. Cells were simultaneously exposed to both drugs for 3 h or sequentially exposed to CPT-11 for 3 h and then exposed to NDP for 3 h (or vice versa) at concentrations near their IC_{50} values.

Fig. 4 illustrates the schedule-dependent synergism between NDP and CPT-11. A remarkable synergistic interaction was observed when the cells were simultaneously exposed to NDP and CPT-11 at concentrations of 0.25–5.0 and 0.5–5.0 μg/ml, respectively, for 3 h. The magnitude of synergism seen in the brief drug exposure experiment was similar to that in the continuous drug exposure experiment (Fig. 3D).

On the other hand, sequential exposure to CPT-11 followed by NDP resulted in only an additive effect, with a broad dose effect (Fig. 4B). A similar tendency was also observed with sequential exposure to NDP followed by CPT-11, with no synergistic effect induced at restricted doses (Fig. 4C). Results such as complete loss of potentiation on sequential exposure to CPT-11 followed by NDP and the lower degree of synergism observed on sequential exposure to NDP followed by CPT-11 imply that the synergistic interaction between both drugs is schedule dependent.

Effect of NDP on the Inhibitory Effect of SN-38 on Topoisomerase I. To analyze the synergistic interaction between the drugs, we examined the effect of NDP and SN-38 (the active form of CPT-11) on the activities of DNA topoisomerase I in the nuclear protein extract of SBC-3 cells.

First, the topoisomerase I activity in control SBC-3 cells was measured by the relaxation assay in a serial dilution and compared with that in cells pretreated with NDP or CDDP at 0.25 and 2.5 μg/ml, respectively, for 3 h. The supercoiled pBR322 DNA was completely relaxed with 0.1 μg of the nuclear protein extract from SBC-3 cells and partially relaxed with 0.01 and 0.001 μg of the nuclear protein extract from SBC-3 cells, and the relaxation activity did not change after pretreatment with NDP, as shown in Fig. 5A. The inhibitory effect of SN-38 on the catalytic activity of topoisomerase I from SBC-3 cells treated with NDP was also compared with that on the enzyme from control cells. The inhibitory activity of SN-38 was similar in both and was the concentration-dependent, as shown in Fig. 5B. These results confirm that neither the catalytic activities of topoisomerase I nor its susceptibility to topoisomerase I inhibitors was affected by pretreatment with NDP.

We examined the effect of NDP on the SN-38-induced inhibition of topoisomerase I. As shown in Fig. 5C, 0.1 μg of the nuclear protein extract from SBC-3 cells relaxed the supercoiled pBR322 DNA, and this relaxation was inhibited by treatment with SN-38 at 1.0 μg/ml, but not by treatment with SN-38 at <0.3 μg/ml. In the presence of NDP (25 μg/ml), on the other hand, 0.03 μg/ml SN-38 was sufficient to inhibit the DNA relaxing activity of topoisomerase I. The inhibiting effect...
of NDP on the SN-38-induced inhibition of topoisomerase I was also observed in PC-14 cells (data not shown). These results suggest that in vitro, the effect of the combination of NDP and CPT-11 correlated with the effect of NDP on the CPT-11-induced inhibition of topoisomerase I.

**DISCUSSION**

The combination of CDDP and VP-16 is one of the regimens used for the treatment of SCLC. However, the administration of CDDP is often associated with nausea, vomiting, and other adverse effects, such as renal and neural toxicity, which hinder the administration of high doses of CDDP. The combination of NDP and VP-16 was expected to have an effect similar to that of the CDDP and VP-16 combination. Because NDP is a second-generation platinum complex with low nephrotoxicity, a higher dose of the platinum compounds can be administered in this combination. Previous in vivo studies on NDP and VP-16 have been reported by Uchida et al. (23), who concluded that the combination of NDP with VP-16 was more potent than that of CDDP with VP-16 with respect to both the inhibition of growth and survival of mice bearing Lewis lung carcinoma.

In the present study, whose object was to seek drugs superior to VP-16 for combination with NDP, it was demonstrated that a NDP and CPT-11 combination chemotherapy resulted in synergism against human lung cancer and that the effect of the combination of NDP and CPT-11 was superior to the effect of the combination of CDDP and VP-16. It is worth in clinically to substitute CDDP with non-nephrotoxic NDP in combination with CPT-11.

The three-dimensional graphs also demonstrated previously that a synergistic interaction was observed for the CDDP and VP-16 combination at higher concentrations of both drugs (17). This is in contrast to the synergistic interaction between NDP and CPT-11 at lower concentrations in this study. These
inhibition of topoisomerase I were examined. The topoisomerase I-inhibitory activity of SN-38 was enhanced 10-fold by coexposure to NDP. These biochemical interactions might be responsible for the synergistic interaction between NDP and CPT-11 and are also thought to be a common underlying mechanism for the synergistic interaction between platinum analogues and topoisomerase I inhibitors (15). Furthermore, the concentration of NDP required to enhance the activity of CPT-11 was 25 μg/ml, which is lower than that of CDDP (150 μg/ml; Ref. 29). This is sufficient to explain the greater synergism observed between NDP and CPT-11 as compared with CDDP and CPT-11. The cytotoxicity of platinum analogues such as CDDP is believed to be due to the formation of DNA adducts, which include DNA protein cross-links, DNA monoadducts, and interstrand and intrastrand DNA cross-links (30). Furthermore, X-ray diffraction of the cross-linked dinucleotide cis-Pt(NH3)2(d(pGpG)) has revealed that the two guanines are completely destacked and that the deoxyribose sugar of the 5′-deoxyguanosine is in a C3′-endo pucker (29, 31). Thus, the intrastrand platinum cross-link produces a severe local distortion in the DNA double helix, leading to unwinding and linking, which may modulate the stability of topoisomerase I-drug-DNA cleavable complexes.

The opposite results observed for CDDP + VP-16 and NDP + CPT-11 with regard to the drug concentrations inducing the synergistic interaction seem to be due to differences in the cytotoxic mechanisms between CPT-11 and VP-16 rather than NDP and CDDP because both NDP and CDDP are known to form DNA adducts and lead to cell death as a common cytotoxic mechanism (30), whereas both CPT-11 and VP-16 are well known inhibitors of different targets, topoisomerase I and II, respectively. However, there is no evidence of any determinant being due to concentration of CPT-11 or VP-16 on the synergistic interaction in literature.

Study of treatment schedules for NDP and CPT-11 demonstrated that a remarkable synergistic interaction was produced by concurrent exposure to NDP and CPT-11; in contrast, sequential exposure treatments led to only an additive effect. These results provided important preliminary information for a future in vivo study.

Potent in vitro and in vivo cytotoxicity, relatively low neurotoxicity and nephrotoxicity, and a large in vivo bioavailability have ensured the position of NDP as a promising platinum analogue for further clinical development as a salvage and primary chemotherapeutic agent for the treatment of patients with advanced lung cancer. Also, high clinical response rates of lung cancer to CPT-11 have been reported previously (32). Based on these findings, the combination of NDP and CPT-11 seems to be a very promising one for cancer chemotherapy. Further examination of these drug interactions in in vivo preclinical studies should provide useful information for future combination chemotherapy regimens.

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In Vitro Synergistic Interactions between the Cisplatin Analogue Nedaplatin and the DNA Topoisomerase I Inhibitor Irinotecan and the Mechanism of this Interaction

Fumihiko Kanzawa, Fumiaki Koizumi, Yasuhiro Koh, et al.


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