Antitumor Activity of NK012 Combined with Cisplatin against Small Cell Lung Cancer and Intestinal Mucosal Changes in Tumor-Bearing Mouse after Treatment

Tatsuya Nagano,1,2,3 Masahiro Yasunaga,1 Koichi Goto,2 Hirotugu Kenmotsu,2 Yoshikatsu Koga,1 Jun-ichi Kuroda,1 Yoshihiro Nishimura,3 Takashi Sugino,4 Yutaka Nishiwaki,2 and Yasuhiro Matsumura1

Abstract Purpose: To investigate the advantages of treatment with the SN-38-incorporating polymeric micelles NK012 over CPT-11 in combination with cisplatin [cis-dichlorodiammineplatinum (II) (CDDP)] in mice bearing a small cell lung cancer xenograft in terms of antitumor activity and toxicity, particularly intestinal toxicity.

Experimental Design: Cytotoxic effects were evaluated in human small cell lung cancer cell lines [H69, H82, and vascular endothelial growth factor (VEGF)–secreting cells (SBC-3/VEGF and its mock transfectant SBC-3/Neo)]. In vivo antitumor effects were evaluated in SBC-3/Neo–bearing and SBC-3/VEGF–bearing mice after NK012/CDDP or CPT-11/CDDP administration on days 0, 7, and 14. Drug distribution was analyzed by high-performance liquid chromatography or fluorescence microscopy, and the small intestine was pathologically examined.

Results: The in vitro growth-inhibitory effects of NK012 were 198- to 532-fold more potent than those of CPT-11. A significant difference in the relative tumor volume on day 30 was found between NK012/CDDP and CPT-11/CDDP treatments (P = 0.0058).

Conclusions: NK012/CDDP combination may be a promising candidate regimen against lung cancer without severe diarrhea toxicity and therefore warrants further clinical evaluation.

SN-38 or 7-ethyl-10-hydroxy-camptothecin is a biologically active metabolite of irinotecan hydrochloride (CPT-11) and is formed through CPT-11 conversion by carboxylesterases. SN-38 is active against various human cancers, such as colorectal, lung, and ovarian cancer (1–4). Although SN-38 shows up to 1,000-fold more potent cytotoxic activity against various cancer cell lines than CPT-11 in vitro (5), it has been clinically unavailable because of its water-insoluble nature, and the conversion rate from CPT-11 to SN-38 is <10% of the original CPT-11 volume in the body (6, 7).

The SN-38–incorporating polymeric micelles NK012 seem to have the advantage of passive targeting of the drug delivery system. In this passive targeting of drug delivery system, the drug accumulates in tumor tissue by using the enhanced permeability and retention effect (8–11). This enhanced permeability and retention effect is based on several pathologic mechanisms, which include hypervascularity, secretion of tumor vascular permeability factors stimulating extravasation of macromolecules including nanoparticles such as liposomes and micelles, and the absence of an effective lymphatic drainage of macromolecules accumulated in solid tumor tissue.

Recent studies showed that NK012 has a significantly more potent antitumor activity than CPT-11 against small cell lung cancer (SCLC; ref. 12), colorectal cancer (13), renal cancer (14), pancreatic cancer (15), stomach cancer (16), and glioma (17).

It was previously reported that the SN-38/cis-dichlorodiammineplatinum (II) (CDDP) combination showed synergistic effects (18). The median survival of SCLC patients treated with the CPT-11/cisplatin (CDDP) combination was significantly longer than that of SCLC patients treated with the etoposide/CDDP combination in a randomized phase III study (P = 0.002) conducted by the Japanese Cooperative Oncology Group.
Translational Relevance
The SN-38-incorporating polymeric micelles NK012 has been shown to have significant antitumor activity against several cancer mouse models compared with CPT-11. The phase I study showed that patients treated with NK012 did not develop grade 3/4 diarrhea, one of the major adverse effects of CPT-11. Here, the antitumor activity of NK012/cisplatin combination was compared with that of CPT-11/cisplatin combination, one of the most active regimens against SCLC and NSCLC in the clinic. We also evaluated the pharmacologic and toxic profiles of the drug combinations, particularly in terms of diarrhea. NK012/cisplatin showed a significant potent antitumor activity against an SBC-3 xenograft compared with CPT-11/cisplatin. Moreover, inflammatory pathologic changes were rarely observed in the small intestinal mucosa of the NK012-treated mouse but were commonly observed in the CPT-11–treated mouse. NK012/cisplatin combination chemotherapy is thus a promising regimen against lung cancer without severe diarrhea toxicity and therefore warrants further clinical evaluation.

Materials and Methods
Drugs and cells. SN-38 and NK012 were prepared by Nippon Kayaku Co. Ltd. CPT-11 was purchased from Yakult Honsha Co. Ltd. CDDP was obtained from WC Heraeus GmbH & Co. KG.

Among the SCLC cell lines used, SBC-3 was kindly provided by Dr. I. Kimura (Okayama University, Okayama, Japan), and H69 and H82 were purchased from the American Type Culture Collection. SBC-3, H69, and H82 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies) and penicillin, streptomycin, and amphotericin B (100 units/mL, 100 µg/mL, and 25 µg/mL, respectively; Sigma) in a humidified atmosphere containing 5% CO2 at 37°C. Vascular endothelial growth factor (VEGF)–secreting cells, SBC-3/VEGF and its mock transfected SBC-3/Neo, were generated from SBC-3 cells transfected with BMG-Neo-VEGF and BMG-Neo, as described (26).

In vitro study. The growth-inhibitory effects of NK012, CPT-11, SN-38, and CDDP were examined by tetrazolium salt–based proliferation assay (WST-8 assay; Wako Chemicals). One hundred microliters of a suspension of exponentially growing cells (1 × 105/mL of SBC-3/Neo and SBC-3/VEGF or 1 × 104/mL of H69 and H82) were placed into the wells of a 96-well plate and incubated for 24 h at 37°C. Then, after medium removal, 100 µL of medium containing various concentrations of each drug were added to the wells and then incubated for 72 h at 37°C. After medium removal, 10 µL of WST-8 solution and 90 µL of medium were added to the wells followed by incubation for 1 h at 37°C. The growth-inhibitory effects of each drug were assessed spectrophotometrically (SpectraMax 190, Molecular Devices Corp.). The IC50 value was determined on the dose-response curves. The nature of interaction between NK012 and CDDP against SCLC cell lines, SBC-3/Neo, SBC-3/VEGF, H69, and H82, was evaluated by median-effect plot analyses and the combination index method of Chou and Talalay (27).

Experimental mice model. Female BALB/c nude mice (6 wk old) were purchased from SLC Japan. Mice were inoculated s.c. in the flank with 1 × 106 cells/150 µL cell suspension of SBC-3/Neo and SBC-3/VEGF cell lines. All animal procedures were done in compliance with the guidelines for the care and use of experimental animals established by the Committee for Animal Experimentation of the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

In vivo growth inhibition assay. When the tumor volume (TV) reached 1,500 mm3, mice were randomly divided into test groups consisting of five mice per group (day 0). Drugs were i.v. administered into the tail vein on days 0, 7, and 14. NK012 was given at SN-38 equivalent doses of 10 and 5 mg/kg/d, which are one third and one sixth of the maximum tolerated dose, respectively. The reference drug, CPT-11, was given at 22 and 10 mg/kg/d, which are one third and one sixth of the maximum tolerated dose, respectively. CDDP was simultaneously given on the same day at 2.5 mg/kg/d based on a previous report (28). In preliminary experiment, NK012 (5 mg/kg) plus CDDP (2.5 mg/kg) seemed to be superior to NK012 (5 mg/kg) alone in these tumors. NaCl solution (0.9%) was administered i.v. as normal control. The length (a) and width (b) of the tumor masses and body weight (BW) were measured twice a week, and TV was calculated using TV = (a × b3) / 2. Relative TV (RTV) on day n was calculated using RTV = TVn / TV0, where TVn is the TV on day n and TV0 is the TV on day 0. Relative BW (RBW) was calculated using RBW = BWn / BW0. Differences in RTV and RBW between the treatment groups on day 30 were analyzed using the unpaired t test.

Pharmacokinetic analysis by high-performance liquid chromatography. Female BALB/c nude mice (n = 3) bearing SBC-3/Neo and SBC-3/VEGF tumors (1,500 mm3) were used for drug pharmacokinetic analysis. NK012 or CPT-11 was administered at an equimolar dose of 20 or 30 mg/kg/day, respectively, as reported (12). CDDP was simultaneously given at 2.5 mg/kg. Mice were sacrificed 1, 6, 24, and 72 h (day 3) after administration. Plasma samples, tumors, upper small intestine, and feces were obtained and stored at -80°C until analysis. SN-38 was extracted for each sample and reversed-phase high-performance liquid chromatography was done as reported (12).

Pathologic studies of small intestinal mucosa. CPT-11 and NK012 were injected to female BALB/c nude mice (n = 3) at the same dose schedules as those used in the treatment experiment. On day 14 after the last dosing, mice were sacrificed and parts of the small intestine were sampled at 5 cm from the pyloric part for the jejunum and 5 cm from the ileocecal junction for the ileum. Samples were fixed in 10% formalin, paraffin embedded, sectioned, and stained with H&E. Inflammation was scored by using an inflammation scale from 0 to ++, with - indicating absent inflammation, + showing mild...
inflammation predominantly infiltrated with lymphocytes, and ++ indicating active inflammation infiltrated with lymphocytes and neutrophils.

Distribution of NK012 or CPT-11 in small intestine by fluorescence microscopy. NK012 or CPT-11 was administered to female BALB/c nude mice at 20 or 30 mg/kg on day 0, respectively. Mice were sacrificed 1, 6, 24, and 72 h after drug injection, and the small intestine was excised at the middle portion and embedded in an OCT compound (Sakura Finetechnochemical Co. Ltd.) and frozen at -80°C. Tissue sections (5 µm thick) were prepared using a cryostatic microtome (Tissue-Tek Cryo3, Sakura Finetechnochemical). Frozen sections were examined under a fluorescence microscope (Biorevo, Keyence) at a 358-nm excitation wavelength and a 461-nm emission wavelength to evaluate NK012 or CPT-11 distribution in the small intestine. Because formulations containing SN-38 bound via ester bonds possess a particular fluorescence, both NK012 and CPT-11 were detected under the same fluorescence conditions.

### Table 1. In vitro growth-inhibitory activity of SN-38, NK012, CPT-11, and CDDP in human SCLC cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (µmol/L)</th>
<th>SN-38</th>
<th>NK012</th>
<th>CPT-11</th>
<th>CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBC-3/VEGF</td>
<td>0.00330 ± 0.00210</td>
<td>0.00365 ± 0.00005</td>
<td>1.11 ± 0.29</td>
<td>2.21 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>SBC-3/Neo</td>
<td>0.00872 ± 0.00063</td>
<td>0.0101 ± 0.0006</td>
<td>5.05 ± 0.08</td>
<td>12.8 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>H69</td>
<td>0.0205 ± 0.0195</td>
<td>0.0417 ± 0.0052</td>
<td>22.2 ± 5.9</td>
<td>6.23 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>H82</td>
<td>0.00716 ± 0.00079</td>
<td>0.00998 ± 0.00328</td>
<td>1.98 ± 0.55</td>
<td>4.08 ± 3.79</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Growth inhibitory effects of NK012/CDDP and CPT-11/CDDP on SBC-3/Neo and SBC-3/VEGF tumor xenografts. A and B, RTV in mice treated with NK012/CDDP or CPT-11/CDDP. SBC-3/Neo (A and C) and SBC-3/VEGF (B and D) tumors were inoculated s.c. into the flank of mice, as described in Materials and Methods. CPT-11 (10 mg/kg/d; ▵), CPT-11 (22 mg/kg/d; □), NK012 (5 mg/kg/d; △), or NK012 (10 mg/kg/d; ▪) combined with CDDP (2.5 mg/kg/d) were i.v. administered on days 0, 7, and 14. ○, NaCl solution (0.9%) was i.v. administered as normal control. Points, mean; bars, SD. *, P < 0.05. C and D, treatment-related BW loss occurred in mice treated with NK012/CDDP and CPT-11/CDDP. Points, mean; bars, SD.
**Statistical analysis.** Data were analyzed with Student’s *t* test when groups showed equal variances (*F* test) or with Welch’s test when they showed unequal variances (*F* test). *P* < 0.05 was considered significant. All statistical tests were two sided, and data were expressed as mean ± SD.

**Results**

**Cellular sensitivity of SCLC cells to NK012, CPT-11, SN-38, and CDDP.** The IC_{50} values of NK012 for the SCLC cell lines ranged from 0.004 μmol/L (SBC-3/VEGF) to 0.041 μmol/L (H69; Table 1). The cytotoxic effects of NK012 were 198- to 532-fold higher than those of CPT-11, whereas those of NK012 were 1.10- to 2.00-fold lower than those of SN-38. These features were comparable with those reported previously (12, 13).

The molar ratios of NK012 to CDDP of 1:600 in SBC-3/VEGF, 1:120 in SBC-3/Neo, 1:150 in H69, and 1:400 in H82 were used for the drug combination studies based on the IC_{50} values of NK012 and CDDP (Table 1). The synergic to additive

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**Fig. 2.** Plasma, tumor, and small intestine concentrations of NK012, CPT-11, and free SN-38. Plasma (A), tumor (B), and small intestine (C) distribution of NK012, CPT-11, and free SN-38 after i.v. administration of CPT-11 (30 mg/kg) combined with CDDP (2.5 mg/kg) or NK012 (20 mg/kg) combined with CDDP (2.5 mg/kg). Left, SBC-3/Neo; right, SBC-3/VEGF. ●, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); △, SN-38 converted from CPT-11; ▲, CPT-11.
effect between NK012 and CDDP was observed in these SCLC cell lines (data not shown).

**Antitumor activity of NK012/CDDP and CPT-11/CDDP against SBC-3/Neo and SBC-3/VEGF tumors.** SBC-3/Neo and SBC-3/VEGF tumors treated with 5 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 10 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 (P = 0.0024, SBC-3/Neo; P = 0.0437, SBC-3/VEGF). Moreover, both tumors treated with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 22 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 (P = 0.0058, SBC-3/Neo; P = 0.0478, SBC-3/VEGF; Fig. 1A and B). Although treatment-related BW loss was observed in mice treated with each drug combination, BW recovered to the normal level in each group by day 30 (Fig. 1C and D). A stronger antitumor activity against SBC-3/VEGF tumors was observed than against SBC-3/Neo tumors. The complete response rates achieved with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were 100% and 0% for SBC-3/VEGF and SBC-3/Neo, respectively. These results further confirm our previous findings that a more potent antitumor effect of NK012 is observed in highly vascularized tumors (12).

**Pharmacokinetics of NK012 and CPT-11 after NK012/CDDP and CPT-11/CDDP administration in mice bearing SBC-3/Neo or SBC-3/VEGF tumors.** After CPT-11/CDDP injection, the plasma concentrations of CPT-11 and SN-38 converted from CPT-11 decreased rapidly within 6 hours in a log-linear fashion (Fig. 2A). Those of NK012 (polymer-bound SN-38) and SN-38 released from NK012 decreased more gradually (Fig. 2A). As for the CPT-11 and free SN-38 concentrations in the SBC-3/Neo and SBC-3/VEGF tumors, they decreased rapidly within 6 hours, and almost no SN-38 converted from CPT-11 was detected at 24 hours in both tumors (Fig. 2B). In the case of NK012/CDDP administration, free SN-38 released from NK012 could be detected in the tumors even at 72 hours after administration (Fig. 2B). In contrast to the case of CPT-11/CDDP administration, the concentrations of free SN-38 released from NK012 were higher in the SBC-3/VEGF tumors than in the SBC-3/Neo tumors at any time point during the observation period (significant at 1 hour; P = 0.013).

Free SN-38 concentrations in the small intestine after NK012/CDDP or CPT-11/CDDP administration were still detectable up to 72 hours in a similar fashion. CPT-11 concentrations 1 hour after CPT-11/CDDP administration were significantly higher than NK012 concentrations after NK012/CDDP administration (P = 0.0056, SBC-3/Neo; P = 0.017, SBC-3/VEGF; Fig. 2C).

These kinetic profiles in liver, spleen, lung, and kidney of free SN-38 after NK012/CDDP or CPT-11/CDDP administration were almost similar to those of NK012 or CPT-11 when administered as a single agent, as described (data not shown; ref. 12).

**Intestinal toxicity of NK012, NK012/CDDP, CPT-11, and CPT-11/CDDP.** Pathologic findings and characteristic mucosal changes are shown in Table 2 and Fig. 3. The small intestinal mucosa of mice in the CPT-11 or CPT-11/CDDP treatment group showed fibrotic changes, and active inflammation with cellular invasion, healed erosion, deformed glandular alignment, and glandular duct disappearance were also found. On the other hand, the small intestinal mucosa of mice in the NK012/CDDP treatment group showed only mild shortening and decreased number of villi or mild inflammatory cell invasion.

We next analyzed the concentrations of NK012, CPT-11, and free SN-38 in the feces. CPT-11 concentrations at 1 hour were significantly higher than NK012 concentrations (P = 0.0021) and decreased rapidly within 24 hours but remained detectable up to 72 hours. On the other hand, NK012 (polymer-bound SN-38) could be detected at a low concentration from 72 hours (Fig. 4A). To evaluate drug distribution over time, sections of the small intestine treated with NK012 or CPT-11 were examined by fluorescence microscopy. In the sections of CPT-11–treated small intestine, strong fluorescence originating from CPT-11 was detected in the epithelium of the small intestine, whereas weaker fluorescence originating from NK012 was distributed uniformly in the mucosal interstitium (Fig. 4B).

**Table 2. Pathologic analysis of small intestine after i.v. administration of drugs**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Treatment group</th>
<th>Site</th>
<th>Fibrosis</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Jejunum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CPT-11</td>
<td>Jejunum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>NK012</td>
<td>Jejunum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>CPT-11</td>
<td>Ileum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>CPT-11</td>
<td>Ileum</td>
<td>+</td>
<td>++ Erosion</td>
</tr>
<tr>
<td>6</td>
<td>CPT-11</td>
<td>Ileum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>CDDP + CPT</td>
<td>Jejunum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>CDDP + CPT</td>
<td>Ileum</td>
<td>-</td>
<td>-</td>
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<td>10</td>
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<td>Ileum</td>
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</table>

Discussion

Here, we compared the antitumor activity of NK012/CDDP with CPT-11/CDDP, the latter being one of the most active regimens against SCLC and NSCLC. The present data showed that when NK012/CDDP was administered, NK012 effectively accumulated in SBC-3/VEGF tumors and sufficiently exerted antitumor effects. This suggests that CDDP did not affect the permeability of tumor vessels and NK012 retention in the tumors. Hasegawa et al. (29) reported that 17 of 24 patients showed positive immunoreactivity for the VEGF protein in tumor specimens and that elevated serum VEGF levels were
associated with poor outcome in SCLC. As for NSCLC, it was reported that the percentage of VEGF-positive cells was 52 ± 33% (95% confidence interval, 41-64%; median, 70%), and this value showed a positive association with high vascular grade (P = 0.008) and poor survival (P = 0.04; ref. 30). Taking all data together, NK012/CDDP may therefore be clinically effective against lung cancers, particularly those with high VEGF production.

Pathologic examinations were also conducted to evaluate changes in the small intestinal mucosa on day 14 after treatment. This is because diarrhea is one of the clinical dose-limiting toxicities of CPT-11, and epithelial apoptosis was reported as a mucosal change induced by CPT-11 (31). This pathologic change was observed on day 6 after i.p. administration of 100 mg/kg CPT-11 daily for 4 days. We found that the CPT-11–induced mucosal change was mainly fibrosis considered to be a form of recovery change from erosion. On the other hand, the small intestinal mucosa of the mice in the NK012/CDDP treatment group showed mild shortening and decreased number of villi or mild inflammatory cell invasion. On comparison of these changes with those caused by CDDP (31), it was found that such alterations were mainly induced by CDDP rather than NK012.

A portion of SN-38 converted from CPT-11 undergoes subsequent conjugation as induced by UDP-glucuronyltransferase to form SN-38 β-glucuronide (SN-38-Glu; ref. 32). CPT-11, SN-38, and SN-38-Glu are excreted into the bile and then reach the small intestinal lumen (32, 33). SN-38-Glu is
deconjugated in the cecum and colon to regenerate SN-38 through bacterial β-glucuronidase (34). In this study, CPT-11 was excreted into feces much more than NK012 and a high CPT-11 concentration was detected in the small intestinal epithelium. It is speculated that the highly excreted CPT-11 is reabsorbed in the small intestinal epithelium and converted to SN-38 to cause damage to the intestinal mucosa. On the other hand, NK012 was uniformly distributed in the mucosal interstitium at a lower concentration, which may be related to the less mucosal damage and diarrhea than those induced by CPT-11, although NK012 was observed for longer period than CPT-11. About other toxic effects including bone marrow, liver,
and kidney toxicities, there was no significant difference between NK012/CDDP and CPT-11/CDDP in the present treatment schedule (data not shown).

In conclusion, NK012/CDDP showed a significantly higher antitumor activity with no severe diarrhea toxicity than CPT-11/CDDP, one of the most active regimens against SCLC and NSCLC. The present data suggest the clinical evaluation of NK012/CDDP in patients with SCLC and NSCLC.

References


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