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Original Article

The Antitumor Activity of NK012, an SN-38–Incorporating Micelle, in Combination With Bevacizumab Against Lung Cancer Xenografts

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BACKGROUND: It has been demonstrated that NK012, a novel 7-ethyl-10-hydroxycamptothecin (SN-38)-incorporating polymeric micelle, exerts significantly more potent antitumor activity against various human tumor xenografts than irinotecan (CPT-11) (a water-soluble prodrug of SN-38). Combination therapy of anticancer agents with bevacizumab (Bv), an anti-vascualr endothelial growth factor humanized monoclonal antibody, has more potently inhibited tumor growth than either agent alone. In the current study, the authors examined the antitumor effect of NK012 in combination with Bv against human lung cancer. **METHODS:** Nude mice bearing lung adenocarcinoma (PC-14 or A549 xenografts) were administered NK012 at SN-38-equivalent doses of 5 mg/kg or 30 mg/kg in combination with or without Bv at 5 mg/kg. CPT-11 at a dose of 66.7 mg/kg was administered with or without Bv at a dose of 5 mg/kg in the same experimental model. To evaluate interaction with Bv, the pharmacokinetics and microvessel density in tumors that were treated on each regimen were analyzed. **RESULT:** In vitro, the growth-inhibitory effect of NK012 was 50-fold more potent than that of CPT-11 and was almost equivalent to that of SN-38. In vivo studies revealed that the combination of NK012 plus Bv had significantly greater antitumor activity against human lung cancer xenografts compared with NK012 alone (PC-14, $P=.0261$; A549, $P < .001$). The pharmacokinetic profile of NK012 revealed that coadministration of Bv did not interfere with the accumulation of NK012. **CONCLUSIONS:** In this study, significant antitumor activity was noted with NK012 in combination with Bv against lung cancer cells. The current results warrant the clinical evaluation of NK012 in lung cancer. ***Cancer* 2010;116:4597–604.** © *2010 American Cancer Society.*

KEYWORDS: NK012, drug-delivery system, lung cancer, lung adenocarcinoma, 7-ethyl-10-hydroxycamptothecin, SN-38, micelles, bevacizumab.

Lung cancer is the leading cause of cancer-related deaths worldwide, and nonsmall cell lung cancer (NSCLC), including adenocarcinoma, accounts for 75% to 80% of lung cancer cases.[1] Currently, cisplatin (CDDP)-based chemotherapy is the recommended first-line treatment for patients with advanced NSCLC.[2,3] Despite recent advances in the treatment of lung cancer, the prognosis for patients with NSCLC remains relatively poor, so attention currently is focused on finding novel agents, including new cytotoxic agents.

Irinotecan (CPT-11), a prodrug of 7-ethyl-10-hydroxycamptothecin (SN-38) (the active metabolite of irinotecan), which is a topoisomerase-I inhibitor, appears to be an effective agent against NSCLC when used as monotherapy or in combination with cisplatin.[4,5] Bevacizumab (Bv) is an antivascular endothelial growth factor (anti-VEGF) humanized monoclonal antibody. Bv reportedly is effective in various cancers, including colorectal cancer,[6] renal cell cancer,[7] and breast cancer.[8] Sandler et al reported that the addition of Bv to paclitaxel plus carboplatin in the treatment of NSCLC had a significant survival benefit.[9] In addition, Reck et al reported that the addition of Bv to gemcitabine plus cisplatin also had a significant clinical benefit in NSCLC.[10]

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We thank Ms. N. Mie and Ms. M. Ohtsu for technical assistance and Ms. K. Shiina and Ms. K. Abe for secretarial assistance.

DOI: 10.1002/cncr.25233, **Received:** November 18, 2009; **Revised:** December 22, 2009; **Accepted:** December 23, 2009, **Published online** June 22, 2010 in Wiley Online Library (wileyonlinelibrary.com)

NK012 is an SN-38-incorporating polymeric micelle and is categorized as a drug-delivery system. We previously demonstrated that NK012 accumulates more efficiently in various human tumor xenografts by using leaky tumor vessels and exerts significantly more potent antitumor activity against various human tumor xenografts compared with CPT-11.[11-17] Since the greater antitumor effect of NK012 may be attributed to its greater accumulation in the tumor using the leaky tumor vasculature, the addition of Bv to NK012 may hinder the efficient accumulation of NK012 in tumors because the permeability of tumor vasculature caused by VEGF is inhibited by Bv. In the current study, we evaluated the antitumor activity of NK012 administered in combination with Bv in experimental models.

MATERIALS AND METHODS

Drugs and Cells

NK012, an SN-38-incorporating polymeric micelle, and SN-38 were obtained from Nippon Kayaku Company, Ltd. (Tokyo, Japan), CPT-11 was purchased from Yakult Honsha Company, Ltd. (Tokyo, Japan), and Bv was purchased from Chugai Seiyaku Company, Ltd. (Tokyo, Japan). The human lung adenocarcinoma cell lines PC-14 and PC-9 kindly were provided by Dr. Y. Hayata (Tokyo Medical University, Tokyo, Japan). Human lung adenocarcinoma cell lines A549, NCI-H23, and NCI-H1975 were purchased from the American Type Culture Collection (Manassas, Va). These cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (25 μg/mL; all from Sigma, St. Louis, Mo) in a humidified, 5% CO_2 atmosphere at 37°C.

In Vitro Growth-Inhibition Assay

PC-14, A549, NCI-H23, and NCI-H1975 cells were seeded in 96-well plates at a density of 10,000 cells per well in a final volume of 100 μL. Twenty-four hours after seeding, the medium was removed, and a graded concentration of SN38, NK012, and CPT-11 was added to the wells. Cultures were maintained in a CO_2 incubator for an additional 72 hours. Then, cell growth inhibition was measured by using a tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). After removal of the medium, WST-8 solution (10 μL) and medium (90 μL) were added to the wells, and the plates were incubated at 37°C for 1 hour. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader. Cell viability was measured and compared with that of the control cells. Each experiment was carried out in triplicate. Data were averaged and normalized against the nontreated controls to generate dose-response curves.

In Vivo Growth-Inhibition Assay

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments from the National Cancer Center.

Female BALB/c mice, 6 weeks old, were obtained from SLC Japan (Shizuoka, Japan). These mice were maintained in a laminar air-flow cabinet and were inoculated subcutaneously with 5×10^6 PC-14 cells or with 5×10^6 A549 cells in the flank region. When tumor volumes (TVs) reached approximately 100 mm^3, the mice were divided randomly into test groups of 5 mice per group (Day 0). The length (a) and width (b) of the tumor mass were measured twice weekly, and the TV was calculated as follows: $TV = (a \times b^2)/2$. The relative TV (RTV) at Day n was calculated as follows: $RTV = TV_n/TV_0$, where TV_n is the TV at Day n, and TV_0 is the TV at Day 0.

Experiment 1: Evaluation of the Antitumor Effect of NK012 and CPT-11

By comparing the data between NK012 and CPT-11, we evaluated their effects as single agents against PC-14 or A549 xenografts. The maximum tolerated dose (MTD) of NK012 (30 mg/kg)[11] or the MTD of CPT-11 (66.7 mg/kg)[18] was administered by intravenous injection into the tail vein on Days 0, 4, and 8.

Experiment 2: Evaluation of the Antitumor Effect of NK012 Alone and NK012 With Bv

By comparing the data between NK012 alone and NK012 plus Bv, we evaluated the combined effect of NK012 plus Bv against PC-14 xenografts. NK012 at a dose of 5 mg/kg was administered intravenously into the tail vein on Days 0, 4, and 8 with or without Bv. In addition, we evaluated the combined effects against A549 xenografts (NK012 [30 mg/kg intravenously] with Bv). When Bv was coadministered with each anticancer agent, Bv was administered intraperitoneally at a dose of 5 mg/kg on Days 0, 4, and 8.

Table 1. Fifty Percent Inhibitory Concentration Values of 7-Ethyl-10-Hydroxycamptothecin (SN-38), the SN-38–Incorporating Polymeric Micelle NK012, and Irinotecan in Various Human Lung Adenocarcinoma Cell Lines

	IC_{50} (μmol/L)[a]		
Cell Line	SN-38	NK012	CPT-11
PC-14	0.050±0.003	0.053±0.002	9.688±1.187
A549	0.506±0.029	0.883±0.840	48.153±4.641
PC-9	0.028±0.011	0.059±0.005	21.782±2.145
NCI-H23	0.025±0.005	0.060±0.002	5.223±1.586
NCI-H1975	0.047±0.084	0.082±0.002	6.330±0.432

IC_{50} indicates 50% inhibitory concentration; CPT-11, irinotecan.
[a] All values shown are the mean values±standard deviation.

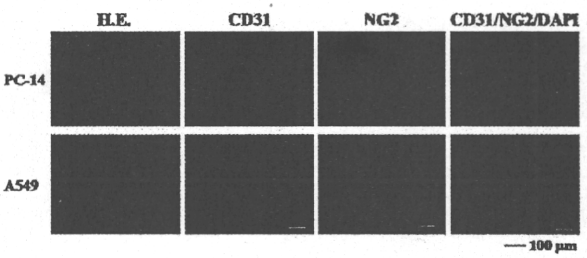

Figure 1. These photomicrographs are from the histologic examination of excised tumors from PC-14 and A549 xenografts that were stained with hematoxylin and eosin (H.E.) or analyzed by immunohistochemistry for cluster of differentiation molecule 31 (CD31) (also called platelet endothelial cell adhesion molecule 1) (red) for the chondroitin sulfate proteoglycan NG2 (green) and for 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bars = 100 μm.

Distribution Studies of Free SN-38, CPT-11, and NK012 in Tumors by High-Performance Liquid Chromatography

When the PC-14 TV reached approximately 100 mm^3, NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was administered intravenously with or without Bv (5 mg/kg intraperitoneally). Twenty-four hours after the injection of NK012 or CPT-11, each tumor was excised under anesthesia. In other experiments, NK012 (5 mg/kg) was administered intravenously with or without Bv (5 mg/kg intraperitoneally), and each tumor was excised under anesthesia at 12 hours, 24 hours, 3 days, 7 days, 10 days, and 14 days after the injection of NK012. The tumor tissues were rinsed with physiologic saline; mixed with 0.1 M glycine-HCl buffer, pH 3.0, in methanol at 5% (weight/weight); and homogenized. To detect free SN-38 and CPT-11, the tumor samples (100 μL) were mixed with 20 μL 1 mM phosphoric acid in methanol (1:1) and 40 μL ultrapure water, and camptothecin was used as the internal standard (10 ng/mL for free SN-38, 12 ng/mL for CPT-11). The samples were vortexed vigorously for 10 seconds and filtered through an Ultrafree-MC centrifugal filter device (Millipore, Bedford, Mass). Reverse-phase high-performance liquid chromatography (HPLC) was conducted at 35°C on a Mightysil RP-18 GP column (150 × 4.6 mm; Kanto Chemical, Tokyo, Japan). Then, the samples were injected into an Alliance Water 2795 HPLC system (Waters, Milford, Mass) equipped with a Waters 2475 multi-λ fluorescence detector. Fluorescence originating from SN-38 was detected at 540 nm with an excitation wavelength of 365 nm.

For the detection of polymer-bound SN-38, SN-38 was released from the polymer as described previously.[11] In brief, 100-μL tissue samples were diluted with 20 μL methanol (50% [weight/weight]) and 20 μL NaOH (0.7 M). The samples were incubated for 15 minutes at room temperature. After incubation, 20 μL HCl (0.7 M) and 60 μL of internal standard solution were added to the samples and the hydrolysate was filtered. The filtrate was applied to the HPLC system. Polymer-bound SN-38 was determined by subtraction of nonpolymer-bound SN-38 from the total SN-38 in the hydrolysate.

Immunofluorescence Study

At Day 14 after the injection of saline, Bv, NK012, or NK012 plus Bv, the PC-14 tumors were excised under anesthesia. Frozen sections of these tumors (10 μm) were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). After blocking with 5% skim milk (BD, Franklin Lakes,. NJ) in PBS, the slides were incubated with anti-cluster of differentiation molecule 31 (anti-CD31) monoclonal antibody (1:100 dilution; PharMingen, San Diego, Calif) and anti-NG2 monoclonal antibody (1:1000 dilution; Chemicon, Temecula, Calif) for 1 hour. After washing with PBS, the slides were stained with Alexa 555-, Alexa 647-conjugated secondary antibodies, antirat (red) and antirabbit immunoglobulin G (green; 1:100 dilution; Invitrogen, Carlsbad, Calif), and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Five areas were chosen randomly from each mouse (n = 2), and the fluorescence intensity was measured and analyzed with BZ-II ANALYZER software (Keyence, Osaka, Japan) for histologic quantification under fluorescence microscopy at 20-fold magnification.

Statistical Analysis

One-way fractional analyses of variance and multiple comparison tests (Scheffe and Bonferroni/Dunn)

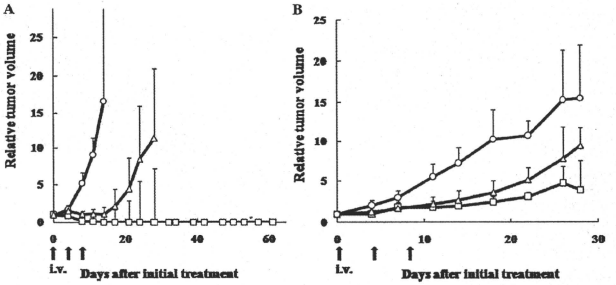

Figure 2. These graphs illustrate (A) the antitumor effects of the novel 7-ethyl-10-hydroxycamptothecin (SN-38)-incorporating polymeric micelle NK012 alone (5 mg/kg daily), bevacizumab (Bv) alone (5 mg/kg daily), and combined NK012 (5 mg/kg daily) plus Bv (5 mg/kg daily) against PC-14 tumor-bearing mice and (B) the effects of NK012 alone (30 mg/kg daily) and combined NK012 (30 mg/kg daily) plus Bv (5 mg/kg daily) against A549 tumor-bearing mice. Squares indicate NK012; open triangles, Bv; solid triangles, NK012 plus Bv; saline, circles. NK012 was administered intravenously (i.v.), and Bv was administered intraperitoneally (i.p.) on Days 0, 4, and 8. Each group included 5 mice. Points indicate mean values; bars, standard deviation.

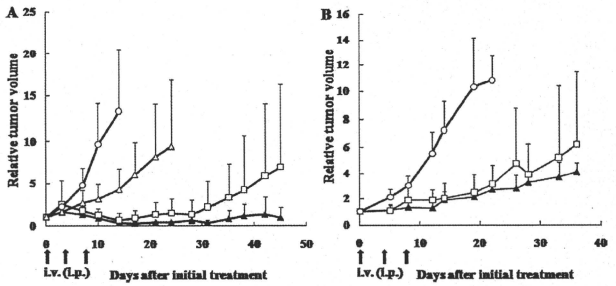

Figure 3. These graphs illustrate the antitumor effects of the novel 7-ethyl-10-hydroxycamptothecin (SN-38)-incorporating polymeric micelle NK012 alone or irinotecan (CPT-11) alone against (A) PC-14 (B) and A549 tumor-bearing mice. The treatment was initiated 11 days after PC-14 inoculation and 13 days after A549 inoculation. NK012 (30 mg/kg daily) (squares), CPT-11 (66.7 mg/kg daily) (triangles), or saline (circles) was administered intravenously (i.v.) on Days 0, 4, and 8. Each group included 5 mice. Points indicate mean values; bars, standard deviation.

conducted with StatView software (version 5.0; SAS Institute, Inc., Cary, NC) were used to compare the different treatment groups of xenografts. Data were expressed as the mean ± standard deviation. Data were analyzed with the Student *t* test when the groups had equal variance (*F* test) or with the Welch test when they had unequal variance (*F* test). *P* values <.05 were regarded as statistically significant. All statistical tests were 2-sided.

RESULTS

Sensitivity of Lung Cancer Cells to SN-38, NK012, and CPT-11

The 50% inhibitory concentration values of NK012 for the cell lines ranged from 0.059 μmol/L to 0.88 μmol/L. The growth-inhibitory effect of NK012 was 50-fold more potent than that of CPT-11 and was almost equivalent to that of SN-38 (Table 1).

Histologic Examination of PC-14 and A549 Xenografts

Hematoxylin and eosin staining of the tumors from PC-14 xenografts revealed that the tumors were poor in stroma, whereas the tumors from A549 xenografts appeared to be stroma-rich. Immunostaining of both tumor tissues with CD31 and NG2 indicated that vasculatures covered with pericytes were more abundant in the A549 xenografts than in the PC-14 xenografts (Fig. 1).

Antitumor Activity of NK012 and CPT-11 on Subcutaneous PC-14 and A549 Xenografts

Experiment 1: Comparison of the antitumor effect of NK012 and CPT-11

In PC-14 xenografts that were treated with NK012 at 30 mg/kg, the tumors started to shrink on Day 4, the tumors disappeared completely by Day 14, and there was no relapse during observation until 60 days after treatment (Fig. 2A). Comparison of the relative TV revealed that the antitumor activity of NK012 was significantly greater than that of CPT-11 ($P = .0267$). Conversely, the TV did not shrink in A549 tumor-bearing mice that were treated with NK012 (Fig. 2B). Although the antitumor activity of NK012 did not differ significantly from that of CPT-11 in A549 xenografts ($P = .0869$), a trend toward a superior antitumor effect against A549 tumors was observed in the NK012 treatment group.

Experiment 2: Comparison of the antitumor effect of NK012 alone and NK012 plus Bv

In PC-14 xenografts, the combination of 5 mg/kg NK012 with 5 mg/kg Bv resulted in a significantly greater inhibition of tumor growth compared with NK012 5 mg/kg alone ($P = .0261$) (Fig. 3A). Also in A549 xenografts, the combination of 30 mg/kg NK012 with 5 mg/kg Bv resulted in significant inhibition of tumor growth compared with NK012 30 mg/kg alone ($P < .0001$) (Fig. 3B).

Distribution Studies of Free SN-38, CPT-11, and NK012 in Tumors Using HPLC

In tumors that were obtained 24 hours after the injection of CPT-11 or NK012, the level of free SN-38 released from NK012 was significantly greater than the level of SN-38 converted from CPT-11 ($P = .003$) (Fig. 4A). Conversely, the level of free SN-38 released from treatment with NK012 plus Bv did not differ significantly from the level released from treatment with NK012 alone. The intratumor concentrations of polymer-bound SN-38 did not differ between NK012 plus Bv and NK012 alone (Fig. 4B). At only 12 hours after injection, intratumor concentrations of polymer-bound SN-38 were significantly greater with NK012 alone than with NK012 plus Bv ($P = .015$). At this time point, however, there was no

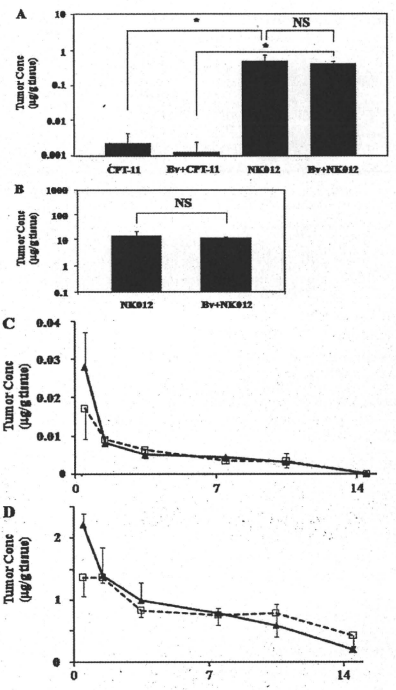

Figure 4. These charts illustrate pharmacokinetics in PC-14 tumor-bearing mice. (A) Polymer-unbound 7-ethyl-10-hydroxycamptothecin (free SN-38) in tumor was quantified by high-performance liquid chromatography (HPLC) 24 hours after the injection of irinotecan (CPT-11) (66.7 mg/kg), combined CPT-11 (66.7 mg/kg) plus bevacizumab (Bv) (5 mg/kg), the SN-38-incorporating micelle NK012 (30 mg/kg), or combined NK012 (30 mg/kg) plus Bv (5 mg/kg). (B) Polymer-bound SN-38 in tumor also was quantified by HPLC 24 hours after the injection of NK012 (30 mg/kg) or combined NK012 (30 mg/kg) plus Bv (5 mg/kg). Free SN-38 (C) and polymer-bound SN-38 (D) in tumor was quantified by HPLC at 12 hours, 24 hours, 3 days, 7 days, 10 days, and 14 days after the injection of NK012 (5 mg/kg daily) (squares) or combined NK012 (5 mg/kg daily) plus Bv (5 mg/kg daily) (triangles). Each group included 3 mice. Points indicate mean values; bars, standard deviation; asterisk, $P < .01$.

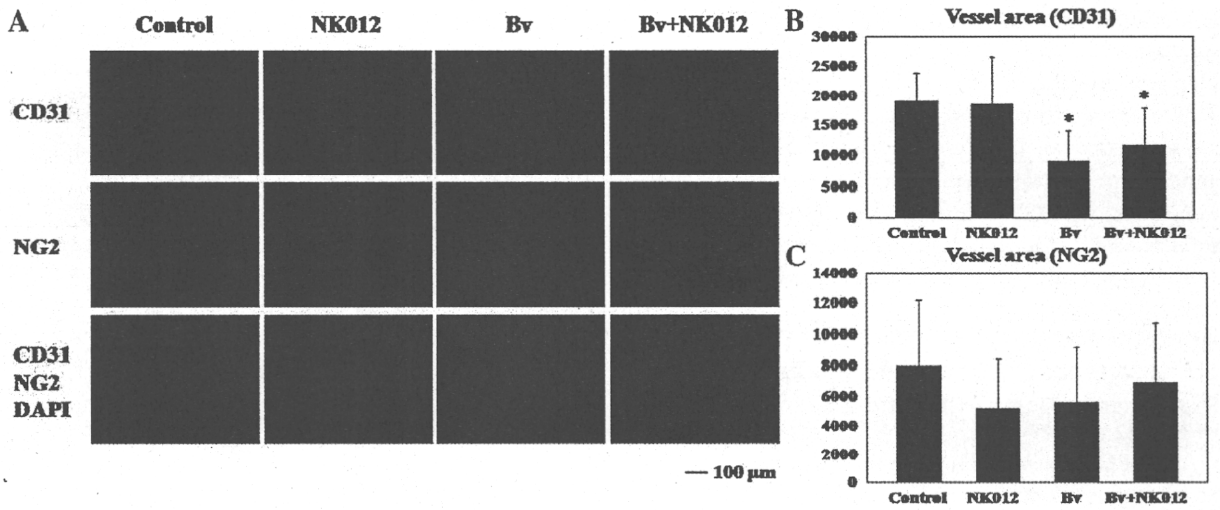

Figure 5. These photomicrographs reveal immunofluorescence staining of cluster of differentiation molecule 31 (CD31)-positive endothelial cells and the chondroitin sulfate proteoglycan NG2-positive pericytes. (A) Fourteen days after the injection of saline, bevacizumab (Bv), the novel 7-ethyl-10-hydroxycamptothecin (SN-38)-incorporating polymeric micelle NK012, or combined NK012 plus Bv, all tumors were excised from the mice. Frozen sections from these tumors (10 μm) were stained with anti-CD31 monoclonal antibody (red), anti-NG2 antibody (green), and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale Bars = 100 μm. Histologic quantification under fluorescence microscopy at 20-fold magnification was performed (B) for CD31-positive areas and (C) for NG2-positive areas. Bars indicate standard deviation; asterisks, $P < .01$ compared with control.

difference in the intratumor concentration of free SN-38 between treatment with NK012 alone and treatment with NK012 plus Bv. Thereafter, the intratumor concentrations of both polymer-bound SN-38 and free SN-38 did not differ between treatment with NK012 alone and treatment with NK012 plus Bv (Fig. 4C,D).

Immunofluorescence Staining to Clarify the Antivascular Effect of Bv

Treatment with Bv in combination with or without NK012 significantly reduced the area of CD31-positive proliferating endothelial cells in the tumors compared with controls on Day 14 ($P < .01$) (Fig. 5A,B). Conversely, the area of NG2-positive pericytes was not significantly different between the groups (Fig. 5A,C).

DISCUSSION

The size of NK012 is approximately 20 nm in diameter, and NK012 is sufficiently large to avoid renal secretion. NK012 can evade nonspecific capture by the reticuloendothelial system in various organs, because the outer shell of NK012 is covered with polyethyleneglycol. Therefore, NK012 is expected to achieve a long plasma half-life, which permits large amounts of SN-38 to reach the tumor site through the enhanced permeability and retention effect.[19]

To date, we have reported that NK012 has significantly greater antitumor activity against various human tumor xenografts including, small cell lung cancer,[11,17] colorectal cancer,[14] renal cancer,[13] pancreatic cancer,[12] gastric cancer,[15] and malignant glioma,[16] compared with CPT-11. In the current study, NK012 also appeared to eradicate PC-14 xenografts completely, but not A549 xenografts. This difference may be because of differences in the sensitivity of each cell line to NK012 and in pericyte coverage on vasculatures. Less pericyte coverage reportedly to results in more leakiness of plasma substances; therefore, the degree of NK012 accumulation may be associated inversely with the degree of pericyte coverage.[20,21]

Angiogenesis, which permits tumors to grow and metastasize, plays a pivotal role in several pathologic disorders.[22] VEGF is 1 of the most potent positive regulators of angiogenesis[23] and is recognized as an attractive target in cancer therapy. Unlike normal vasculature, the microvessels of tumors are hyperpermeable to several substances, including macromolecules and nanoparticles. The permeability, interstitial fluid pressure, and numbers of microvessels are increased by VEGF-induced angiogenesis.[24,25] Anti-VEGF antibody administered in combination with chemotherapeutic agents, including doxorubicin,[26] topotecan,[27,28] paclitaxel,[29] and docetaxel,[30] resulted in more potent inhibition of tumor growth than either agent alone. However, it has not been clarified whether anti-VEGF antibody

administered in combination with drug-incorporating polymeric micelles has an additive effect. In the current study, we demonstrated that the combination of NK012 plus Bv had significantly greater antitumor activity against human lung adenocarcinoma cells (PC-14 and A549) compared with NK012 alone. The concentrations of either polymer-bound SN-38 or free SN-38 after the administration of NK012 plus Bv did not clearly differ from the concentrations after NK012 alone. In addition, after treatment with Bv, the area of vascular endothelial cells stained with CD31 was decreased significantly compared with controls. These results suggest that VEGF inhibition may not disturb NK012 accumulation in the tumors and that the direct effect of NK012 plus Bv produced an additional antitumor effect.

In the current study, we demonstrated that NK012 has significantly greater antitumor activity against human lung adenocarcinoma cells (PC-14 and A549) compared with CPT-11. Therefore, we believe that NK012 is a promising oncologic treatment for patients with NSCLC. In 2 individual phase 1 trials that were conducted in Japan and the United States, the toxic profile of NK012 was favorable, and the dose-limiting toxicity was neutropenia.[31,32] Diarrhea was mild; that is, even the worst diarrhea was grade 2 in the phase 1 setting.

In conclusion, the current study demonstrated the superior antitumor activity of NK012 against NSCLC cells compared with CPT-11. In patients with NSCLC, clinical trials of the combination of NK012 plus Bv may be warranted.

CONFLICT OF INTEREST DISCLOSURES

Supported by a Grant-in-Aid from the Third Term Comprehensive Control Research for Cancer; by Ministry of Health, Labor, and Welfare grant H19-025 (to K. Goto, Y. Nishiwaki, and Y. Matsumura); by Ministry of Education, Culture, Sports, Science, and Technology Scientific Research on Priority Areas grant 17016087 (to Y. Matsumura); by the Japanese Foundation for Multidisciplinary Treatment of Cancer (to Y. Matsumura); and by the Princess Takamatsu Cancer Research Fund (07-23908).

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Synergistic antitumor activity of the SN-38-incorporating polymeric micelles NK012 with S-1 in a mouse model of non-small cell lung cancer

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The combination therapy of CPT-11, a prodrug of SN-38, with S-1, a dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine, shows a high clinical response rate in non-small cell lung cancer (NSCLC). However, this combination causes severe toxicities such as diarrhea. Here, we investigated the advantages of treatment with the SN-38-incorporating polymeric micelles NK012 over CPT-11 in combination with S-1 in mice bearing a NSCLC xenograft in terms of antitumor activity and toxic effects, particularly intestinal toxicity. *In vitro* cytotoxic effects were examined in human NSCLC cell lines (A549, PC-9, PC-14, EBC-1 and H520). *In vivo* antitumor effects were evaluated in PC-14- and EBC-1-bearing mice after NK012 or CPT-11 administration on Days 0 and 7 and S-1 administration on Days 0–13. Pathological changes in the small intestine were also investigated. The *in vitro* growth inhibitory effects of NK012 were 56.8- to 622-fold more potent than those of CPT-11. NK012/S-1 treatment showed significantly higher antitumor activity both in PC-14-bearing ($p = 0.0007$) and EBC-1-bearing mice ($p < 0.0001$) than CPT-11/S-1 treatment. The deformity and decrease in the density of intestinal villi were more severe in CPT-11/S-1-treated mice than in NK012/S-1-treated mice. NK012/S-1 combination is a promising candidate regimen against NSCLC without inducing toxicities such as severe diarrhea and therefore warrants clinical evaluation.

Lung cancer is the leading cause of death from malignancies worldwide in both men and women,[1] and accounted for 31% (male) and 26% (female) of all cancer deaths in 2008.[2] It is histologically classified into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The standard first-line chemotherapy for NSCLC is platinum-based regimens.[3] However, as shown in a randomized phase III study, the response rate to these regimens is only 30–33% and the 1-year survival rate is 48–59%, with a median survival period of 11–14 months for advanced NSCLC patients with PS 0 or 1.[4] Therefore, the development of new chemotherapeutic agents and combination regimens against NSCLC is urgently desired.

Irinotecan hydrochloride (CPT-11), an anticancer drug, is converted to its biologically active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) by carboxylesterases, and SN-38 has been shown to be efficacious against various human cancers such as colorectal, lung and ovarian cancer.[5–8] Although SN-38 has 1,000-fold more potent cytotoxic activity against various cancer cell lines *in vitro* than CPT-11,[9] its conversion rate from CPT-11 to SN-38 is <10% of the original CPT-11 dose in the body.[10,11]

On the other hand, the SN-38-incorporating polymeric micelles NK012 appear to have the advantage of passive targeting of the drug delivery system (DDS). In this passive DDS targeting, the drug accumulates in tumor tissue by utilizing the enhanced permeability and retention (EPR) effect.[12–15] This EPR effect is based on several pathological mechanisms that 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

Key words: NK012, S-1, diarrhea, drug delivery system, non-small cell lung cancer

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology (Scientific Research on Priority Areas); **Grant number:** 17016087; **Grant sponsors:** Third Term Comprehensive Control Research for Cancer, Japanese Foundation for Multidisciplinary Treatment of Cancer, Ministry of Health, Labor and Welfare; **Grant number:** H19-025

DOI: 10.1002/ijc.25282

History: Received 18 Dec 2009; Accepted 26 Jan 2010; Online 2 Mar 2010

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drainage of macromolecules accumulated in solid tumor tissue. In the previous study, we evaluated the antitumor effect by histopathologic evaluation and immunohistochemistry and demonstrated decreased cellularity, increased tumor stroma, and inflammatory cell infiltrations in the tumors treated with NK012. Tumors treated with CPT-11 showed no apparent morphologic differences from control tumors. Concordant with morphologic changes, the number of Ki-67 tumor cells tended to decrease in tumors treated with NK012 compared with CPT-11.[16] Recent studies demonstrated that NK012 is significantly more potent than CPT-11 against SCLC,[17] colorectal cancer,[18] renal cancer,[19] pancreatic cancer,[20] stomach cancer[21] and glioma.[22] Furthermore, in 2 independent phase I clinical trials in Japan[23] and the US,[24] nonhematological toxicities were minimal and grade 3/4 diarrhea, a major clinically important toxic effect or dose-limiting factor of CPT-11, was absent.

CPT-11 causes cell accumulation in the S phase, and 5-fluorouracil (5-FU) infusion induces DNA damage specifically in cells in the S phase.[25] Moreover, CPT-11 reduces thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) mRNA expression,[26] and low gene expression level of TS and DPD had association with the response rate or chemosensitivity to 5-FU in metastatic colorectal cancer.[27,28] Recently, we demonstrated the higher synergistic antitumor activity of the NK012/5-FU combination against a colorectal tumor xenograft than the CPT-11/5-FU combination.[18] However, the use of an indwelling central venous catheter and a portable pump for 5-FU infusion may cause infection or thrombosis, and incur higher healthcare costs.[29]

S-1, on the other hand, is an oral anticancer agent composed of a 5-FU prodrug (tegafur), 5-chloro-2, 4-dihydroxypyrimidine (CDHP), and potassium oxonate (molar ratio = 1:0.4:1) and is categorized under DPD inhibitory fluoropyrimidines.[30] Tegafur generates 5-FU in the blood primarily *via* metabolism by liver enzyme cytochrome P450. CDHP enhances the serum 5-FU concentration by inhibiting the DPD activity competitively. Potassium oxonate is a reversible competitive inhibitor of orotate phosphoribosyl transferase, a phosphoenzyme for 5-FU and attributes to phosphorylation of 5-FU in the gastrointestinal tract and is expected to reduce the intestinal toxicity that is one of the clinical problems of 5-FU.[31]

In this context, we investigated the advantages of NK012/S-1 over CPT-11/S-1 in mice bearing a NSCLC xenograft in terms of antitumor activity and toxic effects, particularly intestinal toxicity.

Material and Methods

Drugs and cells

SN-38 and NK012 were prepared by Nippon Kayaku Co., (Tokyo, Japan). CPT-11 was purchased from Yakult Honsha Co., (Tokyo, Japan). S-1 was obtained from Taiho Pharmaceutical Co. (Tokyo, Japan). 5-FU was purchased from Kyowa Hakko (Tokyo, Japan).

The NSCLC cell lines A549, PC-9, PC-14, EBC-1 and H520 were purchased from the American Type Culture Collection (Rockville, MD). They were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin, streptomycin and amphotericin B (100 units/mL, 100 μg/mL and 25 μg/mL, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

In vitro growth inhibition assay

The growth inhibitory effects of NK012, CPT-11, SN-38 and 5-FU, instead of S-1 that is not suitable for use *in vitro*, because tegafur is a prodrug that is mainly activated in liver were examined by tetrazolum salt-based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan). A suspension (100 mL) of exponentially growing cells (1×10^5/mL) was placed into the wells of a 96-well plate and incubated for 24 hr at 37°C. Following medium removal, 100 μL of medium containing various concentrations of each drug was added to the wells and then incubated for 72 hr 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 hr at 37°C. The growth inhibitory effects of each drug were assessed spectrophotometrically (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). IC_{50} was determined on the dose–response curves. The nature of interaction between NK012 and 5-FU against the NSCLC cell lines A549, PC-9, PC-14, EBC-1 and H520 was evaluated by median-effect plot analyses and the combination index method of Chou and Talalay.[32]

Reverse transcription and real-time PCR analysis

A suspension (2 mL) of exponentially growing cells (1×10^5/mL) was placed into the wells of a 6-well plate and incubated for 24 hr at 37°C. Following medium removal, 2 mL of medium and medium containing NK012 (1 μM) and CPT-11 (1 μM) were added to the wells and then incubated for 24 hr at 37°C ($n = 3$ for each arm), as reported.[33] Total RNA (1 μg) extracted from cells using an RNeasy Mini kit (Qiagen, Valencia, CA) was subjected to reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster, CA). The resulting cDNA was then subjected to real-time PCR analysis using a Taqman PCR Reagent kit and an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). To quantify TS and DPD, we used TaqMan primers and a probe mixture (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Relative quantification of the total RNA in each sample was conducted using the comparative Ct (threshold cycle) method. The formulae for the relative quantification of each gene were as follows: (dCt of each gene) = (Ct of each gene) − (Ct of GAPDH), and (Relative quantification of each gene) = $2^{-(\text{dCt of each gene})}$.

Table 1. *In vitro* growth inhibitory activity of SN-38, NK012, CPT-11 and 5-FU in human non-small cell lung cancer cells

	IC50 (μmol/L)			
Cell line	SN-38	NK012	CPT-11	5-FU
A549	0.500 ± 0.092	0.888 ± 0.096	50.4 ± 2.3	419 ± 44
PC-9	0.0574 ± 0.0414	0.0732 ± 0.0020	8.86 ± 0.43	15.0 ± 4.2
PC-14	0.0488 ± 0.0011	0.0554 ± 0.0118	7.53 ± 4.97	2.99 ± 0.27
EBC-1	0.00374 ± 0.00449	0.00747 ± 0.00053	4.65 ± 0.17	45.8 ± 2.6
H520	0.0721 ± 0.0131	0.0773 ± 0.0071	9.10 ± 0.29	13.6 ± 7.1

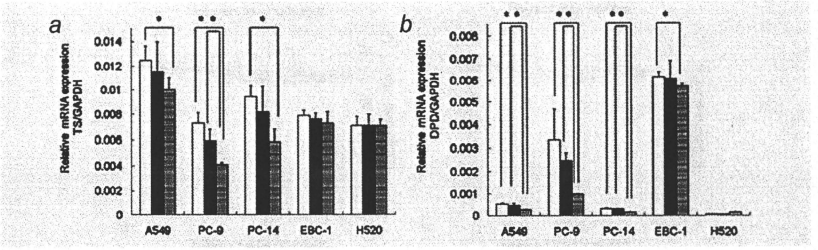

Figure 1. Effects of NK012 and CPT-11 on the expression of TS and DPD mRNA in non-small cell lung cancer (NSCLC) cell lines. (*a, b*) Downregulation of TS (*a*) and DPD (*b*) mRNA by NK012 and CPT-11 in NSCLC cell lines. A549, PC-9, PC-14, EBC-1 and H520 cells were incubated with medium containing 10% serum, medium containing NK012 (1 μmol/L) and 10% serum, and medium containing CPT-11 (1 μmol/L) and 10% serum for 24 hr. Then, total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS and DPD mRNA. The amount of TS and DPD mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Bars, SD. *, $p < 0.05$. □, Medium; ■, CPT-11; ⊟, NK012.

Experimental mice model

Female BALB/c nude mice (6-weeks-old) were purchased from SLC Japan (Shizuoka, Japan). Mice were inoculated subcutaneously in the flank with 2.5×10^6 cells/50 μL cell suspension of PC-14 and 1.0×10^6 cells/50 μL cell suspension of EBC-1.

All animal procedures were performed 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 comply with the guidelines for the use of experimental animals in Japan.

In vivo growth inhibition assay

When the tumor volume (TV) reached 250 mm^3, mice were randomly divided into test groups consisting of 5 mice per group (Day 0). NK012, CPT-11, or NaCl solution (0.9%) was intravenously (i.v.) administered into the tail vein on Days 0 and 7. NK012 was administered at 5 mg/kg/d, which is 1/6 of the maximum tolerated dose (MTD). CPT-11 (reference drug) was administered at 10 mg/kg/d, which is also 1/6 of the MTD. S-1 was singularly or simultaneously administered by oral gavage once a day on Days 0–13 at 10 mg/kg/d, as reported.[34] 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 $TV = (a \times b^2)/2$. Relative tumor volume (RTV) on day n was calculated using $RTV = TV_n/TV_0$, where TV_n is the tumor volume on Day n and TV_0 is the tumor volume on Day 0. Relative body weight (RBW) was calculated using $RBW = BW_n/BW_0$. We evaluated the feces of mice on Days 4, 11 and 18 and considered soft, wet and canescent feces to indicate diarrhea, as reported.[35]

Experiment 1. Evaluation of the synergistic effects of NK012 with S-1. NK012, S-1, NK012/S-1, or NaCl solution (0.9%) was administered following the above dose schedules. We evaluated the effects of NK012/S-1 by comparing the data between NK012/S-1 and the additive effect (expected RTV). Expected RTV was calculated using (RTV of NK012) × (RTV of S-1) / (RTV of control), as reported.[36]

Experiment 2. Comparison of the antitumor effects of NK012/S-1 and CPT-11/S-1. NK012/S-1, CPT-11/S-1, or NaCl solution (0.9%) was administered following the above dose schedules. Two-way analysis of variance (ANOVA) was performed to compare the transitional RTV between NK012/S-1-treated mice and CPT-11/S-1-treated mice.

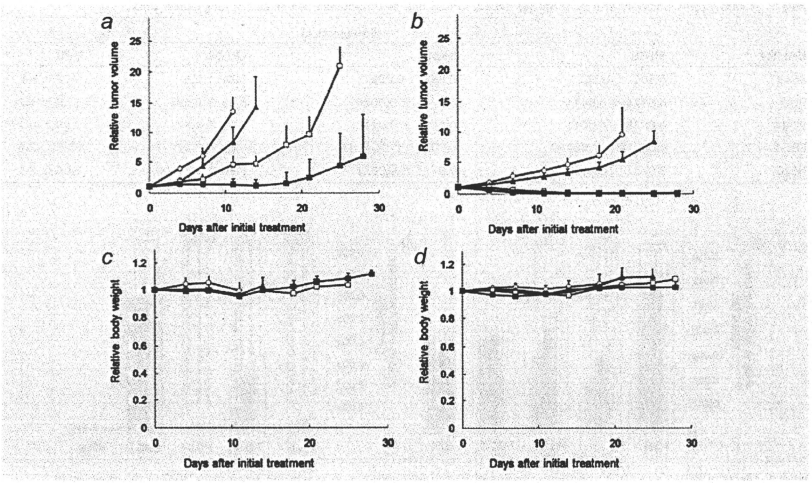

Figure 2. Growth inhibitory effects of NK012, S-1 and NK012/S-1 on PC-14 and EBC-1 tumor xenografts. (*a*, *b*) Relative tumor volume in mice treated with NK012, S-1 and NK012/S-1. PC-14 (*a*, *c*) and EBC-1 (*b*, *d*) cells were inoculated subcutaneously into the flank of mice, as described in Material and Methods. Drug administration was as follows: NK012 (5 mg/kg/d) on Days 0 and 7 (□), S-1 (10 mg/kg/d) on Days 0–13 (▲), NK012 (5 mg/kg/d) on Days 0 and 7 and combined with S-1 (10 mg/kg/d) on Days 0–13 (■), or NaCl solution (0.9%) on Days 0 and 7 as normal control (○). Points, mean; bars, SD. *, $p < 0.05$. (*c*, *d*) Treatment-related body weight loss occurred in mice treated with each drug. Points, mean; bars, SD.

Pathological studies of small intestinal mucosa

NaCl solution, CPT-11, NK012, S-1, CPT-11/S-1 and NK012/S-1 were administered to female BALB/c nude mice ($n = 3$) following the same dose schedules as those used in the treatment experiment. On Day 7 after the last dozing, mice were sacrificed and the small intestine was sampled at the middle portion. Samples were fixed in 10% formalin, paraffin-embedded, sectioned and stained with H&E. Villi density was defined as the number of villi per mm. We also evaluated the fecal condition mice on Days 4, 11 and 18. The extent of diarrhea as well as the appearance and number of villi was scored by independent, 2 blinded researchers.

Statistical analysis

Data were analyzed with Student's *t*-test when groups showed equal variances (*F* test) or Welch's test when they showed unequal variances (*F* test). ANOVA was performed to compare transitional RTV. Differences in the number of mice with diarrhea between NK012/S-1-treated mice and CPT-11/S-1-treated mice were tested for significance using the Pearson χ^2 test or Fisher exact test. All analyses were performed using StatView 5.0, and $p < 0.05$ was considered significant. All statistical tests were 2 sided, and data were expressed as mean ± SD.

Results

Sensitivity of NSCLC cells to NK012, CPT-11, SN-38 and S-1

The IC_{50} values of NK012 for the NSCLC cell lines ranged from 0.00747 μmol/L (EBC-1) to 0.888 μmol/L (A549) (Table 1). The cytotoxic effects of NK012 were 56.8- to 622-fold higher than those of CPT-11, whereas those of NK012 were 1.07- to 2.00-fold lower than those of SN-38. These features were comparable to those reported previously.[17,37] The molar ratios of NK012: 5-FU of 1:500 in A549, 1:200 in PC-9 and H520, 1:50 in PC-14, and 1:6,000 in EBC-1 were used for the drug combination studies based on the IC_{50} values of NK012 and 5-FU (Table 1). The synergic to additive effect between NK012 and 5-FU was observed in these NSCLC cell lines (data not shown).

Effects of NK012 and CPT-11 on the expression of TS and DPD mRNA in NSCLC cell lines

NK012 induced a significant decrease in TS mRNA expression in A549, PC-9 and PC-14 ($p = 0.0487$, $p = 0.0027$ and $p = 0.0095$, respectively) compared with the control, as well as in PC-9 ($p = 0.0225$) compared with CPT-11. NK012 also tended to decrease TS mRNA expression in EBC-1 and H520 compared with the control and CPT-11 (Fig. 1*a*). NK012

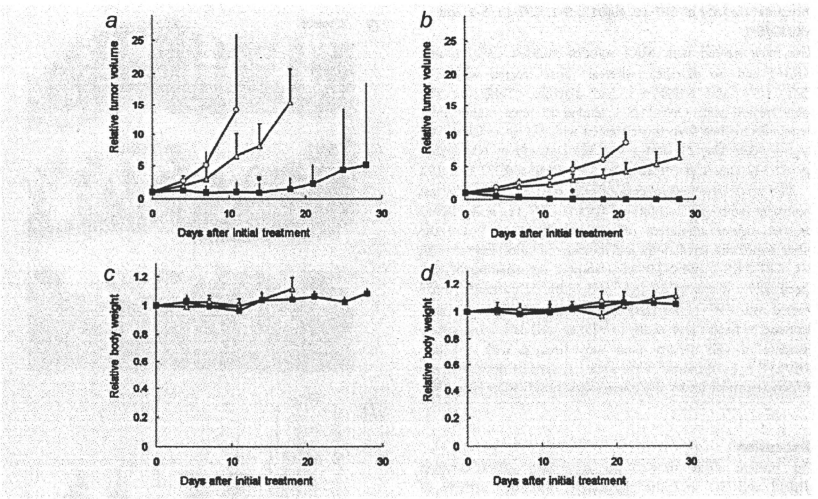

Figure 3. Antitumor effect of combined NK012/S-1 and CPT-11/S-1 treatment on PC-14 and EBC-1 tumor xenografts. (*a*, *b*) Relative tumor volume in mice treated with NK012/S-1 or CPT-11/S-1. PC-14 (*a*, *c*) and EBC-1 (*b*, *d*) cells were inoculated subcutaneously into the flank of mice, as described in Material and Methods. Drug administration was as follows: NK012 (5 mg/kg/d) on Days 0 and 7 and combined with S-1 (10 mg/kg/d) on Days 0–13 (■), CPT-11 (10 mg/kg/d) on Days 0 and 7 and combined with S-1 (10 mg/kg/d) on Days 0–13 (△), or NaCl solution (0.9%) on Days 0 and 7 as normal control (○). Points, mean; bars, SD. *, $p < 0.05$. (*c*, *d*) Treatment-related body weight loss occurred in each treated-mouse. Points, mean; bars, SD.

Table 2. Diarrhea after i.v. administrations of drugs

	Control	S-1	NK012	S-1 + NK012	CPT-11	S-1 + CPT-11
Day 4	0/24 (0)	1/13 (7.7)	0/10 (0)	1/24 (4.2)	0/4 (0)	3/14 (21.4)
Day 11	0/24 (0)	2/13 (15.4)	0/10 (0)	6/24 (25)	0/4 (0)	7/14 (50.0)
Day 18	0/24 (0)	0/13 (0)	0/10 (0)	2/24 (8.3)	0/4 (0)	3/14 (21.4)

Values in parentheses indicate percentage.

induced a significant decrease in DPD mRNA expression in A549, PC-9, PC-14 and EBC-1 ($p = 0.0019$, $p = 0.0358$, $p = 0.0020$ and $p = 0.0399$, respectively) compared with the control, as well as in A549, PC-9 and PC-14 ($p = 0.0373$, $p = 0.0013$ and $p = 0.0001$, respectively) compared with CPT-11. NK012 also tended to decrease DPD mRNA expression in EBC-1 compared with CPT-11, but not in H520 (Fig. 1*b*).

Antitumor activity of S-1, NK012, NK012/S-1, CPT-11 and CPT-11/S-1 against PC-14 and EBC-1 tumors

The therapeutic effect of NK012/S-1 was significantly superior to that of NK012 both in PC-14 ($p = 0.0013$) (Fig. 2*a*) and EBC-1 ($p = 0.0017$) (Fig. 2*b*), and this combination demonstrated a synergistic efficacy. The complete response rates achieved with NK012 and NK012/S-1 were 0 and 20% for PC-14 and 40 and 100% for EBC-1, respectively. 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 21 (Figs. 2*c* and 2*d*).

The therapeutic effect of NK012/S-1 was significantly superior to that of CPT-11/S-1 in PC-14-bearing ($p = 0.0007$) (Fig. 3*a*) and EBC-1-bearing mice ($p < 0.0001$) (Fig. 3*b*). The complete response rates achieved with NK012/S-1 were 40 and 100% for PC-14 and EBC-1, respectively. Although slight treatment-related BW loss was observed in mice treated with each drug combination, there was no significant difference between NK012/S-1 and CPT-11/S-1, and BW recovered to the normal level in each group by Day 21 (Figs. 3*c* and 3*d*).

Intestinal toxicity of CPT-11, NK012, S-1, CPT-11/S-1 and NK012/S-1

The mice treated with NaCl solution (0.9%), CPT-11, and NK012 had no diarrhea, whereas those treated with S-1, CPT-11/S-1 and NK012/S-1 had diarrhea (Table 2). The mice treated with CPT-11/S-1 tended to have higher incidence of diarrhea than those treated with S-1 ($p = 0.596$ Day 4, $p = 0.103$ Day 11, and $p = 0.222$ Day 18) or NK012/S-1 ($p = 0.132$ Day 4, $p = 0.163$ Day 11, and $p = 0.337$ Day 18).

The small intestinal mucosa of mice on Day 7 after the last treatment with NaCl solution (0.9%), CPT-11, and NK012 showed regular alignment of normal villi (Fig. 4). On the other hand, the small intestinal mucosa of mice treated with S-1, CPT-11/S-1 and NK012/S-1 showed deformation of villi, specifically a decrease in height and width. In particular, mice treated with CPT-11/S-1 showed more severe deformation and decrease in height and width of villi, as well as a more severe decrease in villi density than those treated with S-1 and NK012/S-1. Furthermore, CPT-11/S-1 treatment decreased villi density the most under the microscopic observation (Fig. 4*b*).

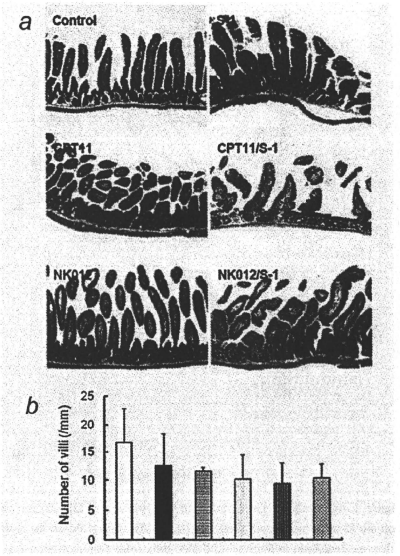

Figure 4. Pathological findings in intestinal mucosa. Mice were administered the following: NaCl solution (0.9%) on Days 0 and 7 as normal control, CPT-11 (10 mg/kg/d) on Days 0 and 7, NK012 (5 mg/kg/d) on Days 0 and 7, S-1 (10 mg/kg/d) on Days 0–13, CPT-11 (10 mg/kg/d) on Days 0 and 7 and combined with S-1 (10 mg/kg/d) on Days 0–13, or NK012 (5 mg/kg/d) on Days 0 and 7 and combined with S-1 (10 mg/kg/d) on Days 0–13. (*a*) Mice were sacrificed on Day 21 and the small intestine was sampled at the middle portion. Samples were fixed in 10% formalin, paraffin-embedded, sectioned and stained with H&E. In the NaCl-, CPT-11- and NK012-treated mice, the small intestinal mucosa showed regular alignment of normal villi. In the S-1- and NK012/S-1-treated mice, the small intestinal mucosa showed deformation of villi, specifically decreased height and width. This was also observed in CPT-11/S-1-treated mice with accompanying severe decrease in villi density. (*b*) Villi density indicates the number of villi per mm. Villi density was decreased the most with CPT-11/S-1 treatment. □, NaCl solution; ■, CPT-11; ⊟, NK012; ⊡, S-1; ◫, CPT-11/S-1; ▨, NK012 /S-1.

Discussion

The present study showed the synergistic effect between NK012 and S-1 and the significant antitumor activity of NK012/S-1 compared with CPT-11/S-1, the latter being one of the promising combinations against several cancers including NSCLC.[38] Indeed, CPT-11 combined with S-1 also exhibits potentially promising clinical activity with favorable toxic profile not only in NSCLC, but also advanced colorectal cancer,[29] and metastatic advanced gastric cancer.[39] Previously, we studied the differences in the effects between NK012 and CPT-11 on the cell cycle and demonstrated that NK012 induced a more prolonged accumulation of tumor cells in the S phase than CPT-11,[18] and this may explain the higher synergistic effect of NK012/5-FU than CPT-11/5-FU. Here, NK012 caused a larger decrease in TS and DPD mRNA expression than CPT-11. TS and DPD mRNA is thought to be associated with fluoropyrimidine sensitivity in lung cancer,[40] and a greater synergistic effect is expected between NK012 and fluoropyrimidines than between CPT-11 and fluoropyrimidines. In a phase II study of CPT-11/S-1 for advanced NSCLC, the grade 3/4 hematologic toxicities observed included neutropenia (25%), thrombocytopenia (3.6%) and anemia (3.6%), and the most common grade 3/4 nonhematologic toxicities were anorexia (14.3%), fatigue (8.9%) and diarrhea (8.9%).[38] Severe late-onset diarrhea is a major clinically important toxic effect or dose-limiting factor of CPT-11.[41–43] Diarrhea is also a clinical problem in S-1 treatment.[44] We previously demonstrated that a large amount of CPT-11 was excreted into the feces and high CPT-11 concentration was detected in the small intestinal epithelium. In contrast, a small amount of NK012 was found in the feces and NK012 was weakly and uniformly distributed in the mucosal interstitium. Furthermore, inflammatory changes in the small intestinal mucosa were rare in all NK012-treated mice, but were commonly observed in CPT-11-treated mice.[45] Here, in the present study, we used the same nude mice bearing human tumor xenografts in order to compare the present data with the previous data and demonstrated CPT-11/S-1 treatment induced more severe deformation of villi,

specifically decreased height and width, and severe decrease in villi density than NK012/S-1. Furthermore, villi density in CPT-11/S-1-treated mice was less than that in NK012/S-1-treated mice. The incidence of CPT-11/S-1-induced diarrhea was higher than that of NK012/S-1-induced diarrhea, although the difference was not significant ($p = 0.132$–0.337). There was no significant difference in other toxic effects including bone marrow and liver toxicities between NK012/S-1 and CPT-11/S-1 in the present treatment schedule (data not shown).

In conclusion, NK012/S-1 showed a significantly higher antitumor activity with less intestinal damage than CPT-11/S-1, one of the promising regimens against NSCLC, advanced colorectal cancer and metastatic advanced gastric cancer. Phase II clinical trials of NK012 monotherapy are now underway in patients with colorectal cancer in Japan and patients with triple negative breast cancer or SCLC in the USA. The present results suggest the clinical evaluation of NK012/S-1 in NSCLC patients.

Acknowledgements

This work was supported partly by a Grant-in-Aid from the Third Term Comprehensive Control Research for Cancer, the Ministry of Health, Labor and Welfare (Goto, Nishiwaki, and Matsumura), Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (Matsumura), and the Japanese Foundation for Multidisciplinary Treatment of Cancer (Matsumura). The authors thank Ms. N. Mie and Ms. M. Ohtsu for technical assistance and Ms. K. Shiina and Ms. K. Abe for secretarial assistance.

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Detailed Distribution of NK012, an SN-38–Incorporating Micelle, in the Liver and Its Potent Antitumor Effects in Mice Bearing Liver Metastases

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Abstract

Purpose: To clarify and compare the antitumor effects and specific biodistribution of NK012, an SN-38–incorporating polymeric micelle, in mice bearing multiple liver metastases of human colon cancer HT-29 cells with irinotecan hydrochloride (CPT-11).

Experimental Design: The maximum tolerable dose of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was i.v. administered three times every 4 days to mice bearing metastases to the liver colonized 7 days after the portal administration of HT-29 cells ($n = 6$). *In vivo* antitumor effects were evaluated by bioluminescence imaging and histopathologic examination. Drug biodistribution was analyzed by high-performance liquid chromatography and fluorescence microscopy ($n = 3$).

Results: NK012 eradicated the liver metastases and produced a significant longer survival rate than CPT-11 ($P = 0.0006$). High-performance liquid chromatography showed the prolonged distribution of NK012 and free SN-38 released from NK012 in the tumors, liver, and spleen for weeks after NK012 administration. On the other hand, the accumulation levels of CPT-11 and free SN-38 converted from CPT-11 rapidly decreased within 1 day after CPT-11 administration. In the liver metastases, fluorescence microscopy and immunohistochemistry showed that administered NK012 was distributed mainly adjacent to tumor vessels after 1 day. As for the normal liver, NK012 was distributed in Kupffer cells instead of hepatocytes for at least 7 days after administration.

Conclusion: This study suggests that NK012 is strongly effective against liver metastases and does not damage the liver despite the long retention time of NK012 in Kupffer cells. *Clin Cancer Res; 16(19); 4822–31.*

Colorectal cancer is one of the most common malignancies in the world, and colorectal liver metastasis (CLM) is the most frequent distant metastatic pattern. Although resection of the lesion in the liver is the most effective curative treatment, patients with initially resectable disease include only 15% to 20% of all CLM (1–3). Recently, the development of effective regimens including irinotecan (CPT-11), oxaliplatin, and molecular targeted agents has improved the median survival time of patients with advanced colorectal cancer. This improvement enables 13% of patients with unresectable CLM to undergo resection, which yields a similar outcome to patients with initially resectable CLM (4). However, the median survival time of patients with advanced colorectal cancer still ranges from 15.6 to 20.8 months (5, 6).

On the other hand, intensive or prolonged preoperative chemotherapy causes liver toxicities such as chemotherapy-associated steatohepatitis or sinusoidal dilation (7, 8). These hepatic toxicities increase the risk of perioperative mortality and morbidity of hepatic resection (9, 10). Additionally, although molecular agents yield certain benefits against CLM, unexpected serious adverse effects and the high expense of drugs should be carefully taken into consideration (5, 11, 12). To address this situation, it is necessary to develop a new effective therapy against CLM without causing serious adverse effects on postchemotherapy hepatic resection.

7-Ethyl-10-hydroxy-camptothecin (SN-38) is a biologically active metabolite of CPT-11 and a broad-spectrum anticancer agent that targets DNA tomoisomerase I. Although SN-38 has 1,000-fold more potent cytotoxic activity than CPT-11 (13), it has been clinically unavailable due to its water-insoluble nature. Additionally, its metabolic conversion rate is only <10% of the original volume of CPT-11

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doi: 10.1158/1078-0432.CCR-10-1467

Translational Relevance

CPT-11 is one of the key drugs for the treatment of advanced colorectal cancer including liver metastases. However, chemotherapy-induced liver toxicity caused by preoperative intensive use of CPT-11 is of important concern. Therefore, a more effective and less toxic drug must be developed.

We previously showed the stronger antitumor effects of NK012, an SN-38–incorporating polymeric micelle, against various cancer mouse models than CPT-11. Two phase I trials in Japan and the United States revealed that patients treated with NK012 did not develop grade 3/4 diarrhea, a major adverse effect of CPT-11.

Here, we showed the advantage of NK012 over CPT-11 in mice bearing multiple liver metastases of human colon cancer HT-29 cells. Moreover, we investigated the detailed biodistribution of NK012 in the liver and metastatic tumors. The results provide a strong basis for the clinical evaluation of NK012 as treatment for advanced colorectal cancer particularly with accompanying liver metastases.

(14, 15). On the other hand, NK012, an SN-38–incorporating polymeric micelle, is also a prodrug of SN-38 that is categorized under drug delivery system (DDS) agents. The mean particle size of NK012 is 20 nm in diameter with a relatively narrow range. This DDS agent known as macromolecule polymeric micelles has two major advantages over traditional small-molecule agents. First, NK012 can deliver more SN-38 to tumor tissue and is retained via the enhanced permeability and retention effect (16). Second, NK012 has potential for the sustained release of SN-38, which has time-dependent cytotoxic effects following its accumulation in tumor tissue. The release rates of SN-38 from NK012 under biological condition are 57% and 74% at 24 and 48 hours, respectively, and the release proceeds nonenzymatically (17). To date, we have reported the potent antitumor effects and less toxicity of NK012 than CPT-11 (17–20).

However, the detailed biodistribution of micellar nanoparticles in a biological liver metastasis model has scarcely been reported. In particular, the biodistribution of DDS agents is closely related to the vascularity and specificity of each organ or tumor. The liver is highly vascularized and has many vital functions, including phagocytosis, which is involved in the clearance of DDS agents (21–24). In this specific liver environment, it is conceivable that some differences in the delivery of DDS agents to a hypovascular metastatic tumor exist. To further clarify underlying mechanisms, it is necessary to confirm the biodistribution of DDS agents in an orthotopic model close to the biological environment of real human CLM and not in a s.c. xenograft model.

In this study, we evaluated the antitumor effects of NK012 against CLM using a mouse model of multiple liver metastases of human colon cancer HT-29 cells. Moreover, we examined the detailed biodistribution of NK012 to elucidate its behavior in biological organs, particularly the liver, as well as its effects on liver metastases.

Materials and Methods

Drugs

NK012 was supplied by Nippon Kayaku Co. Ltd. and stored in the freeze-dried state and dissolved in distilled water at a 5 mg/mL SN-38 equivalent dose immediately before administration to mice. NK012 is stable under either low temperature (~20°C) or acidic condition (pH ~4.6) and gradually begins to release the encapsulated SN-38 under *in vivo* conditions. We previously reported precise information about NK012 including preparation and pharmacokinetic analysis (17). CPT-11 was purchased from Yakult Honsha Co. Ltd.

Cell culture

The human colon cancer cell line HT-29 was obtained directly from the American Type Culture Collection. HT-29 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Cell Culture Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Sigma) in humidified 5% CO_2 at 37°C.

Establishment of HT-29 cell line stably expressing firefly luciferase

For the *in vivo* bioluminescence imaging of liver metastatic tumors, an HT-29 cell line stably expressing firefly luciferase (HT-29/Luc) was established. In brief, the coding sequence for firefly luciferase was subcloned into the pcDNA3.1(+) vector (Invitrogen) to generate plasmids of pcDNA3.1/luciferase. HT-29 cells (2×10^5) were seeded onto 3-cm dishes 24 hours before transfection. The cells were transfected with 2.5 μg of plasmid DNA using Lipofectamine LTX Reagent and PLUS Reagent (Invitrogen) according to the manufacturer's instructions and then incubated for 48 hours at 37°C. The cells were then passaged in medium containing 1 mg/mL G418 (Invitrogen) to select for the neomycin resistance gene integrated in the pcDNA3.1(+) plasmids. The accuracy of a quantitative bioluminescence image as an indicator of HT-29/Luc cell number was analyzed using the Photon Imager animal imaging system (BioSpace) *in vitro*, as described under *in vivo* growth inhibition assay. This analysis showed a clear correlation between a quantitative bioluminescence image and cell number. The sensitivity of HT-29/Luc cells to each drug was almost similar to that of parental HT-29 cells (data not shown).

Liver metastasis model

Six- to 8-week-old female athymic BALB/c nude mice (CLEA Japan) weighing 17 to 20 g at the time of surgery were used for this study. The animals were maintained under specific pathogen-free conditions and provided with sterile food, water, and cages. The mice were anesthetized

by i.p. injection of 0.15 mL/g body weight of an anesthetic agent, which consisted of dissolved 1.0 g of 2,2,2-tribromoethanol (Wako) in 1.0 mL of 2-methyl-2-butanol (Sigma)/40 mL of H_2O. A median laparotomy was done following disinfection of the abdominal skin. After mobilization of the duodenum, 2×10^6 HT-29/Luc cells suspended in 100 μL of PBS were injected into the portal vein using a Hamilton syringe (30-gauge needle). To prevent either bleeding or dissemination of tumor cells, puncture sites were gently pressed for several minutes. Sterile measures were taken during the operation. All animal procedures were done in compliance with the Guidelines for the Care and Use of Experimental Animals of the National Cancer Center, Japan; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Bioluminescence imaging

To evaluate and visualize the hepatic metastases of HT-29/Luc cells, *in vivo* bioluminescence imaging was done using the Photon Imager animal imaging system. Mice were i.p. administered D-luciferin potassium salt (Synchem) at 2.5 mg/mouse and anesthetized with isoflurane during imaging. For photon quantification, a region of interest was encircled manually using Photon Vision software (Bio-Space), and the total number of photon per minute [counts per minute (cpm)] was recorded.

In vivo tumor growth inhibition assay

Seven days after the portal injection of HT-29/Luc cells, mice were randomly divided into three test groups consisting of six mice per group (day 0). Randomization was done based on a bioluminescence image (>20,000 cpm), and the mean cpm was confirmed to be statistically identical between groups. The mice were given i.v. injections of 30 mg/kg NK012 and 66.7 mg/kg CPT-11 via the lateral tail vein (200 μL) on days 0, 4, and 8. The dose of NK012 represents the equivalent dose of SN-38 incorporated in the micelle. Control mice were injected with 200 μL of PBS following the same schedule. *In vivo* bioluminescence imaging was done every 7 days from the day of treatment initiation, and the body weight of each mouse was also measured. Mortality and morbidity were checked daily, and the mice were maintained until each mice showed signs of morbidity (massive ascites or observable hepatic tumor, jaundice, and 20% weight loss), at which point they were sacrificed in consideration of animal welfare.

Pharmacokinetics analysis by high-performance liquid chromatography

To assess the biodistribution in each organ, tissue concentrations of NK012, CPT-11, and free SN-38 were measured using high-performance liquid chromatography. Over 14 days after the portal injection of HT-29/Luc cells, female BALB/c nude mice with $>1 \times 10^6$ cpm were used for pharmacokinetics analysis. NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was i.v. administered on day 0, as reported (17). After blood removal from the inferior vena cava, the

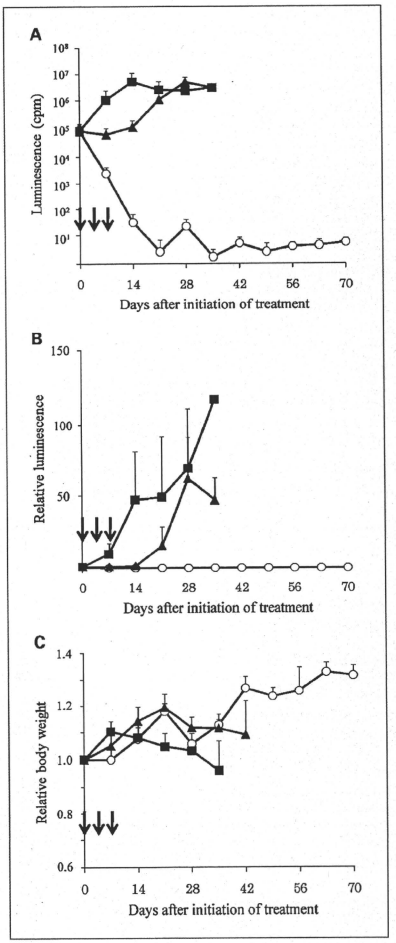

Fig. 1. Effects of NK012 and CPT-11 in HT-29/Luc liver metastasis mouse models. Each treatment was initiated on days 0, 4, and 8 (○, NK012, 30 mg/kg/d, three times; ▲, CPT-11, 66.7 mg/kg/d, three times; ■, control, 200 μL/d, three times). Day 0 indicates 7 d after the portal vein injection of HT-29/Luc cells ($n = 6$). The antitumor activity of NK012 or CPT-11 was evaluated by determining the absolute number (A) and relative number (B) of photon using a Photon Imager system. C, treatment-related body weight loss did not occur in all groups.