Novel SN-38–Incorporating Polymeric Micelles, NK012, Eradicate Vascular Endothelial Growth Factor–Secreting Bulky Tumors

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Abstract

7-Ethyl-10-hydroxy-camptothecin (SN-38), a biological active metabolite of irinotecan hydrochloride (CPT-11), has potent antitumor activity but has not been used clinically because it is a water-insoluble drug. For delivery by i.v. injection, we have successfully developed NK012, a SN-38-releasing nanodevice. The purpose of this study is to investigate the pharmacologic character of NK012 as an anticancer agent, especially in a vascular endothelial growth factor (VEGF)–secreting tumor model. The particle size of NK012 was ~20 nm with a narrow size distribution. NK012 exhibited a much higher cytotoxic effect against lung and colon cancer cell lines as compared with CPT-11. NK012 showed significantly potent antitumor activity against a human colorectal cancer HT-29 xenograft as compared with CPT-11. Enhanced and prolonged distribution of free SN-38 in the tumor was observed after the injection of NK012. NK012 also had significant antitumor activity against bulky SBC-3/Neo (1,533.1 ± 1,204.7 mm3) and SBC-3/VEGF tumors (1,620.7 ± 834.0 mm3) compared with CPT-11. Furthermore, NK012 eradicated bulky SBC-3/VEGF tumors in all mice but did not eradicate SBC-3/Neo tumors. In the drug distribution analysis, an increased accumulation of SN-38 in SBC-3/VEGF tumors was observed as compared with that in SBC-3/Neo tumors. NK012 markedly enhanced the antitumor activity of SN-38, especially in highly VEGF-secreting tumors, and could be a promising SN-38-based formulation. (Cancer Res 2006; 66(20): 10048-56)

Introduction

The antitumor plant alkaloid camptothecin (CPT) is a broad-spectrum anticancer agent that targets DNA topoisomerase I. Although CPT has shown promising antitumor activity in vitro and in vivo (1, 2), it has not been clinically used because of its low therapeutic efficacy and severe toxicity (3, 4). Among CPT analogues, irinotecan hydrochloride (CPT-11) has recently been shown to be active against colorectal, lung, and ovarian cancer (5–9). CPT-11 itself is a prodrug and is converted to 7-ethyl-10-hydroxy-CPT (SN-38), a biologically active metabolite of CPT-11, by carboxylesterases. SN-38 exhibits up to 1,000-fold more potent cytotoxic activity against various cancer cells in vitro than CPT-11 (10). Although CPT-11 is converted to SN-38 in the liver and tumor, the metabolic conversion rate is <10% of the original volume of CPT-11 (11, 12). In addition, the conversion of CPT-11 to SN-38 depends on the genetic interindividual variability of carboxylesterase activity (13). Thus, direct use of SN-38 might be of great advantage and attractive for cancer treatment. For the clinical use of SN-38, however, it is essential to develop a soluble form of water-insoluble SN-38. The progress of the manufacturing technology of “micellar nanoparticles” may make it possible to use SN-38 for in vivo experiments and further clinical use.

Passive targeting of drug delivery system is based on the pathophysiologic characteristics that are observed in many solid tumors: hypervascularity, irregular vascular architecture, potential for secretion of vascular permeability factors, and the absence of effective lymphatic drainage that prevents efficient clearance of macromolecules. These characteristics, unique to solid tumors, are believed to be the basis of the enhanced permeability and retention effect (14–17). Supramolecular structures, such as liposomes and polymeric micelles, are expected to increase the accumulation of drugs in tumor tissue through these pathophysiologic features. Polymeric micelle–based anticancer drugs have been developed in recent years (18–20), and some of them have been under evaluation for clinical trials (21–23). This carrier system can incorporate various kinds of drugs into the inner core by chemical conjugation or physical entrapment with relatively high stability, and the size can be controlled within the range of 20 to 100 nm in diameter. This range of diameters is too large to pass through normal vessel walls; therefore, the drug can be expected to reduce side effects due to a decrease in volume of distribution.

Angiogenesis is essential for the growth and metastasis of solid tumors (24). The clinical importance of angiogenesis in human tumors was shown by several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis for survival in patients with various types of cancers (25–28). Furthermore, Natsume et al. (29) reported that the antitumor activities of anticancer agents, including cis-diammine-dichloroplatinum, vincristine, and docetaxel, were less active against vascular endothelial growth factor (VEGF)–secreting cells, SBC-3/VEGF, in vivo as compared with its mock transfectant (SBC-3/Neo), although the high vascularity should have been favorable for the drug delivery.

VEGF is also well known as a potent vascular permeability factor (30). The ability of supramolecular structures to accumulate in target tissue is based on the enhanced tumor angiogenesis and tumor vascular permeability that occur in solid tumors. Therefore, we hypothesized that a polymeric micelle–based drug carrier would increase its accumulation and deliver enhanced therapeutic efficacy in tumors that secrete higher levels of VEGF. In the present study, we present the superiority of NK012 over CPT-11 in a tumor model,
especially in a VEGF-secreting tumor, and we illustrate the outstanding advantage of polymeric micelle–based drug carriers.

Materials and Methods

Drugs and Cells
SN-38 was synthesized by Nippon Kayaku Co., Ltd. (Tokyo, Japan). CPT-11 was purchased from Yakult Honsha Co., Ltd. (Tokyo, Japan). Human colon cancer cell lines WiDr, SW480, Lovo, and HT-29 and human non–small-cell lung cancer cell line A549 were purchased from American Type Culture Collection (Rockville, MD). Human small-cell lung cancer cell line SBC-3 and human non–small-cell lung cancer cell line PC-14 were kindly provided by Dr. I. Kimura (Okayama University, Okayama, Japan) and Dr. Y. Hayata (Tokyo Medical University, Tokyo, Japan), respectively. SBC-3 and PC-14 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 of 5% CO₂ at 37°C. Other cell lines were maintained in DMEM (Nikkos Bio Med. Lab., Kyoto, Japan) supplemented with 10% fetal bovine serum, SBC-3/Neo and SBC-3/VEGF were generated from SBC-3 cells that were transfected with BMG-Neo and BMG-Neo/VEGF as previously reported (29). The full-length sequence of human VEGF expressing 206 amino acids (31) was selected. SBC-3/VEGF cells express 100 times more soluble VEGF than SBC-3/Neo and SBC-3 cells in the supernatant of cultured cells as shown by ELISA (29).

Preparation of an SN-38–Conjugated Poly(Ethylene Glycol)-Poly(Glutamic Acid) Block Copolymer for NK012

Construction
Poly(ethylene glycol)-poly(glutamic acid) block copolymer (PEG-PGlu(SN-38)) was synthesized as follows: A poly(ethylene glycol)-poly(glutamic acid) block copolymer (PEG-PGlu) was prepared according to the previously reported technique (32, 33). SN-38 was covalently introduced into the PGlu segment by the condensation reaction between the carboxylic acid on PGlu and the phenol on SN-38 with 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole (HOBt)-N,N′-diisopropylcarbodiimide and phenoxycarboxylic acid (31). SN-38 was covalently introduced into the PGlu segment by the condensation reaction between the carboxylic acid on PGlu and the phenol on SN-38 with 1,3-diisopropylcarbodiimide and N,N′-diisopropylcarbodiimide and phenoxycarboxylic acid (31). Consequently, the PGlu segment obtained sufficient hydrophobicity. Accordingly, NK012 was constructed with self-assembling PEG-PGlu(SN-38) amphiphilic block copolymers in an aqueous milieu.

Determination of the Size Distribution of NK012 and Drug Release Behavior of SN-38 from NK012

The size distribution of NK012 was measured with the dynamic light scattering method at 25°C using a Particle Sizer NICOMP 380ZLS (Particle Sizing Systems, Santa Barbara, CA). The release behavior of SN-38 from NK012 was investigated in vitro at 20°C or 37°C in PBS (pH 7.3) or 5% glucose solution (pH 4.6). The concentration was 0.1 mg/mL. The amount of SN-38 released from NK012 was estimated by UV measurement at 265 nm.

In vitro Growth Inhibition Assay

The growth inhibitory effects of NK012, SN-38, and CPT-11 were examined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. One hundred microliters of an exponentially growing cell suspension (6 × 10⁴/mL) were seeded into a 96-well microtiter plate, and 20 μL of various concentrations of each drug were added. After incubation for 72 hours at 37°C, 20 μL of MTT solution (5 mg/mL in PBS) were added to each well, and the plates were incubated for an additional 4 hours at 37°C. After centrifuging the plates at 200 × g for 5 minutes, the medium was aspirated from each well, and 180 μL of DMSO were added to each well to dissolve the formazan. The growth inhibitory effect of each drug was assessed spectrophotometrically (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

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 in the National Cancer Center or Nippon Kayaku.

Experiment 1. Female BALB/c nude mice, 7 weeks old, were purchased from CLEA Japan (Tokyo, Japan). Human colorectal cancer HT-29 cells were grown as s.c. tumor in the flank of the mice. The tumors were excised from the mice and fragments were inoculated s.c. in the mouse flank. When the tumor volume reached 70 to 170 mm³, mice were randomly divided into test groups consisting of six mice per group (day 0). Drugs were administered on days 0, 4, and 8 by i.v. injection into the tail vein. NK012 was given at doses of 30 (maximum tolerated dose), 15, and 7.5 mg/kg/d. The reference drug, CPT-11, was given at the maximum tolerated dose, 66.7 mg/kg/d, in the optimal schedule reported (34). Relative tumor volumes at day n were calculated according to the following formula: \( \text{RTV}_n = \frac{V_n}{V_0} \). Relative tumor volumes at day n, where \( V_n \) is the tumor volume at day n, and \( V_0 \) is the tumor volume at day 0. Differences in relative tumor sizes between the treatment groups at day 21 were analyzed with an unpaired \( t \) test.

Experiment 2. As a hypervascular tumor model, we used SBC-3/VEGF cells. SBC-3/Neo or SBC-3/VEGF cells (10⁶) were s.c. injected into the back of mice. NK012 or CPT-11 was administered when the mean tumor volumes \( n = 4 \) reached a mass of 1,500 mm³, which gave tumors almost 1.5 cm in length. It took ~65 days for SBC-3/Neo and 30 days for SBC-3/VEGF to reach the tumor volume of 1,500 mm³ from the day of inoculation. NK012 at a dose of 10 or 20 mg/kg/d and CPT-11 at a dose of 15 or 30 mg/kg/d were administered i.v. on days 0, 4, and 8. Differences in tumor sizes between the treatment groups and control group at day 14 were analyzed with an unpaired \( t \) test.

Histologic and Immunohistochemical Analysis

Histologic sections were taken from SBC-3/Neo and SBC-3/VEGF tumor tissues when the volumes reached 1,500 mm³. After extirpation, tissues were fixed with 3.9% formalin in PBS (pH 7.4), and the subsequent preparations and H&E staining were done by Tokyo Histopathologic Laboratory Co., Ltd. (Tokyo, Japan). For detection of tumor blood vessels, polyclonal anti–von Willebrand factor antibody (Dako, Glostrup, Denmark) was used.

Assay for SN-38 and CPT-11 in Plasma and Tissues

Female BALB/c nude mice bearing HT-29 (as mentioned in experiment 1; \( n = 3 \)) were used for the analysis of the biodistribution of NK012 and CPT-11. NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was administered i.v. to the mice. Under anesthesia, blood and tumor samples were taken at 5 minutes, 1, 6, 24, 48, 72, and 168 hours after administration of NK012 and at 5 minutes, 1, 3, and 24 hours after administration of CPT-11. The blood samples were collected in microtubes and immediately centrifuged at 1,600 × g for 15 minutes. The plasma and tumor samples were stored at −80°C until analysis.

For the biodistribution study in hypervascular tumors (experiment 2), female BALB/c nude mice (\( n = 3 \)) bearing 1,500-mm³ massive SBC-3/Neo and SBC-3/VEGF tumors were used. NK012 (20 mg/kg) and CPT-11 (30 mg/kg) were administered on day 0. The mice were sacrificed at 1, 6, 24, and 72 hours (day 3) after administration. The tumor, liver, spleen, upper small intestine, lung, and blood were taken and stored at −80°C until analysis.

Preparation of the free SN-38 (polymer-unbound SN-38) and CPT-11. Tumor samples were homogenized on ice using a Digital homogenizer (luchi, Osaka, Japan) and suspended in the mixture of 100 mmol/L glucose-HCl buffer (pH 3) in methanol (1:1, v/v) at a concentration of 5% w/w. The concentrations of free SN-38 and CPT-11 in the plasma and tumor from aliquots of the homogenates (100 μL) and plasma (50 μL) were determined by high-performance liquid chromatography. For free SN-38 (polymer-unbound SN-38) and CPT-11, proteins were precipitated with an ice-cold mixture of methanol/H₂O/HIClO₄ (50:45:5, v/v/v) containing CPT as an internal standard. The sample was vortexed for 10 seconds, filtered through a MultiScreen Solvent (Millipore Corp., Bedford, MA), and analyzed.
Preparation of the polymer-bound SN-38 (SN-38 remaining bound to PEG-PGlu). To permit complete release of SN-38 from the conjugate, 20 μL of plasma and 100 μL of tissue samples were diluted with 20 μL of methanol (50%, v/v) and 20 μL of NaOH (0.3 mol/L for plasma and 0.7 mol/L for tissue). The samples were incubated for 15 minutes at 25°C. After incubation, 20 μL of HCl (0.3 mol/L for plasma and 0.7 mol/L for tissue) and 60 μL of internal standard solution were added to the samples, and then the hydrolysis was filtered through a MultiScreen Solvinert. The filtrate was applied to the high-performance liquid chromatography system.

High-performance liquid chromatography. Reversed-phase high-performance liquid chromatography was done at 35°C on a Mightsyl RP-18 GP column 150 × 4.6 mm (Kanto Chemical Co., Inc., Tokyo, Japan). The samples were injected into an Alliance Waters 2795 high-performance liquid chromatography system (Waters, Milford, MA) equipped with a Waters 2475 multi λ fluorescence detector. The detector was set at 365 and 430 nm (excitation and emission, respectively) for CPT-11 and CPT, and at 365 and 540 nm for SN-38. A reversed-phase column was used at 35°C. The mobile phase was a mixture of 100 mmol/L ammonium acetate (pH 4.2) and methanol [119 (v/v) for SN-38 in plasma and tumor, 3:2 (v/v) for CPT-11 in plasma, and 63:37 (v/v) for CPT-11 in tumor]. The flow rate was 1.0 mL/min. Peak data were recorded with a chromatography management system (Empower, Waters). Polymer-bound SN-38 was determined by subtraction of polymer-unbound SN-38 from the total SN-38 of the hydrolysate.

Pharmacokinetic and Statistical Analyses

The concentrations of SN-38 and CPT-11 in plasma and tissue were fitted to a pharmacokinetic model by the nonlinear least-square method using WinNonlin Professional software (version 4.1; Pharsight Corp., Palo Alto, CA). We used a noncompartmental analysis. The pharmacokinetic variables were calculated using the following equations (AUC last was determined to be 108 for polymer-bound and 11.0 for free SN-38 (Table 3)). These results indicate that NK012 can remain in the tumor tissue for a longer period and release free SN-38.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>AUC (μmol*h/L)</th>
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<tbody>
<tr>
<td>0-24</td>
<td>15</td>
</tr>
<tr>
<td>24-48</td>
<td>10</td>
</tr>
<tr>
<td>49-120</td>
<td>5</td>
</tr>
</tbody>
</table>

The terminal half-life (t1/2) was calculated as 0.693/λz (λz is first-order rate constant associated with the terminal portion of the curve).

\[
\text{AUC}_{\text{inf}} = \int_{0}^{\infty} C(t) \, dt \\
T_{1/2} = (\text{terminal half - life}) = 0.693/\lambda z \\
\text{CL}_{\text{tot}} = \text{Dose} / \text{AUC}_{\text{inf}} \\
V_{\text{tot}} = \text{MRT} \times \text{CL}_{\text{tot}} (\text{MRT, mean residence time})
\]

Data were expressed as mean ± SD. Data were analyzed with the Student's t test when the groups showed equal variances (F test) or with Welch's t test when they showed unequal variances (F test). P < 0.05 was regarded as statistically significant. All statistical tests were two sided.

Results

Preparation and characterization of NK012. NK012 is an SN-38-loaded polymeric micelle constructed in an aqueous milieu by the self-assembly of an amphiphilic block copolymers, PEG-PGlu(SN-38). The molecular weight of PEG-PGlu(SN-38) was determined to be ~19,000 (PEG segment, 12,000; SN-38-conjugated PGlu segment, 7,000). NK012 was obtained as a freeze-dried formulation and contained ca. 20% (w/w) of SN-38 (Fig. 1A). The mean particle size of NK012 was 20 nm in diameter with a relatively narrow range (Fig. 1B). The releasing rates of SN-38 from NK012 in PBS at 37°C were 57% and 74% at 24 and 48 hours, respectively, and those in 5% glucose solution at 37°C were 1% and 3% at 24 and 48 hours, respectively (Fig. 1C). SN-38 is loaded by chemical bonding to the block copolymer. The bonding is phenyl ester bond, which is stable under acidic condition and labile under mild alkaline condition. These results indicate that NK012 can release SN-38 under neutral condition even without the presence of a hydrolytic enzyme and is stable in 5% glucose solution. It is suggested that NK012 is stable before administration and starts to release SN-38, the active component, under physiologic conditions after administration.

Cellular sensitivity of non–small-cell lung cancer and colon cancer cells to SN-38, NK012, and CPT-11. The IC50 values of NK012 for the cell lines ranged from 0.009 μmol/L (SBC-3 cells) to 0.16 μmol/L (WiDR cells). The growth inhibitory effects of NK012 are 43- to 340-fold more potent than those of CPT-11, whereas the IC50 values of NK012 were 2.3- to 5.8-fold higher than those of SN-38. NK012 exhibited a higher cytotoxic effect against each cell line as compared with CPT-11 (43- to 340-fold sensitivity). On the other hand, the IC50 values of NK012 were a little higher than those of SN-38, similar to the cytotoxic feature also reported in a previous study about micellar drugs (ref. 23; Table 1).

Antitumor activity and pharmacokinetic analysis of NK012 and CPT-11 using HT-29–bearing nude mice (experiment 1). Potent activity was observed in mice treated with NK012 at doses of 15 and 30 mg/kg (Fig. 2A), although neither CPT-11 at a dose of 66.7 mg/kg/d nor NK012 at a dose of 7.5 mg/kg/d exerted any significant antitumor activity in vivo. Comparison of the relative tumor volume at day 21 revealed significant differences between 15 mg/kg/d NK012 and 66.7 mg/kg/d CPT-11 and between 30 mg/kg/d NK012 and 66.7 mg/kg/d CPT-11 (P < 0.05). Although treatment-related body weight loss was observed in mice treated with each drug, body weight recovered by day 21 (Fig. 2B). These results clearly show the significant in vivo activity of NK012 against HT-29.

After injection of CPT-11, the concentrations of CPT-11 and SN-38 for plasma declined rapidly with time in a log-linear fashion. On the other hand, NK012 (polymer-bound SN-38) exhibited slower clearance (Fig. 3A). The clearance of NK012 in the HT-29 tumor was significantly slower and the concentration of free SN-38 was maintained at >30 ng/g even at 168 hours after injection (Fig. 3B). The pharmacokinetic variables of each drug in the plasma and tumor are depicted in Table 2.

Tumor-to-plasma concentration ratios (Kp) of polymer-bound and free SN-38 increased during the observation period. The highest value of Kp was achieved at 168 hours after administration, 108 for polymer-bound and 11.0 for free SN-38 (Table 3). These results indicate that NK012 can remain in the tumor tissue for a longer period and release free SN-38.

Antitumor activity and the distribution of NK012 and CPT-11 in SBC-3/Neo or SBC-3/VEGF tumors (experiment 2). To determine whether the potent antitumor effect of NK012 is enhanced in the tumors with high vascularity, we used VEGF-secreting cells SBC-3/VEGF. There was no significant difference in the in vitro cytotoxic activity of each drug between SBC-3/Neo and SBC-3/VEGF (Fig. 4A). SBC-3/VEGF tumors are reddish by gross evaluation as compared with SBC-3/Neo tumors (Fig. 4B). Histologic and immunohistochemical (von Willebrand factor) examination revealed that prominent leakage of erythrocytes and high vascularity were observed in SBC-3/VEGF tumor xenografts. On the other hand, SBC-3/Neo tumors have less tumor vasculatures and more interstitial space as compared with SBC-3/VEGF tumors.
Deviating from the ordinary experimental tumor model, tumors were allowed to grow until they became massive in size, ~1.5 cm (Fig. 4C), and then the treatment was initiated. NK012 at doses of 15 and 30 mg/kg showed potent antitumor activity against bulky SBC-3/Neo tumors (1,533.1 ± 1,204.7 mm³) as compared with CPT-11 (Fig. 4C). Striking antitumor activity was observed in mice treated with NK012 (Fig. 4C) when we compared the antitumor activity of NK012 with that of CPT-11 using SBC-3/VEGF cells. SBC-3/VEGF bulky masses (1,620.7 ± 834.0 mm³) disappeared in all mice, although relapse 3 months after treatment was noted in one mouse treated with NK012 20 mg/kg. On the other hand, SBC-3/VEGF were not eradicated and rapidly regrew after a partial response in mice treated with CPT-11. Approximately 10% body weight loss was observed in mice treated with 20 mg/kg NK012, but no significant difference was observed in comparison with mice treated with 30 mg/kg CPT-11.

We then examined the distribution of free SN-38 in the SBC-3/Neo and SBC-3/VEGF masses after administration of NK012 and CPT-11. In the case of CPT-11 administration, the concentrations at 1 and 6 hours after the administration were <100 ng/g both in the SBC-3/Neo and SBC-3/VEGF tumors and were almost negligible at 24 hours in both tumors (Fig. 5A). There was no significant difference in the concentration between the SBC-3/Neo and SBC-3/VEGF tumors. On the other hand, in the case of NK012 administration, free SN-38 was detectable in the tumors even at 72 hours after the administration. The concentrations of free SN-38 were higher in the SBC-3/VEGF tumors than those in the SBC-3/Neo tumors at any time point during the period of observation (significant at 1, 6, and 24 hours; P < 0.05; Fig. 5A).

Tissue distribution of SN-38 after administration of NK012 and CPT-11. We examined the concentration-time profile of free SN-38 in various tissues after i.v. administration of NK012 and

Table 1. In vitro growth inhibitory activity of SN-38, NK012, and CPT-11 in human lung and colorectal cancer cells (MTT assay)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (μmol/L)</th>
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<tbody>
<tr>
<td></td>
<td>SN-38</td>
</tr>
<tr>
<td>WIDR</td>
<td>0.046 ± 0.008</td>
</tr>
<tr>
<td>SW480</td>
<td>0.025 ± 0.003</td>
</tr>
<tr>
<td>Lovo</td>
<td>0.0067 ± 0.0012</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.016 ± 0.003</td>
</tr>
<tr>
<td>PC-14</td>
<td>0.044 ± 0.025</td>
</tr>
<tr>
<td>SBC-3</td>
<td>0.0016 ± 0.001</td>
</tr>
<tr>
<td>A431</td>
<td>0.0081 ± 0.002</td>
</tr>
</tbody>
</table>
CPT-11. All organs measured exhibited the highest concentration of SN-38 at 1 hour after administration in mice given CPT-11 (Fig. 5B). On the other hand, mice given NK012 exhibited prolonged distribution in the liver and spleen (Fig. 5B). In a similar manner to other micellar drugs (19, 23), NK012 showed relatively higher accumulation in organs of the reticuloendothelial system. In the lung, kidney, and small intestine, the highest concentration of free SN-38 was achieved at 1 hour after injection of NK012 and the concentration was almost negligible at 24 hours. Although relatively high at 1 hour after administration of NK012 and CPT-11, the concentrations of free SN-38 in the small intestine rapidly decreased. Interestingly, there was no significant difference in the kinetic character of free SN-38 in the small intestine between mice treated with NK012 and CPT-11.

Discussion

The drug-incorporating polymeric micelle has characteristic pharmacokinetic features. These structures are too large to pass through normal vessel walls and evade renal excretion. The outer shell of the drug with PEG diminishes nonspecific capture by the reticuloendothelial system. Therefore, the drug can be expected to achieve a long half-life, which permits a large amount of the drug-incorporating micelles to reach the tumor site through the enhanced permeability and retention effect. The pharmacokinetic study revealed that the plasma AUC of polymer-bound SN-38 after administration of NK012 at a dose of 30 mg/kg to the HT-29-bearing mice was ~200-fold higher than that of CPT-11 at a dose of 66.7 mg/kg. A 14-fold higher AUC of the free SN-38 was achieved in mice given NK012 compared with mice given CPT-11. Prolonged circulation of NK012 in the blood might increase the accumulation of NK012 in a tumor tissue due to the enhanced permeability and retention effect. In fact, the tumor concentration of free SN-38 at 24 hours after administration of NK012 reached 90.4 ng/g and high concentrations were maintained up to 168 hours (53.1 ng/g for 48 hours, 42.6 ng/g for 72 hours, and 35.8 ng/g for 168 hours). This range of concentrations can exert sufficient antitumor activity against tumor cells. On the other hand, the concentration of CPT-11 was only 4.5 ng/g at 24 hours. These results indicate that the enhancement of tumor distribution closely contributes to the potent antitumor activity of NK012 in vivo.
Several preclinical studies on cytotoxic agent–incorporating polymeric micelles show their advantage as anticancer agents in vivo as compared with drugs of small molecular size (19, 22, 23). Because the advantage of passive targeting has been explained by the enhanced permeability and retention theory, it is essential to elucidate the correlation between the effectiveness of micellar drugs and tumor hypervascularity and hyperpermeability. We hypothesized that a polymeric micelle–based drug carrier could increase its accumulation in the tumor site and could thus enhance the therapeutic efficacy in tumors with high vascularity. To ascertain the hypothesis, we used SBC-3/VEGF. We adopted a bulky tumor model for our in vivo experiment to clarify the difference in activity against SBC-3/Neo and SBC-3/VEGF tumors. Histologic examination of SBC-3/VEGF showed hypervascularity and prominent leakage of erythrocytes. On the other hand, SBC-3/Neo showed hypovascularity. Our in vivo experiment showed that NK012 obviously enhanced its antitumor activity in SBC-3/VEGF-inplanted mice and eradicated bulky masses. It was thought that the sensitivity of cells to NK012 might not change in vivo because the in vitro sensitivity of NK012 was almost equivalent between SBC-3/Neo and SBC-3/VEGF cells. When we compared the distribution of NK012 (free SN-38) in the tumor sites, significantly enhanced accumulation was observed in the SBC-3/VEGF tumors. This strongly suggested that the drug distribution throughout the tumor site was enhanced by the hypervascularity and hyperpermeability induced by VEGF, and, subsequently, higher antitumor activity was achieved. High vascular density and enhanced vascular permeability might also be favorable for drug delivery of low molecular weight drugs. However, the SN-38 concentration was not significantly high in SBC-3/VEGF tumors after the administration of CPT-11, and tumors exhibited rapid regrowth after the treatment. We assume that such conventional low molecular size anticancer agents almost disappear from the bloodstream without being subjected to the enhanced permeability and retention effect before they can reach the target organs (solid tumor). The fact of correlation between the blood vessel density in

<table>
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<th>Test article</th>
<th>Chmax (μg/mL)</th>
<th>Tmax (h)</th>
<th>T12/2 (h)</th>
<th>AUClast (μg h/mL)</th>
<th>AUCinf (μg h/mL)</th>
<th>Cmax (mL/h/kg)</th>
<th>Vss (mL/kg)</th>
<th>MRTlast (h)</th>
<th>MRTinf (h)</th>
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<tr>
<td>Plasma NK012 P-b SN-38</td>
<td>—</td>
<td>—</td>
<td>31.4</td>
<td>5,000</td>
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<td>Tumor NK012 P-b SN-38</td>
<td>13.8</td>
<td>6</td>
<td>—</td>
<td>1,010</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>62.8</td>
<td>—</td>
</tr>
<tr>
<td>P-u SN-38</td>
<td>0.188</td>
<td>6</td>
<td>—</td>
<td>10.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>58.1</td>
<td>—</td>
</tr>
<tr>
<td>CPT-11</td>
<td>12.6</td>
<td>3</td>
<td>3.36</td>
<td>99.7</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>4.41</td>
<td>4.55</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.108</td>
<td>1</td>
<td>4.75</td>
<td>1.07</td>
<td>1.10</td>
<td>—</td>
<td>—</td>
<td>5.20</td>
<td>5.92</td>
</tr>
</tbody>
</table>

NOTE: Three female nude mice were used for the analysis of biodistribution of SN-38 and CPT-11 in plasma and tissues. Data were expressed as means.
*Polymer-bound SN-38; SN-38 remaining bound to PEG-PGlu.
†Not determined.
‡Polymer-unbound SN-38; free SN-38 from PEG-PGlu.

Several preclinical studies on cytotoxic agent–incorporating polymeric micelles show their advantage as anticancer agents in vivo as compared with drugs of small molecular size (19, 22, 23). Because the advantage of passive targeting has been explained by the enhanced permeability and retention theory, it is essential to elucidate the correlation between the effectiveness of micellar drugs and tumor hypervascularity and hyperpermeability. We hypothesized that a polymeric micelle–based drug carrier could increase its accumulation in the tumor site and could thus enhance the therapeutic efficacy in tumors with high vascularity. To ascertain the hypothesis, we used SBC-3/VEGF. We adopted a bulky tumor model for our in vivo experiment to clarify the difference in activity against SBC-3/Neo and SBC-3/VEGF tumors. Histologic examination of SBC-3/VEGF showed hypervascularity and prominent leakage of erythrocytes. On the other hand, SBC-3/Neo showed hypovascularity. Our in vivo experiment showed that NK012 obviously enhanced its antitumor activity in SBC-3/VEGF-inplanted mice and eradicated bulky masses. It was thought that the sensitivity of cells to NK012 might not change in vivo because the in vitro sensitivity of NK012 was almost equivalent between SBC-3/Neo and SBC-3/VEGF cells. When we compared the distribution of NK012 (free SN-38) in the tumor sites, significantly enhanced accumulation was observed in the SBC-3/VEGF tumors. This strongly suggested that the drug distribution throughout the tumor site was enhanced by the hypervascularity and hyperpermeability induced by VEGF, and, subsequently, higher antitumor activity was achieved. High vascular density and enhanced vascular permeability might also be favorable for drug delivery of low molecular weight drugs. However, the SN-38 concentration was not significantly high in SBC-3/VEGF tumors after the administration of CPT-11, and tumors exhibited rapid regrowth after the treatment. We assume that such conventional low molecular size anticancer agents almost disappear from the bloodstream without being subjected to the enhanced permeability and retention effect before they can reach the target organs (solid tumor). The fact of correlation between the blood vessel density in

Table 2. Pharmacokinetic variables of analytes in plasma and tumor after an i.v. administration of NK012 or CPT-11 to nude mice bearing human colon cancer HT-29 cells (NK012, 30 mg/kg; CPT-11, 66.7 mg/kg)

<table>
<thead>
<tr>
<th>Test article</th>
<th>Analyte</th>
<th>Time after administration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK012 P-b SN-38</td>
<td>Plasma (μg/mL)</td>
<td>0.0833</td>
</tr>
<tr>
<td>NK012 P-b SN-38</td>
<td>Tumor (μg/g)</td>
<td>4.99</td>
</tr>
<tr>
<td>NK012 P-b SN-38</td>
<td>Kp (mL/g)</td>
<td>0.00815</td>
</tr>
<tr>
<td>NK012 P-u SN-38</td>
<td>Plasma (μg/mL)</td>
<td>3.10</td>
</tr>
<tr>
<td>NK012 P-u SN-38</td>
<td>Tumor (μg/g)</td>
<td>0.0763</td>
</tr>
<tr>
<td>NK012 P-u SN-38</td>
<td>Kp (mL/g)</td>
<td>0.0246</td>
</tr>
</tbody>
</table>

NOTE: Data were expressed as means of three mice.
*Polymer-bound SN-38; SN-38 remaining bound to PEG-PGlu.
†Kp values were calculated on the mean concentrations of three mice.
‡Polymer-unbound SN-38; free SN-38 from PEG-PGlu.

Table 3. Tumor-to-plasma concentration ratio (Kp) of analytes after an i.v. administration of NK012 (30 mg/kg) to nude mice bearing human colon cancer HT-29 cells

<table>
<thead>
<tr>
<th>Test article</th>
<th>Analyte</th>
<th>Time after administration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK012 P-b SN-38</td>
<td>Plasma (μg/mL)</td>
<td>612</td>
</tr>
<tr>
<td>NK012 P-b SN-38</td>
<td>Tumor (μg/g)</td>
<td>4.99</td>
</tr>
<tr>
<td>NK012 P-b SN-38</td>
<td>Kp (mL/g)</td>
<td>0.00815</td>
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<tr>
<td>NK012 P-u SN-38</td>
<td>Plasma (μg/mL)</td>
<td>3.10</td>
</tr>
<tr>
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<td>Tumor (μg/g)</td>
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</tr>
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<td>NK012 P-u SN-38</td>
<td>Kp (mL/g)</td>
<td>0.0246</td>
</tr>
</tbody>
</table>

NOTE: Data were expressed as means of three mice.
*Polymer-bound SN-38; SN-38 remaining bound to PEG-PGlu.
†Kp values were calculated on the mean concentrations of three mice.
‡Polymer-unbound SN-38; free SN-38 from PEG-PGlu.
Figure 4. Growth inhibitory effect of NK012, SN-38, and CPT-11 on SBC-3/Neo and SBC-3/VEGF cells. A, in vitro experiment, the cells were exposed to the indicated concentrations of each drug for 72 hours. The growth inhibition curves and IC50 values for NK012 (△), SN-38 (●), and CPT-11 (■) are shown. B, representative photographs of massive tumors developed from SBC-3/Neo and SBC-3/VEGF at the time just before treatment initiation. Histologic (H&E, ×20) and immunohistochemical (von Willebrand factor, ×20) examinations for each tumor are shown. C, i.v. administration of NK012 or CPT-11 was started when the mean tumor volumes of groups reached a massive size of 1,500 mm3. The mice were divided into test groups (○, control; □, CPT-11 15 mg/kg/d; ○, CPT-11 30 mg/kg/d; △, NK012 10 mg/kg/d; ●, NK012 20 mg/kg/d). NK012 or CPT-11 was administered i.v. on days 0, 4, and 8. Each group consisted of four mice. *, P < 0.05.
the tumor mass and poor prognosis for survival in people with various types of cancers (25–28) supports the idea that low molecular weight drugs are not so effective in the treatment of solid tumors, which are rich in blood vessels.

Jain (35) reported that the convective passage of large drug molecules into the core of solid tumors could be impeded by abnormally high interstitial pressures in solid tumors. However, he also considered that low molecular weight anticancer agents might be harmful to normal organs because they can leak out of normal blood vessels freely; he finally concluded that one useful strategy for evading the barriers to drug dispersion would be to inject patients with drug carriers, such as liposome, filled with low molecular weight drugs. NK012 has the potential to allow the effective sustained release of SN-38 inside a tumor following the accumulation of NK012 into tumor tissue. As a matter of fact, substantial amount of SN-38 is expected to be released from the polymeric micelle. Consequently, released SN-38 becomes distributed throughout the tumor tissue and internalizes into cancer cells to kill them.

In recent years, the novel liposome-based formulation of SN-38 (LE-SN38) has been developed (36). LE-SN38 shows promising antitumor activity against various cancer cell lines (37, 38) and a clinical trial to assess its efficacy is now under way (39). The release of SN-38 from LE-SN38 is very slow as compared with NK012, ~1.9% of the drug being released from LE-SN38 in PBS buffer over 120 hours (36). The size of LE-N38 ranges from 150 to 200 nm. On the other hand, the particle size of NK012 is ~20 nm. Interestingly, Unezaki et al. (40) reported that fluorescence-labeled PEG liposomes were densely located outside the tumor vessels and stayed around the vessel walls for 2 days after i.v. injection. These data suggest that the PEG liposome is too large to move freely in the tumor interstitium and too stable to be released easily. The difference in size distribution and the character of the drug release between NK012 and LE-SN38 might influence their clinical effectiveness in the treatment of solid tumors.

One of the major toxicities associated with CPT-11 administration is severe diarrhea. Although the mechanism of the diarrhea has not yet been elucidated, one possible explanation is structural and functional injuries to the gastrointestinal tract owing to the mitotic inhibitory activity of SN-38 and CPT-11. It was reported that the number of episodes of diarrhea had a better correlation with the plasma AUC of SN-38 than with CPT-11 (41). In the present study, no difference in SN-38 accumulations in the small intestine was seen when equimolar NK012 (20 mg/kg) and CPT-11 (30 mg/kg) were administered. We also reported, using a rat mammary tumor model, that NK012 showed significant antitumor effect with diminishing incidence of diarrhea as compared with CPT-11 (42). These results suggest that diarrhea, one of the dose-limiting toxicities of CPT-11, is not augmented by the administration of NK012.

In conclusion, the present data suggest that NK012 possesses a treatment advantage over CPT-11, especially in hypervascular tumors such as renal cell carcinomas, medulloblastomas, and hepatocellular carcinomas. We have now started a phase I clinical trial for NK012 in patients with advanced solid tumors.

Acknowledgments

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References

Novel SN-38—Incorporating Polymeric Micelles, NK012, Eradicate Vascular Endothelial Growth Factor—Secreting Bulky Tumors

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