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Antitumor Effect of SN-38–Releasing Polymeric Micelles, NK012, on Spontaneous Peritoneal Metastases from Orthotopic Gastric Cancer in Mice Compared with Irinotecan

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Abstract

7-Ethyl-10-hydroxy-camptothecin (SN-38), an active metabolite of irinotecan hydrochloride (CPT-11), has potent antitumor activity. Moreover, we have reported the strong antitumor activity of NK012 (i.e., SN-38–releasing polymeric micelles) against human cancer xenografts compared with CPT-11. Here, we investigated the advantages of NK012 over CPT-11 treatment in mouse models of gastric cancer with peritoneal dissemination. NK012 or CPT-11 was i.v. administered thrice every 4 days at their respective maximum tolerable doses (NK012, 30 mg/kg/day; CPT-11, 67 mg/kg/day) to mice receiving orthotopic transplants of gastric cancer cell lines (44As3Luc and 58As1mLuc) transfected with the luciferase gene (n = 5). Antitumor effect was evaluated using the photon counting technique. SN-38 concentration in gastric tumors and peritoneal nodules was examined by high-performance liquid chromatography (HPLC) 1, 24, and 72 hours after each drug injection. NK012 or CPT-11 distribution in these tumors was evaluated using a fluorescence microscope on the same schedule. In both models, the antitumor activity of NK012 was superior to that of CPT-11. High concentrations of SN-38 released from NK012 were detected in gastric tumors and peritoneal nodules up to 72 hours by HPLC. Only a slight conversion from CPT-11 to SN-38 was observed from 1 to 24 hours. Fluorescence originating from NK012 was detected up to 72 hours, whereas that from CPT-11 disappeared until 24 hours. NK012 also showed antitumor activity against peritoneal nodules. Thus, NK012 showing enhanced distribution with prolonged SN-38 release may be ideal for cancer treatment because the antitumor activity of SN-38 is time dependent. [Cancer Res 2008;68(22):9318–22]

Introduction

Gastric cancer is the second most common cause of death from cancer in the world. The survival rate has remained low in patients with advanced gastric cancer, with a median survival rate of 13 months having been recently reported in a phase III trial, which has been the best outcome thus far (1). Patients with gastric cancer with scirrhous type stroma particularly showed poor prognosis even after curative resection, as well as highly progressed peritoneal dissemination (2). Because peritoneal dissemination causes several refractory symptoms such as massive ascites, intestinal obstruction, hydronephrosis, and obstructive jaundice, the quality of life of patients at the end stage of cancer is severely impaired.

Poor delivery of anticancer drugs to peritoneal metastatic cells may be one of the reasons for the poor prognosis of patients with peritoneal dissemination (3). In peritoneal nodules, the distribution and eventual diffusion of drugs to cancer cells tend to be impeded because of several obstacles such as severe fibrosis and high interstitial pressure (4, 5). On the other hand, angiogenesis was reported to be an essential factor in the development of peritoneal metastasis, and the high expression level of vascular endothelial growth factor (VEGF) in primary gastric tumors or ascitic fluid, which can enhance tumor vascular permeability, was found to be directly associated with the development of ascites and peritoneal dissemination (6–10). In addition, several factors such as kinins and nitric oxide are involved in tumor vascular permeability (11–13). Polymer-conjugated drugs and nanoparticles categorized under drug delivery system agents are favorably extravasated from the vessels into the interstitium of tumors due to the enhanced permeability and retention effect (EPR effect; refs. 14, 15). The EPR effect is based on the following pathophysiologic characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Moreover, macromolecules cannot freely leak out from normal vessels, and thus, the side effects of an anticancer agent can be reduced. Very recently, we have shown that NK012 (i.e., SN-38–releasing polymeric micelles) exerted superior antitumor activity and less toxicity than CPT-11 (15–17). In a series of studies, we showed that NK012 markedly enhanced the antitumor activity of SN-38, particularly against highly VEGF-secreting SBC-3/VEGF tumors compared with SBC-3/Neo tumors. On the other hand, it is conceivable that satisfactory drug delivery cannot be achieved in less-vascularized and highly fibrotic tumors, particularly for macromolecules. However, we observed that NK012 showed a strong antitumor activity even in the xenograft of Capan1 cells, which are pancreatic cancer cells with abundant stromal tissue, compared with CPT-11. This result suggests that NK012 can selectively accumulate in both hypervascular and hypovascular tumors with high interstitial pressure, and then induce sustained

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release of SN-38, followed by SN-38 distribution throughout the entire tumor tissues. In the present study, we evaluated the antitumor activity of NK012 against peritoneal tumor dissemination compared with that of CPT-11 using mouse models orthotopically transplanted with scirrhous gastric cancer cells, as well as against spontaneously progressing peritoneal dissemination (18, 19).

Materials and Methods

Cell cultures. 44As3 and 58As1m were previously reported as human signet-ring cell gastric cancer cell lines that spontaneously metastasize to the peritoneal cavity and produce large volumes of bloody ascites after orthotopic implantion in the gastric wall (18–21). Here, 44As3 and 58As1m cells were transfected with a complex of 4 μg of pEGF-PLuc plasmid DNA (Clontech) and 24 μL of GeneJammer reagent (Stratagene; Cloning Systems) in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (400 μg/mL; Invitrogen), and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system (Xenogen). Clones expressing the luciferase gene were named 44As3Luc and 58As1mLuc. 44As3Luc and 58As1mLuc cells were maintained in RPMI 1640 supplemented with 10% FCS (Sigma), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Immuno-Biological Laboratories) in a humidified atmosphere containing 5% CO_2 at 37°C.

Orthotopic models *in vivo*. Six-week-old female BALB/c *nu/nu* mice were purchased from CLEA Japan, Inc., and maintained under specific pathogen-free conditions and provided with sterile food, water, and cages. Ambient light was controlled to provide regular cycles of 12 h of light and 12 h of darkness. A total of 1×10^6 cells of 44As3Luc or 58As1Luc were inoculated into the gastric wall of each mouse after laparotomy, as described previously (18–21). *In vivo* photon counting analysis was conducted on a cryogenically cooled IVIS system using Living Image acquisition and analysis software (Xenogen). 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 conform to the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Drugs. NK012 was prepared by Nippon Kayaku Co., Ltd. (15). CPT-11 was purchased from Yakult Honsha Co., Ltd.

***In vivo* growth inhibition assay.** After inoculation of 44As3Luc or 58As1mLuc cells into the gastric wall (day 0), mice were randomly divided into test groups consisting of 5 mice per group. 44As3Luc mice were i.v. administered the maximum tolerated dose (MTD) of the 2 drugs via the tail vein on days 20, 24, and 28 as previously reported, that is, at 66.7 mg/kg/d for CPT-11 and 30 mg/kg/d for NK012 (15). 58As1mLuc mice were given the drugs in the same manner on days 18, 22, and 26. Photon counting analysis and body weight were measured twice a week. "Visible ascites," which was evident a few days before death in this mouse model, was used as a surrogate for survival time in consideration of animal welfare. Mice were euthanized when ascites became visible, and colonization of gastric wall by cancer cells and metastasis to the peritoneal cavity were confirmed in all the euthanized mice. Differences in relative photon counts between the treatment groups at day 42 in 44As3Luc mice and at day 81 in 58As1mLuc mice were analyzed using the unpaired *t* test.

Assay of free SN-38 in tissues. We next analyzed the biodistributions of NK012 and CPT-11 to orthotopic gastric tumors and peritoneal nodules. Twenty-six days after the inoculation of 44As3Luc cells into the gastric wall of mice, NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was administered via the tail vein. Under anesthesia, orthotopic gastric tumor and peritoneal nodule samples were excised 1, 24, and 72 hours after injection.

Measurements of tissue concentration of free SN-38 by high-performance liquid chromatography. Samples were rinsed with physiologic saline, mixed with 0.1 mol/L glycine-HCl buffer (pH 3.0)/methanol at 5 w/w%, and then homogenized. To analyze the concentration of free SN-38, 100 μL of the tumor samples were mixed with 20 μL of 1 mmol/L phosphoric acid/methanol (1:1) and 40 μL of ultrapure water, and camptothecin (CPT) was used as the internal standard (10 ng/mL for free SN-38). The samples were vortexed vigorously for 10 s, and then filtered

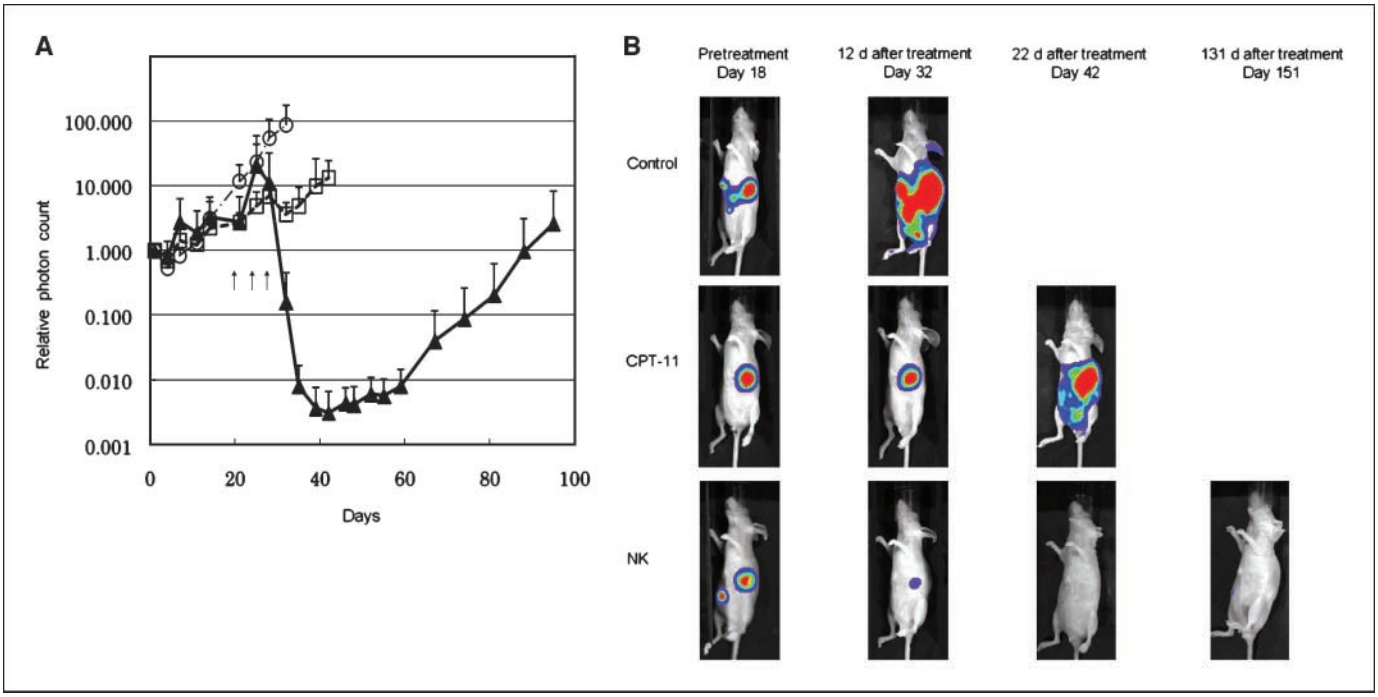

Figure 1. Effects of NK012 and CPT-11 in 44As3Luc mouse models. *A,* antitumor activity of NK012 or CPT-11 was evaluated by counting the number of photons using the IVIS system (*points,* mean; *bars,* SD; *arrows,* drug injections). Antitumor effect of each regimen on days 20, 24, and 28. (○), control; (□), CPT-11 (66.7 mg/kg/d, ×3); and (▲), NK012 (30 mg/kg/d, ×3) in 44As3Luc mouse model. *B,* images of 44As3Luc mouse model administered NK012 taken using the IVIS system on days 18, 32, 42, and 151 after inoculation of 44As3Luc cells. Data were derived from the same mice as those used in the present study.

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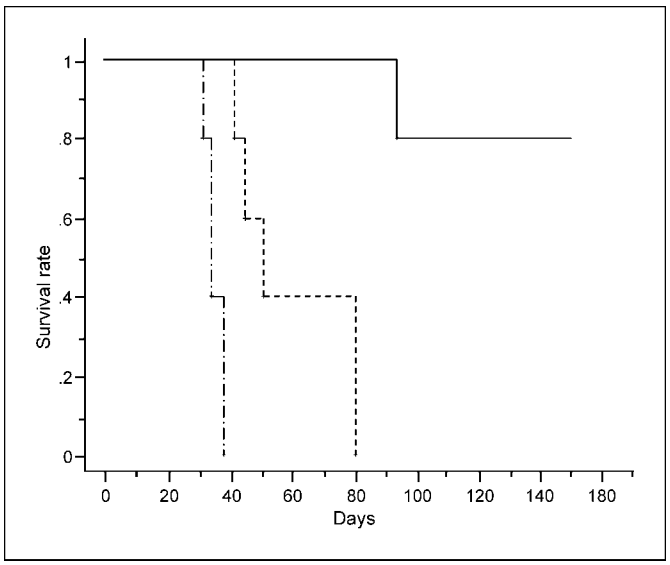

Figure 2. Survival curves of 44As3Luc mouse models. Survival curves of 44As3Luc mouse model in each regimen on days 20, 24, and 28. (–·–·–), control; (– – –), CPT-11 given at 66.7 mg/kg/d ×3; and (——), NK012 given at 30 mg/kg/d ×3.

through Ultrafree-MC Centrifugal Filter Devices with a cutoff molecular diameter of 0.45 μm (Millipore Co.). Reversed-phase high-performance liquid chromatography (HPLC) was conducted at 35°C on a Mightysil RP-18 GP column (150 × 4.6 mm; Kanto Chemical Co., Inc.). Fifty microliters of a sample were injected into an Alliance Water 2795 HPLC system (Waters) 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. The mobile phase was a mixture of 100 mmol/l ammonium acetate (pH 4.2) and methanol [11:9 (v/v)], and the flow rate was 1.0 mL/min. The content of SN-38 was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the CPT internal standard. Peak data were recorded using a chromatography management system (MassLynx v4.0; Waters).

Visualization of distribution of NK012 and CPT-11 by fluorescence microscopy. Mice were given fluorescein *Lycopersicon esculentum* lectin (100 μL per mouse; Vector Laboratories) to visualize tumor vasculature in the samples 5 min before anesthesia. The samples were then excised and embedded in an optimal cutting temperature compound (Sakura Finetechnochemical Co., Ltd.) and frozen at −80°C. Six-μm-thick tumor sections were then prepared using a cryostatic microtome, Tissue-Tek Cryo3 (Sakura Finetechnochemical Co., Ltd.). Frozen sections were examined under a fluorescence microscope, BIOZERO (KEYENCE), at an excitation wavelength of 377 nm and an emission wavelength 447 nm to evaluate the distribution of NK012 and CPT-11. Both drugs could be detected under the same fluorescence conditions because formulations containing SN-38 bound via ester bonds possess a particular fluorescence.

Statistical analyses. Data were expressed as mean ± SD. Data were analyzed using the Student's *t* test when groups showed equal variances (*F* test), or the Welch's test when they showed unequal variances (*F* test). *P* value of <0.05 was considered as significant. All statistical tests were two sided.

Results

Antitumor activities of NK012 and CPT-11. Comparison of the relative photon counts on day 42 in the 44As3Luc mouse model revealed significant differences in counts between mice given with NK012 and those given with CPT-11 ($P = 0.0282$; Fig. 1*A* and *B*). Similar result was obtained in the experiment with 58As gastric tumor (data not shown). The survival rates on day 150 in the 44As3Luc mouse model were 80% and 0% for the NK012 group and CPT-11 group, respectively (Fig. 2). Similar result was obtained in the experiment with 58As gastric tumor (data not shown). No marked toxic effects in terms of body weight changes were observed in any groups for any mouse models (data not shown). Only 1 mouse in the CPT-11 group of 44As1 mouse models showed diarrhea for 3 d, and any other clinical symptoms were not observed.

Tissue concentrations of free SN-38 after administration of NK012 and CPT-11. We examined the concentration-time profile of free SN-38 in orthotopic gastric tumors and peritoneal nodules in the 44As3Luc mouse model after the administration of NK012 and CPT-11 (Fig. 3*A* and *B*). Either orthotopic gastric tumors or peritoneal nodules exhibited the highest concentration of free SN-38 24 hours after NK012 administration, and 1 hour after CPT-11 administration. The highest concentrations of free SN-38 in the NK012 group were much higher than those in the CPT-11 group in either orthotopic gastric tumors or peritoneal nodules. The concentrations of free SN-38 released from NK012 in orthotopic gastric tumors were higher than those in peritoneal nodules.

Tumor tissue distribution of NK012 and CPT-11 as determined by fluorescence microscopy. Results showed that NK012 accumulation in either orthotopic gastric tumors or peritoneal nodules had been maintained from 1 hour to 72 hours after injection (Fig. 4*A*). On the other hand, CPT-11 showed maximum accumulation in either orthotopic gastric tumors or peritoneal nodules 1 hour after injection and disappeared within 24 hours (Fig. 4*B*).

Discussion

The main purpose of this study was to clarify the advantages of NK012 over CPT-11 as treatment against peritoneal metastasis spontaneously disseminated from orthotopically transplanted scirrhous gastric cancer cells in mouse models. We showed that NK012 exerted more potent antitumor activity in the mouse models used than CPT-11. Therefore, NK012 is considered promising in terms of providing clinical benefit to patients with gastric cancer showing progressing peritoneal dissemination.

CPT-11 is converted to SN-38, a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases (CE) in the liver and tumors. However, only 2% to 8% of administered CPT-11 is converted by CE in the liver and tumors to the active form SN-38 (22, 23). The conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of CE (24). Thus, the direct use of SN-38 might be of great advantage and is attractive for cancer treatment. We have recently shown that NK012 (i.e., SN-38–releasing polymeric micelles) exerted superior antitumor activity and less toxicity than CPT-11 (15–17). The mean particle size of NK012 is 20 nm in diameter. NK012 can release SN-38 under neutral conditions even in the absence of CE because SN-38, which is bound to the blockcopolymer by phenolic ester binding, is stable under acidic conditions but relatively labile under neutral and mild alkaline conditions. The release rate of SN-38 from NK012 under physiologic conditions is quite high, that is, >70% of SN-38 is gradually released within 48 hours.

In this study, we used mouse models with orthotopically transplanted human scirrhous gastric cancer cells showing spontaneously progressing peritoneal dissemination, which we

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reported previously (18, 19, 21). These models can imitate more realistically the progressing mode of human peritoneal dissemination of gastric cancer than conventional experimental models directly transplanted with cancer cells i.p. Moreover, our models enabled us to quantitatively evaluate drug antitumor effect even against peritoneal dissemination without having to sacrifice the animal and perform autopsy through the use of gastric cancer cells transfected with the luciferase gene and by applying photon counting analysis, having already verified the significant correlation between tumor volume and photon counts in a previous report (19).

For *in vivo* growth inhibition assay, drug administration was started on day 18 or 20 after cell inoculation into the gastric wall, when small peritoneal metastatic nodules and a small degree of ascites had appeared. The present results showed that NK012 had more potent antitumor activity than CPT-11 in the mouse models tested, suggesting its effectiveness against peritoneal dissemination of gastric cancer in the clinical setting.

In the pharmacologic evaluation, we could confirm the more enhanced distribution of NK012 than CPT-11 to not only orthotopic gastric tumors but also peritoneal nodules by quantifying SN-38 concentration in the tumors and visualization of fluorescence originating from NK012 or CPT-11 distributed in the tumors. Because CPT-11 or SN-38 has been reported to possess time-dependent growth-inhibitory activity against tumor cells, this prolonged retention of NK012 in the tumors and the sustained release of free SN-38 from NK012 may be responsible for its more potent antitumor activity observed in the present study (25). On the other hand, CPT-11 disappeared from the tumors before exerting sufficient antitumor activity. For both drugs, however, the concentrations of SN-38 in orthotopic gastric tumors were higher than those

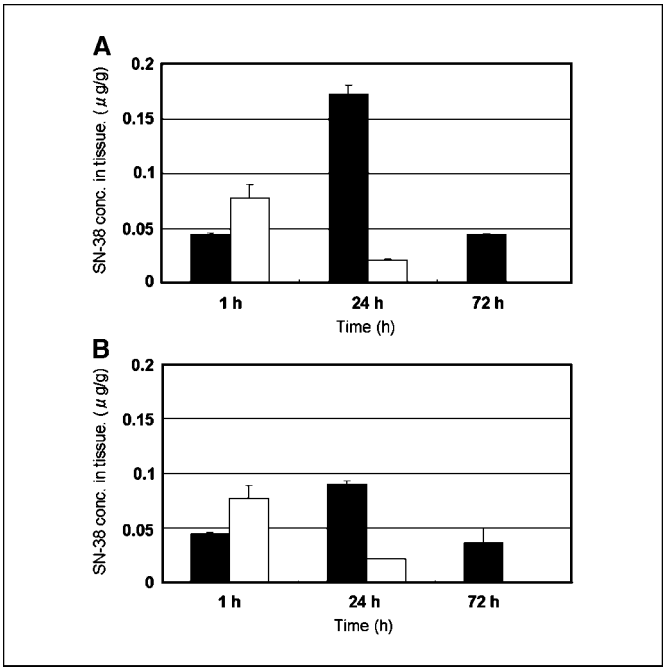

Figure 3. Concentration-time profile of free SN-38. NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was injected 26 d after implantation of 44As3Luc gastric cancer cells (*columns,* mean; *bars,* SD). *A,* concentration (*conc.*) of free SN-38 in orthotopic gastric tumor tissue of 44As3Luc mouse model after administration of NK012 (*black column*) and CPT-11 (*white column*). *B,* concentration of free SN-38 in peritoneal nodules of 44As3Luc mouse model after administration of NK012 (*black column*) and CPT-11 (*white column*).

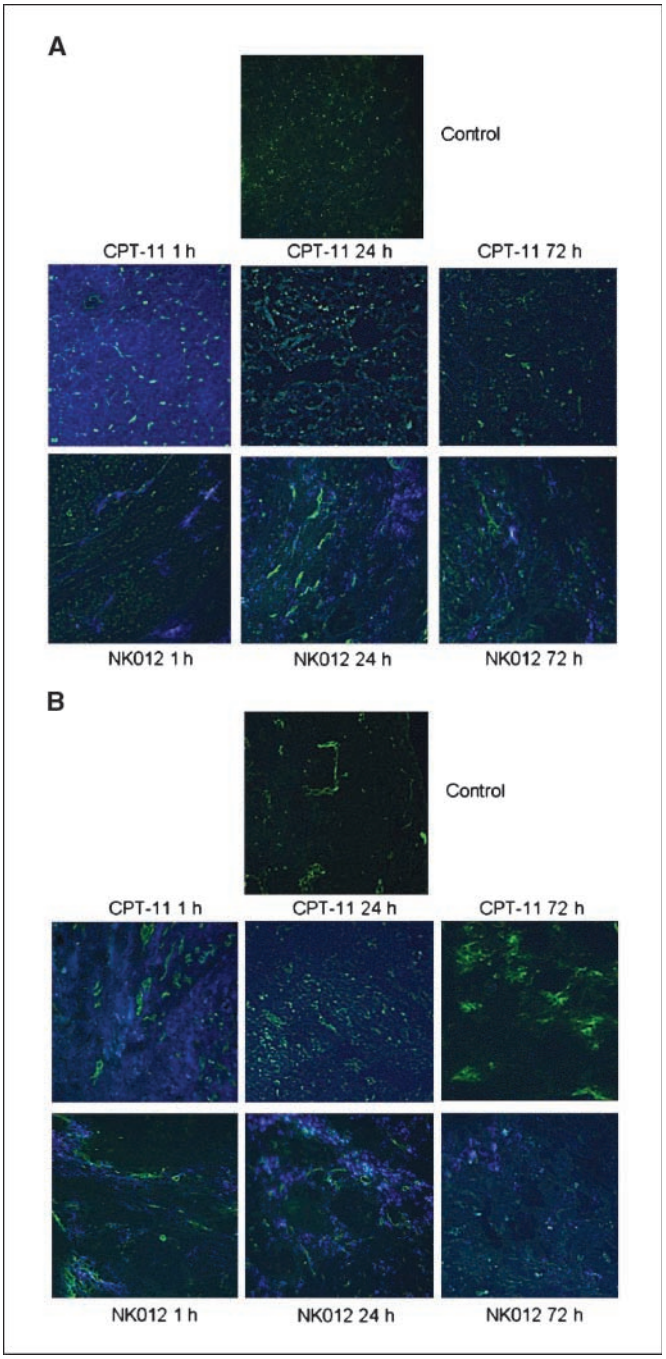

Figure 4. Tissue distribution of NK012 and CPT-11 as determined by fluorescence microscopy. Orthotopic gastric tumors or peritoneal nodules of 44As3Luc mouse model were excised 1, 24, and 72 h after i.v. injection of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg). Each mouse was i.v. administered with fluorescein-labeled *Lycopersicon esculentum* lectin just before being sacrificed to detect tumor blood vessels. Frozen sections were examined under a fluorescence microscope at an excitation wavelength of 377 nm and an emission wavelength of 447 nm. The same fluorescence condition can be applied for visualizing NK012 and CPT-11 fluorescence. Free SN-38 cannot be detected under this fluorescence condition. *A,* distribution of NK012 or CPT-11 in orthotopic gastric tumors (×100). *B,* distribution of NK012 or CPT-11 in peritoneal nodules (×100).

in peritoneal nodules. This is consistent with previous reports stating the poor delivery of anticancer drugs to peritoneal metastatic cells probably because of some obstacles such as abundant interstitium or high interstitial pressure. To date, we reported that NK012 can

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more selectively accumulate and retain longer in various tumor xenografts transplanted s.c. compared with CPT-11 (15–17). In the present study, we succeeded in demonstrating higher accumulation and longer retention of NK012 compared with CPT-11 in orthotopic and peritoneal disseminated gastric cancer model that is closer to human gastric cancer in clinics.

Peritoneal dissemination sometimes causes intestinal obstruction, which enhances the enterohepatic circulation of SN-38 after direct damage to the small intestine, and makes the use of CPT-11 difficult (26, 27). In the present study, no mouse in the NK012 group developed diarrhea. The dose-limiting toxic effects of CPT-11 seem to be neutropenia and diarrhea. In our previous data, however, there was no significant difference in the level of SN-38 in the small intestine between mice treated with NK012 and mice treated with CPT-11 despite the higher plasma area under the concentration of NK012 than CPT-11 (15). Moreover, no serious diarrhea has been reported even at the MTD dose in two phase I clinical trials against advanced solid tumors in Japan and the US (28, 29).

In conclusion, we showed that NK012 exerts significantly more potent antitumor activity against peritoneal dissemination of scirrhous gastric cancer cells than CPT-11, indicating the possibility of the clinical evaluation of this drug in patients with disseminated grastic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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