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. ©2010 AACR.

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 topoisoisomerase 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.
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% CO2 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 × 105) 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 H2O. A median laparotomy was done following disinfection of the abdominal skin. After mobilization of the duodenum, \(2 \times 10^6\) 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 (BioSpace), 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 mouse 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 × 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
metastatic liver tumor, normal liver parenchyma, kidney, and spleen were excised under anesthesia. The analysis time points were 2, 12, 24, 72, 168, 336, 504, 672, and 1,008 hours after NK012 or CPT-11 administration. Pharmacokinetic analysis was conducted using three mice for each time point. The samples were rinsed sufficiently with 0.9% NaCl solution, mixed with 0.1 mol/L glycine-HCl buffer (pH 3.0)/methanol at 5% (w/w), and then homogenized using Precellys 24 (Bertin Technologies). Free SN-38, NK012, and CPT-11 were extracted from each sample, and the extracted sample was analyzed by reversed-phase high-performance liquid chromatography as described previously (17, 19).

Histopathologic analysis
For conventional histopathologic analysis, the liver with a metastatic tumor was excised from the mice bearing liver metastases as described above. The liver was excised after 15 days from the initiation of each treatment and fixed in buffered 4% paraformaldehyde for 72 hours and embedded in paraffin. Then, 3-μm-thick sections were prepared and stained with H&E.

Biodistribution of NK012 as determined by immunohistochemistry and immunofluorescence microscopy
To evaluate the detailed biodistribution of NK012 in immunohistologic sections, we used 20-nm-sized fixable fluorospheres that have equal diameter to NK012 (FluoSpheres, red fluorescent latex microspheres; Molecular Probes) as a reference of positional relationship within the tissue. Because NK012 could neither be fixed nor stained with other antibodies, we applied this fluorescent-labeled macro-molecular substance whose surface is carboxylate modified to decrease nonspecific binding. Five minutes after NK012 administration (30 mg/kg) to mice with liver metastases, fluorospheres (0.25 ml/animal; concentration, 2.5 mg/ml) were also injected i.v. The mice were sacrificed under deep anesthesia and perfused with 0.9% NaCl through the inferior vena cava to prevent blood stasis, and then the liver with metastases was excised 2 hours and 1, 3, and 7 days from the administration of NK012 and fluorospheres. The samples were embedded in an OCT compound (Sakura Finetechnochemical) and quickly frozen in liquid nitrogen.

For direct observation of the fluorescence of NK012 and fluorospheres, 10-μm-thick frozen sections were prepared using a cryostat and examined under a fluorescence microscope (BIOREVO BZ9000; Keyence) at an excitation wavelength of 377 nm and an emission wavelength of 447 nm to evaluate NK012 distribution. For immunohistchemical analysis, frozen sections were prepared as described earlier and fixed in 4% paraformaldehyde in PBS (pH 7.4). After blocking, sections were incubated for 1 hour at room temperature with primary antibodies. Anti-CD31 goat antibody for endothelial cells (R&D Systems) was used at 1:200 dilution, and anti-CD68 rat antibody for macrophages (Kupffer cells; AbD Serotec) was used at 1:200 dilution. The sections were then incubated with the following secondary antibodies at 1:500 dilution: Alexa 647 donkey anti-goat IgG, Alexa 488 goat anti-rat IgG, or Alexa 647 goat anti-rat IgG. Nuclei were

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**Fig. 1. Continued.** D, images of an HT-29/Luc mouse model treated with each regimen taken using a Photon Imager system on days 0, 7, 21, and 35 after therapy initiation. Points, mean; bars, SD. Arrows, drug injections. Statistical comparisons between the NK012 group and the CPT-11 group were done by ANOVA on day 28 after treatment initiation (P < 0.0001).
counterstained with 4′,6-diamidino-2-phenylindole at 1:1,000 dilution (Roche).

Statistical analysis

Data were expressed as mean ± SD. To evaluate changes in the photon count of each treatment group, repeated-measures ANOVA was used. Survival was assessed using the Kaplan-Meier method. For all tests, \( P \) values of <0.05 were considered significant using SPSS software version 12.0 (SPSS, Inc.). All statistical tests were two-sided.

Results

Antitumor activity of NK012 and CPT-11 against HT-29/Luc liver metastasis model

Comparison of the relative photon count on day 28 in the HT-29/Luc liver metastasis model revealed significant differences between mice given NK012 and those given CPT-11 (\( P = 0.002; \) Fig. 1A, B, and D). The survival rates on day 140 in the three test groups were 100%, 0%, and 0% for the NK012, CPT-11, and control groups, respectively (Fig. 2). Moreover, neither relapse nor any other clinical problems were observed in the NK012 group until day 140. Kaplan-Meier analysis showed that a significant improvement in the survival rate in the NK012 group compared with the CPT-11 group (\( P = 0.0006 \)), whereas there was no significant improvement between the CPT-11 group and the control group (\( P = 0.1556 \)). There was no severe body weight loss or toxic death for any treatment used in this study (Fig. 1C).

Histopathologic findings

Histopathologic observation of liver metastases after NK012 administration showed the disappearance of tumor cells. Tumor tissue was replaced with fibrotic or granulomatous tissue with mild infiltration of inflammatory cells. On the other hand, liver metastases treated with CPT-11 showed slight degeneration of cancer cells and few apoptotic cells (Fig. 3). At the liver parenchyma, sinusoidal dilation or steatosis that was a characteristic feature of chemotherapy-associated liver toxicity was not observed after a single or triple administration of NK012.

Tissue concentration and transition of SN-38 after NK012 and CPT-11 administration

We investigated the concentration-time profile of NK012, CPT-11, and free SN-38 in various tissues after single i.v. administration more precisely compared with a previous report (17). The accumulation of free SN-38 converted from CPT-11 was rapidly decreased within 24 hours and could not be detected thereafter in the plasma (Fig. 4A), liver, spleen, kidney, and liver tumor (Fig. 4B). In contrast, the accumulation of free SN-38 released by NK012 was maintained at a relatively high level for weeks after administration. Notably, prolonged higher accumulation of free SN-38 and NK012 was observed in the liver and spleen, which are organs categorized under the reticuloendothelial system. The concentrations of free SN-38 and NK012 gradually decreased over 6 weeks.

Biodistribution of NK012 in hepatic metastases and liver parenchyma

First, we observed directly the distribution and relationship of NK012 and fluorospheres to confirm the detailed biodistribution of NK012 in tissue. In the metastatic tumor, the biodistribution of both substances was similar after 24 hours following their administration, whereas a discrepancy was found at 2 hours (Fig. 5A). For the normal liver parenchyma, fluorescence microscopy showed a similar biodistribution of NK012 and fluorospheres with the exception of the weak accumulation of NK012 2 hours after administration (Fig. 5B).

Second, we observed fluorospheres as a reference of NK012 biodistribution by immunohistochemistry. Anti-CD31 and anti-CD68 antibodies were used as vascular endothelial cell and macrophage (Kupffer) cell markers, respectively. Fluorospheres distributed around tumor vessels in the metastatic liver tumor in all observation points (Fig. 6A). For the liver parenchyma, fluorospheres were not observed in the hepatocytes but were well phagocytized by CD68-positive Kupffer cells in all observation points (Fig. 6B). In addition, the number of CD68-positive cells did not decrease significantly (data not shown).

Discussion

This study highlights four novel findings. First, NK012 was strongly effective in mice bearing liver metastases of human colorectal cancer cells. Second, free SN-38 released from NK012 showed high accumulation and NK012 was detected in the liver metastatic tumor for a long time. Third, free SN-38 and NK012 had been retained in the liver and spleen for weeks with no accompanying toxicity to normal
organs either symptomatically or pathologically. Fourth, NK012 entrapment by macrophages in the liver and spleen led to its prolonged accumulation, and NK012 retention around tumor vessels also resulted in its relatively high accumulation lasting for weeks.

To our knowledge, this is the first study to show that NK012 completely eradicated orthotopic tumors in an orthotopic model, in addition to our previously reported potent antitumor effects of NK012 against various models using human cancer cell lines (17–20, 25). The strong antitumor activity of NK012 is attributable to the high concentration of free SN-38 released from NK012 in the tumor, which is higher than any other concentrations previously reported. Liver metastasis in a specific organ has an adequate potential to be a target of DDS agents, although it presents a disadvantage for such agents because it is considered a hypovascular tumor.

The accumulation and metabolism of DDS agents are closely associated with their biodistribution. The two major differences about the distribution between NK012 and fluorospheres 2 hours after administration in direct fluorescence observation are the lower accumulation of NK012 in the liver parenchyma and discrepancy in metastatic liver tumor (Fig. 5A and B). In agreement with previous reports, these data indicate that NK012 extravasation was successfully achieved and NK012 uptake by Kupffer cells was less than fluorosphere uptake during the early phase after administration because of the higher biocompatibility of the outer shell, which was enveloped by polyethylene glycol (26–28). NK012 biodistribution was similar to fluorosphere biodistribution after 24 hours following administration, and therefore, this fixable fluorescence-labeled nanoparticle can be considered as a substitute for NK012 observation in histochemical studies after such period. Considering these facts, NK012 was retained around CD31-positive tumor vessels in the metastatic tumors and stored in CD68-positive macrophage cells in the liver parenchyma during the late phase after administration, thereby resulting in the high concentration of free SN-38 in the tumor and liver for weeks.

The metabolic pathway of NK012 also plays an important role in organ toxicities. Specifically, the mechanism of liver injury has not yet been clarified, but it is speculated that mitochondrial damage due to cytotoxic agents and the resulting reactive oxygen species have some effect on liver toxicities (29). As for NK012 and CPT-11, SN-38 is detoxified mainly in normal liver cells to form SN-38β-glucuronide (inactive form) by UDP-glucuronosyltransferase, and the detoxification capability may be related to liver toxicities (30). In this study, the single or triple administration of NK012 at the maximum tolerable dose showed no chemotherapy-associated liver toxicity in terms of pathologic changes (Fig. 3) and blood biochemical findings, which reflects hepatocyte injury (data not shown). Daemen et al. (31) reported severe depletion of liver macrophages 24 hours after the administration of liposomal doxorubicin and emphasized the possibility of developing infection due to depletion of the phagocytic capacity of the reticuloendothelial system. Notably, our present data are not in agreement with those of Daemen et al. The number of CD68-positive cells did not decrease because NK012, which is endocytosed in Kupffer cells, is rapidly transported to an acidic environment (pH <5.0), and this novel polymeric micelle agent is very stable at such an acidic condition, indicating that NK012 gradually releases free SN-38 after it accumulates in Kupffer cells (17, 32). Given the metabolite pathway of SN-38 and present data, NK012 causes neither injury of hepatocytes nor depletion of Kupffer cells. However, further studies of liver toxicity with repeated NK012 administrations and the detoxification capability of SN-38, which is gradually released from NK012 for a long period, are needed to clarify whether the prolonged use of NK012 causes chemotherapy-associated liver toxicity or not.

A limitation of this study is the difficulty in the observation of NK012 or free SN-38 by fluorescence microscopy to show their detailed biodistribution. This is because...
Fig. 4. Transition of plasma and tissue concentration after single administration of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) to HT-29/Luc liver metastasis mouse models (n = 3). A, plasma. B, tissue. •, NK012; ○, free SN-38 released from NK012; ▲, CPT-11; △, free SN-38 converted from CPT-11. Points, mean; bars, SD. Arrows, drug injections.
Fig. 5. Biodistribution of NK012 and fluorospheres in hepatic metastasis and liver parenchyma for assessment of distributional relationship of the two substances. Frozen sections of livers from HT-29/Luc liver metastasis nude mice administered NK012 and fluorospheres were directly observed by fluorescence microscopy.

A, liver metastatic tumor. Scale bars, 50 μm.
B, liver parenchyma. Scale bars, 100 μm.
NK012 cannot be fixed and the fluorescence intensities of NK012 and free SN-38 are very weak. Here, we used 20-nm-sized fixable fluorospheres as a substitute for NK012, although direct observation of the object substance remains the best method to verify NK012 biodistribution. There is, however, a technical difficulty in developing a fixable fluorescence substance with entirely the same properties as those of developed DDS agents.

CPT-11 has been approved for the treatment of advanced colorectal cancer in combination with other agents such as fluorouracil, leucovorin, and molecular targeting agents. However, the intensive use of CPT-11 induces chemotherapy-associated steatohepatitis, which affects mortality or morbidity of hepatectomy. NK012 is a biocompatible agent for the treatment of CLM and an excellent SN-38 vehicle because SN-38 is a time-dependent anticancer agent, and the appropriate SN-38 concentration released by NK012 is well maintained for a long time in liver metastases. In terms of liver toxicity, NK012 seems to be nontoxic as shown in this study regardless of the long-term storage of NK012 in Kupffer cells.

In conclusion, NK012 showed strong antitumor effects against liver metastatic tumor. NK012 well infiltrates metastatic tumor tissue and cannot be easily endocytosed by...
macrophages during the early phase after administration. The excellent biodistribution and metabolic pathway of NK012 corroborates both its strong antitumor effects and low toxicity. Our data warrant clinical evaluation of NK012 for the treatment of liver metastases of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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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

Amane Takahashi, Nobuhiro Ohkohchi, Masahiro Yasunaga, et al.

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