Novel SN-38–Incorporated Polymeric Micelle, NK012, Strongly Suppresses Renal Cancer Progression

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Abstract
It has been recently reported that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)–releasing nanodevice, markedly enhances the antitumor activity of SN-38, especially in hypervascular tumors through the enhanced permeability and retention effect. Renal cell carcinoma (RCC) is a typical hypervascular tumor with an irregular vascular architecture. We therefore investigated the antitumor activity of NK012 in a hypervascular tumor model from RCC. Immunohistochemical examination revealed that Renca tumors contained much more CD34-positive neovessels than SKRC-49 tumors. Compared with CPT-11, NK012 had significant antitumor activity against both bulky Renca and SKRC-49 tumors. Notably, NK012 eradicated rapid-growing Renca tumors in 6 of 10 mice, whereas it failed to eradicate SKRC-49 tumors. In the pulmonary metastasis treatment model, an enhanced and prolonged distribution of free SN-38 was observed in metastatic lung tissues but not in nonmetastatic lung tissues after NK012 administration. NK012 treatment resulted in a significant decrease in metastatic nodule number and was of benefit to survival. Our study shows the outstanding advantage of polymeric micelle-based drug carriers and suggests that NK012 would be effective in treating disseminated RCCs with irregular vascular architectures.


Introduction
Passive targeting of the drug delivery system is suited to combating the pathophysiologic characteristics present 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 (EPR) effect (1). Polymeric micelle-based anticancer drugs have recently been developed (2, 3), and some were put under evaluation for clinical trials (4, 5).

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 unstable and has a water-insoluble drug. It has been recently shown that novel SN38-incorporated polymeric micelles, NK012, have the potential to allow effective sustained release of SN-38 inside a tumor and possess potent antitumor activities especially in a vascular endothelial growth factor (VEGF)–secreting hypervascular tumor (6), because the supramolecular structures of NK012 which enable SN-38 to accumulate in the target tissue are based on the EPR effect (1).

Renal cell carcinoma (RCC) is a typical hypervascular tumor with an irregular vascular architecture. We therefore conducted an investigation to determine whether NK012 would be effective in treating RCC by using established RCC tumor models with pulmonary metastasis.

Materials and Methods
Drugs and cells. CPT-11 was purchased from Yakult Honsha Co., Ltd. SN-38 and NK012 was prepared and supplied by Nippon Kayaku Co., Ltd. (6). Five human RCC lines (SKRC-49, Caki-1, 769P, 786O, and KU19-20) and murine Renca cells were maintained in DMEM or MEM supplemented with 2 mmol/L glutamine, 1% nonessential amino acids, 100 units/mL streptomycin and penicillin, and 10% FCS.

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, as described previously (6).

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. Athymic nude mice (3–4 wk old) were maintained in a laminar air flow cabinet under aseptic conditions. 107 RCC cells were s.c. injected into the backs of the mice. NK012 at doses of 10 mg/kg/d or 20 mg/kg/d and CPT-11 at doses of 15 mg/kg/d or 30 mg/kg/d were given i.v. on days 0 (when tumors were allowed to grow until they became massive in size, around 1.5 cm), 4, and 8. Tumor volume was determined by direct measurement with calipers and calculated as π/6 × (large diameter) × (small diameter)2.

Assessment of treatment effects of NK012 on murine pulmonary metastasis model. A total of 1 × 105 Renca cells were inoculated into male BALB/c mice via the tail vein. The mice were randomly divided into three groups of 10. NK012 at dose of 20 mg/kg/d and CPT-11 at dose of 30 mg/kg/d were given i.v. on days 0 (7 d after inoculation), 4, and 8. After that, the mice were sacrificed, their lungs were stained intratracheally with 15% India black ink solution, and the number of metastatic nodules in each mouse was counted. To determine the effect of NK012 on survival, an identical experiment to the one described above was done. After treatment, mice were maintained until each animal showed signs of morbidity (i.e., over 10% weight loss compared with untreated controls), at which point they were sacrificed. Kaplan-Meier analysis was done to determine the effect on time to morbidity, and statistical differences were ranked according to a Mantel-Cox log-rank test using the StatView 5.0 software package.

Histologic and immunohistochemical analysis. Histologic sections were taken from Renca tumor tissues. After extirpation, tissues were fixed with 3.9% formalin in PBS (pH 7.4), and the subsequent preparations and H&E staining were performed by Tokyo Histopathological Laboratory Co.
Differences were calculated using the unpaired Student’s t-test with repeated measures. Data were expressed as mean ± SD. Significance of differences was calculated using the unpaired t test with repeated measures of StatView 5.0.

**ASSAY FOR FREE (POLYMER-UNBOUND) SN-38 IN LUNG TISSUES.** The Renca pulmonary metastasis model described above was used for the analysis of the biodistribution of NK012 and CPT-11. Ten days after Renca inoculation, NK012 (20 mg/kg) or CPT-11 (30 mg/kg) was given i.v. to the mice. The mice were sacrificed at 0, 24, 48, and 72 h after administration, and lung samples were taken and stored at −80°C until analysis. We prepared control mice without Renca inoculation as the nonmetastatic model; NK012 was administered as well, and lung samples were stored. Samples were then homogenized on ice using a Digital homogenizer (Iuchi) and centrifuged at 7000 rpm for 10 min. The supernatants containing camptothecin as an I.S. The sample was vortexed for 10 s and filtered through a MultiScreen Solvinert (Millipore Corporation), and the concentration of free SN-38 in the aliquots of the homogenates (100 μL) was determined using the high-performance liquid chromatography method (6).

**Statistical analysis.** Data were expressed as mean ± SD. Significance of differences was calculated using the unpaired t test with repeated measures of StatView 5.0. *P < 0.05* was regarded as statistically significant.

**RESULTS AND DISCUSSION**

We first evaluated in vitro cellular sensitivity of RCC lines to SN-38, NK012, and CPT-11. The IC50 values of each agent for RCC lines are shown in Table 1. NK012 exhibited higher cytotoxic effect against each cell line compared with CPT-11 (96-fold to 406-fold sensitive).

It is essential to elucidate the correlation between the effectiveness of micellar drugs and tumor hypervascularity and hyperpermeability. Gross evaluation of those RCC tumors s.c. injected into the backs of mice revealed that Renca tumors were much reddish and grew faster than SKRC-49 tumors, and immunohistochemical examination showed that Renca tumors contained much more CD34-positive neovessels than SKRC-49 tumors (Fig. 1).

We allowed the tumors to grow until they became massive, around 1.5 cm, and then initiated treatment. A striking decrease in Renca tumor volume was observed on day 15 in mice treated with NK012 at 20 mg/kg/d compared with the untreated control (Fig. 2A). Renca bulky masses completely disappeared on day 21 in 6 of 10 mice treated with NK012 at 20 mg/kg/d. On the other hand, Renca tumors in mice treated with CPT-11 at 30 mg/kg/d were not eradicated and rapidly regrew after a partial response at day 15. An approximate 10% body weight loss occurred in mice treated with NK012 20 mg/kg, compared with the untreated controls, but there was no significant difference in comparison with tumor-free mice treated with NK012, suggesting that the decrease in body weight was likely to be due to tumor shrinkage rather than toxic effects. We next compared the antitumor activities of the NK012 and CPT-11 treatment in SKRC-49 and Renca tumors. The SKRC-49 tumor volume in mice treated with NK012 at 20 mg/kg/d on day 21 was over 70% smaller than in the untreated controls on day 21 and ~50% smaller than in mice on day 0 (Fig. 2B). However, the SKRC-49 tumors were not eradicated in mice treated with NK012.

Considering that equivalent in vitro growth inhibitory effects by NK012 were observed for SKRC-49 and Renca cells (Table 1), our results suggest that the antitumor activity of NK012 in vivo might be affected by tumor environment factors, such as tumor vascularity.

We next examined the distribution of free SN-38 in the metastatic or nonmetastatic (no inoculation of Renca cells) lung tissues after administration of NK012 or CPT-11. In the case of NK012 administration in mice with lung metastasis, free SN-38 was detectable at the concentration of >100 ng/g in metastatic lung tissues with a typical microvascular architecture (Fig. 3A) even at 72 hours after administration, whereas the concentrations of free SN-38 in nonmetastatic lung tissues after NK012 administration were much lower than those in metastatic lung tissues after treatment with NK012 (significant at 24, 48, and 72 hours; *P < 0.05.*

![Comparison of tumor angiogenesiness of Renca and SKRC-49 in athymic nude mice. A, representative photographs of massive tumors developed from Renca and SKRC-49 at 28 d after s.c. injection (magnification ×400). Immunohistochemical (CD34, ×400) examinations for each tumor are shown. B, tumor neovascularization in each tumor was quantified by counting CD34-positive neovessels. Bars, SD. Experiments were repeated twice with similar results.](image-url)

| Table 1. In vitro growth inhibitory activity of SN-38, NK012, and CPT-11 in RCC lines (MTT assay) |
|-----------------|-----------------|-----------------|-----------------|
| **Cell line**   | **IC50 (μmol/L)** |
| **SN-38**       | **NK012**       | **CPT-11**      |
| SKRC-49         | 0.0064 ± 0.005  | 0.011 ± 0.008   | 4.14 ± 0.45     |
| Caki-1          | 0.0062 ± 0.009  | 0.032 ± 0.006   | 8.45 ± 0.85     |
| 769P            | 0.015 ± 0.007   | 0.085 ± 0.014   | 34.54 ± 3.76    |
| 786O            | 0.031 ± 0.007   | 0.12 ± 0.012    | 28.14 ± 1.21    |
| KU19-20         | 0.10 ± 0.006    | 0.34 ± 0.014    | 32.65 ± 1.25    |
| Renca           | 0.045 ± 0.005   | 0.0096 ± 0.008  | 2.26 ± 0.05     |

*The dose of NK012 is expressed as a dose equivalent to SN-38.*
On the other hand, the concentrations of free SN-38 after administration of CPT-11 were almost negligible in metastatic lung tissues at all time points (data not shown). These results strongly suggest that SN-38 could be selectively released from NK012 and maintained in metastatic Renca tumor tissues.

Deviating from the ordinary experimental pulmonary metastasis prevention model, we initiated treatment 7 days after inoculation (day 0) when multiple lung nodules derived from Renca were observed in all mice in our preliminary study (Fig. 4A). On day 21, there was no significant difference between the mean number of metastatic nodules in the control group (287 ± 56 nodules, n = 10) and in the group receiving CPT-11 treatment (236 ± 59 nodules, n = 10). Significant treatment effects were found, however, in the group receiving NK012 treatment (32 ± 18 nodules, n = 10) on day 21 compared with the control group on day 21 (P < 0.0001). Notably, a dramatic decrease in metastatic nodule number was observed in the NK012 treatment group on day 21 compared with the control group on day 0 (126 ± 23 nodules, n = 10, P < 0.001; Fig. 4A). Kaplan-Meier analysis showed that a significant survival benefit was obtained in the NK012 treatment group compared with
the control group \((P < 0.001)\), but no significant survival benefit was obtained in CPT-11 treatment group \((P = 0.239; \text{Fig. 4B})\). Although no severe toxic effects were observed in any mouse treated with NK012, 3 of 10 mice treated with NK012 were sacrificed during the observation period according to the Guidelines for Animal Experiments because their body weights had become 10% lower than those of the other mice. However, the sacrificed mice were a little bit smaller than others when they started treatment, and they showed no disseminated lung metastasis (data not shown).

Our results presented here strongly support recent findings reported by us that the macromolecular drug distribution throughout the tumor site was enhanced by the hypervascularity and hyperpermeability, and subsequently higher antitumor activity was achieved \((6)\). We assume that conventional low molecular size anticancer agents almost disappear from the bloodstream without being subjected to the EPR effect before they can reach the target organs (solid tumor). The clinical importance of angiogenesis in human tumors has been shown in several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis with chemoresistance in patients with various cancers \((7-9)\). Furthermore, recent reports showing that anticancer agents were less active against VEGF-overexpressing tumors \((10, 11)\) may support the idea that low-molecular drugs are not so effective in the treatment of solid tumors which are rich in blood vessels.

Our study thus far has several limitations about clarifying whether extensive angiogenesis in the tumor is an essential determinant for the susceptibility to NK012. In our ongoing study, we found that NK012 also has a striking antitumor activity against some hypovascular tumor models of human pancreatic cancer xenografts.\(^5\) It also remains unclear whether NK012 possesses strong antitumor activity in other metastatic sites besides the lung. It is known that the EPR effect is affected by various permeability factors, such as bradykinin \((12)\), nitric oxide \((13)\), and various cytokines independent of VEGF and hypervascularity \((14)\). Among solid tumors with rapid progression potential, irregularity occurs not only in blood flow and vascular density, but also in the vascular network and anatomic architecture \((15, 16)\), suggesting that EPR effect may be predominantly promoted in rapid-progressive tumor phenotypes and influenced by organ-specific tumor microenvironment. Hoffman and coworkers \((17, 18)\) have developed a technique of surgical orthotopic implantation (SOI) with more clinical features of systemic and aggressive metastases than our conventional animal models. Further preclinical studies using such models as SOI might clarify cancer phenotypes and metastatic organs to which we can apply NK012 more precisely.

The results of chemotherapy in RCCs have been disappointing, as indicated by the low response proportions. However, clinical trials using gemcitabine-containing regimens have been encouraging, with major responses occurring in 5% to 17% of patients \((19, 20)\), suggesting the possibility that chemotherapy is promising as a modality for RCC therapy if anticancer agents can be selectively delivered, released, and maintained around tumor tissues. Our current report highlights the advantages of polymeric micelle-based drug carriers like NK012 as promising modalities for treatment, rather than prevention, of disseminated RCCs with abnormal vascular architecture. The results of our ongoing phase-I

clinical trial and future phase-II trials of NK012 in patients with advanced solid tumors including RCC might meet or even exceed our expectations.

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References


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