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

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

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

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

Material and Methods

Drugs and cells

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

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

In vitro growth inhibition assay

The growth inhibitory effects of NK012, CPT-11, SN-38 and 5-FU, instead of S-1 that is not suitable for use *in vitro*, because tegafur is a prodrug that is mainly activated in liver were examined by tetrazolum salt-based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan). A suspension (100 mL) of exponentially growing cells (1×10^5/mL) was placed into the wells of a 96-well plate and incubated for 24 hr at 37°C. Following medium removal, 100 μL of medium containing various concentrations of each drug was added to the wells and then incubated for 72 hr at 37°C. After medium removal, 10 μL of WST-8 solution and 90 μL of medium were added to the wells, followed by incubation for 1 hr at 37°C. The growth inhibitory effects of each drug were assessed spectrophotometrically (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). IC_{50} was determined on the dose–response curves. The nature of interaction between NK012 and 5-FU against the NSCLC cell lines A549, PC-9, PC-14, EBC-1 and H520 was evaluated by median-effect plot analyses and the combination index method of Chou and Talalay.[32]

Reverse transcription and real-time PCR analysis

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

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

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

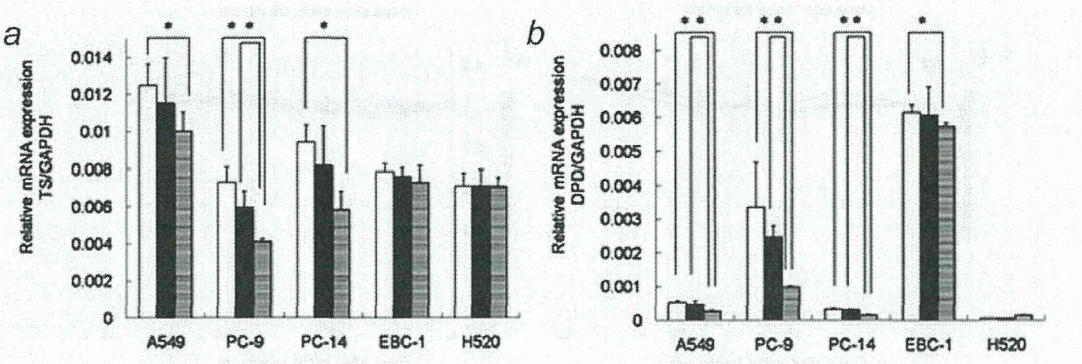

Figure 1. Effects of NK012 and CPT-11 on the expression of TS and DPD mRNA in non-small cell lung cancer (NSCLC) cell lines. (*a, b*) Downregulation of TS (*a*) and DPD (*b*) mRNA by NK012 and CPT-11 in NSCLC cell lines. A549, PC-9, PC-14, EBC-1 and H520 cells were incubated with medium containing 10%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

Experimental mice model

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

All animal procedures were performed in compliance with the guidelines for the care and use of experimental animals established by the Committee for Animal Experimentation of the National Cancer Center. These guidelines meet the ethical standards required by law and comply with the guidelines for the use of experimental animals in Japan.

In vivo growth inhibition assay

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

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

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

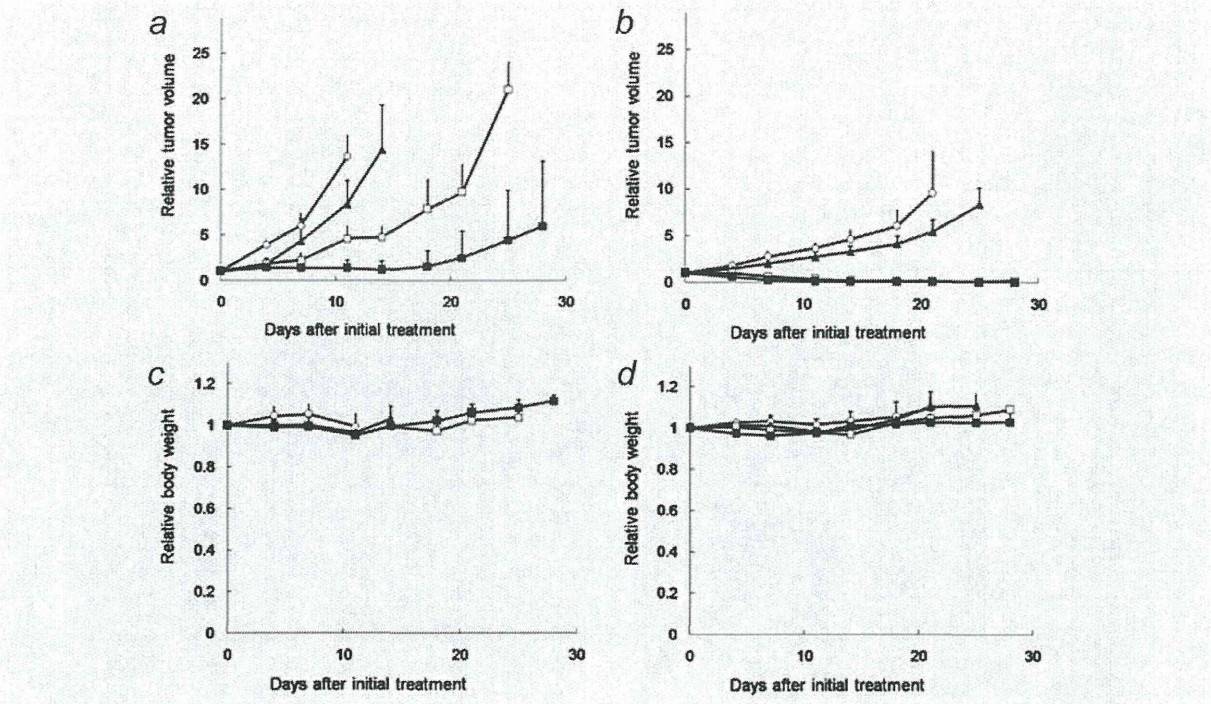

Figure 2. Growth inhibitory effects of NK012, S-1 and NK012/S-1 on PC-14 and EBC-1 tumor xenografts. (*a*, *b*) Relative tumor volume in mice treated with NK012, S-1 and NK012/S-1. PC-14 (*a*, *c*) and EBC-1 (*b*, *d*) cells were inoculated subcutaneously into the flank of mice, as described in Material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

Pathological studies of small intestinal mucosa

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

Statistical analysis

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

Results

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

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

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

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

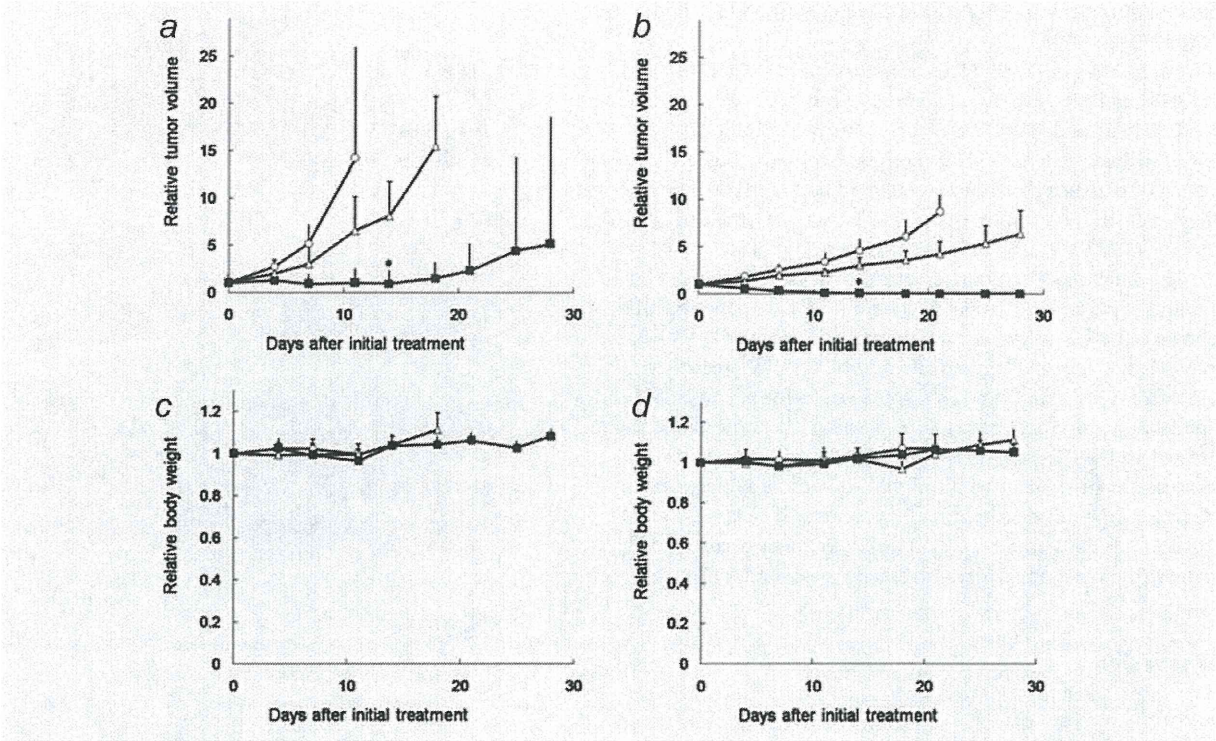

Figure 3. Antitumor effect of combined NK012/S-1 and CPT-11/S-1 treatment on PC-14 and EBC-1 tumor xenografts. (*a*, *b*) Relative tumor volume in mice treated with NK012/S-1 or CPT-11/S-1. PC-14 (*a*, *c*) and EBC-1 (*b*, *d*) cells were inoculated subcutaneously into the flank of mice, as described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

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

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

Values in parentheses indicate percentage.

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

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

The therapeutic effect of NK012/S-1 was significantly superior to that of NK012 both in PC-14 ($p = 0.0013$) (Fig. 2*a*) and EBC-1 ($p = 0.0017$) (Fig. 2*b*), and this combination demonstrated a synergistic efficacy. The complete response rates achieved with NK012 and NK012/S-1 were 0 and 20% for PC-14 and 40 and 100% for EBC-1, respectively. Although treatment-related BW loss was observed in mice treated with each drug combination, BW recovered to the normal level in each group by Day 21 (Figs. 2*c* and 2*d*).

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

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

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

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

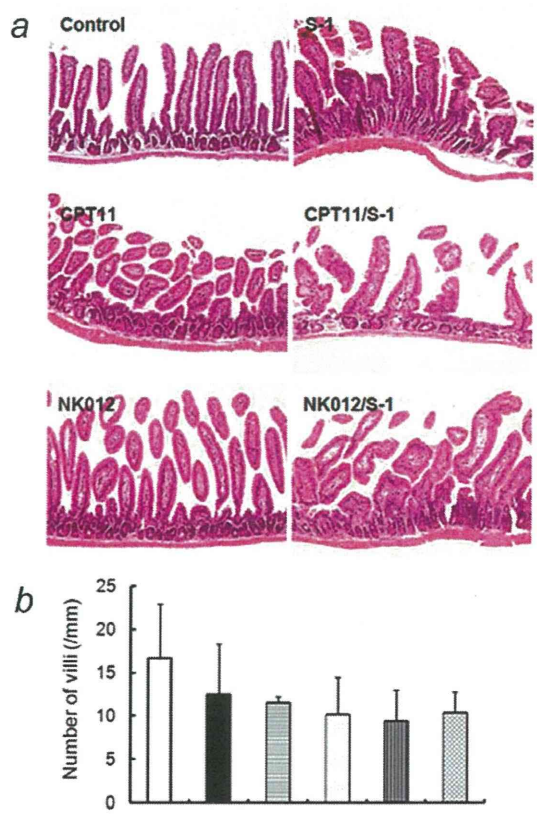

Figure 4. Pathological findings in intestinal mucosa. Mice were administered the following: NaCl solution (0.9%) on Days 0 and 7 as normal control, CPT-11 (10 mg/kg/d) on Days 0 and 7, NK012 (5 mg/kg/d) on Days 0 and 7, S-1 (10 mg/kg/d) on Days 0–13, CPT-11 (10 mg/kg/d) on Days 0 and 7 and combined with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

Discussion

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

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

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

Acknowledgements

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References

1. Spiro SG, Porter JC. Lung cancer—where are we today? Current advances in staging and nonsurgical treatment. *Am J Respir Crit Care Med* 2002;166:1166–96.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
3. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–8.
4. Ohe Y, Ohashi Y, Kubota K, Tamura T, Nakagawa K, Negoro S, Nishiwaki Y, Saijo N, Ariyoshi Y, Fukuoka M. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* 2007;18:317–23.
5. Argiris A, Murren JR. Advances in chemotherapy for small cell lung cancer: single-agent activity of newer agents. *Cancer J* 2001;7:228–35.
6. Bodurka DC, Levenback C, Wolf JK, Gano J, Wharton JT, Kavanagh JJ, Gershenson DM. Phase II trial of irinotecan in patients with metastatic epithelial ovarian cancer or peritoneal cancer. *J Clin Oncol* 2003;21:291–7.
7. Cunningham D, Pyrhonen S, James RD, Punt CJ, Hickish TF, Heikkila R, Johannesen TB, Starkhammar H, Topham CA, Awad L, Jacques C, Herait P. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet* 1998;352:1413–18.
8. Negoro S, Masuda N, Takada Y, Sugiura T, Kudoh S, Katakami N, Ariyoshi Y, Ohashi Y, Niitani H, Fukuoka M. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. *Br J Cancer* 2003;88:335–41.
9. Mathijssen RH, van Alphen RJ, Verweij J, Loos WJ, Nooter K, Stoter G, Sparreboom A. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). *Clin Cancer Res* 2001;7:2182–94.
10. Rothenberg ML, Kuhn JG, Burris HA, III, Nelson J, Eckardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI, Rock MK, Von Holf DD. Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993;11:2194–204.
11. Slatter JG, Schaaf LJ, Sams JP, Feenstra KL, Johnson MG, Bombardt PA, Cathcart KS, Verburg MT, Pearson LK, Compton LD, Miller LL, Baker DS, et al. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients. *Drug Metab Dispos* 2000;28:423–33.
12. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol* 1988;133:95–109.
13. Maeda H, Matsumura Y. Tumoritropic and lymphotropic principles of macromolecular drugs. *Crit Rev Ther Drug Carrier Syst* 1989;6:193–210.
14. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986;46:6387–92.
15. Matsumura Y, Maruo K, Kimura M, Yamamoto T, Konno T, Maeda H. Kinin-generating cascade in advanced cancer patients and in vitro study. *Jpn J Cancer Res* 1991;82:732–41.
16. Kuroda J, Kuratsu J, Yasunaga M, Koga Y, Kenmotsu H, Sugino T, Matsumura Y. Antitumor effect of NK012, a 7-ethyl-10-hydroxycamptothecin-incorporating polymeric micelle, on U87MG orthotopic glioblastoma in mice compared with irinotecan hydrochloride in combination with bevacizumab. *Clin Cancer Res* 2010;16:521–9.
17. Koizumi F, Kitagawa M, Negishi T, Onda T, Matsumoto S, Hamaguchi T, Matsumura Y. Novel SN-38-incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor-secreting bulky tumors. *Cancer Res* 2006;66:10048–56.
18. Nakajima TE, Yasunaga M, Kano Y, Koizumi F, Kato K, Hamaguchi T, Yamada Y, Shirao K, Shimada Y, Matsumura Y. Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil. *Int J Cancer* 2008;122:2148–53.
19. Sumitomo M, Koizumi F, Asano T, Horiguchi A, Ito K, Asano T, Kakizoe T, Hayakawa M, Matsumura Y. Novel SN-38-incorporated polymeric micelle, NK012, strongly suppresses renal cancer progression. *Cancer Res* 2008;68:1631–5.
20. Saito Y, Yasunaga M, Kuroda J, Koga Y, Matsumura Y. Enhanced distribution of NK012, a polymeric micelle-encapsulated SN-38, and sustained release of SN-38 within tumors can beat a hypovascular tumor. *Cancer Sci* 2008;99:1258–64.
21. Eguchi Nakajima T, Yanagihara K, Takigahira M, Yasunaga M, Kato K, Hamaguchi T, Yamada Y, Shimada Y, Mihara K, Ochiya T, Matsumura Y. Antitumor effect of SN-38-releasing polymeric micelles, NK012, on spontaneous peritoneal metastases from orthotopic gastric cancer in mice compared with irinotecan. *Cancer Res* 2008;68:9318–22.

22. Kuroda J, Kuratsu J, Yasunaga M, Koga Y, Saito Y, Matsumura Y. Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. *Int J Cancer* 2009;124:2505–11
23. Kato K, Hamaguchi T, Shirao K, Shimada Y, Doi T, Ohtsu A, Matsumura Y, Yamada Y. Interim analysis of phase I study of NK012, polymer micelle SN-38, in patients with advanced cancer. *Proc Am Soc Clin Oncol* 2008 (Abstract No. 485).
24. Burris HA, III, Infante JR, Spigel DR, Greco FA, Thompson DS, Matsumoto S, Kawamura S, Jones SF. A phase I dose-escalation study of NK012. *Proc Am Soc Clin Oncol* 2008 (Abstract No. 2538).
25. Azrak RG, Cao S, Slocum HK, Toth K, Durrani FA, Yin MB, Pendyala L, Zhang W, McLeod HL, Rustum YM. Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts. *Clin Cancer Res* 2004;10: 1121–9.
26. Takiuchi H, Kawabe S-i, Gotoh M, Katsu K-i. Thymidylate synthase gene expression in primary tumors predicts activity of S-1-based chemotherapy for advanced gastric cancer. *Gastrointest Cancer Res* 2007;1: 171–6.
27. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000;6:1322–7.
28. Metzger R, Danenberg K, Leichman CG, Salonga D, Schwartz EL, Wadler S, Lenz HJ, Groshen S, Leichman L, Danenberg PV. High basal level gene expression of thymidine phosphorylase (platelet-derived endothelial cell growth factor) in colorectal tumors is associated with nonresponse to 5-fluorouracil. *Clin Cancer Res* 1998;4: 2371–6.
29. Goto A, Yamada Y, Yasui H, Kato K, Hamaguchi T, Muro K, Shimada Y, Shirao K. Phase II study of combination therapy with S-1 and irinotecan in patients with advanced colorectal cancer. *Ann Oncol* 2006;17:968–73.
30. Shirasaka T, Shimamoto Y, Fukushima M. Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. *Cancer Res* 1993;53:4004–9.
31. Muraoka A, Suehiro I, Fujii M, Nagata K, Kusunoki H, Kumon Y, Shirasaka D, Hosooka T, Murakami K. Acute gastric anisakiasis: 28 cases during the last 10 years. *Dig Dis Sci* 1996;41:2362–5.
32. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22: 27–55.
33. Kamiyama H, Takano S, Tsuboi K, Matsumura A. Anti-angiogenic effects of SN38 (active metabolite of irinotecan): inhibition of hypoxia-inducible factor 1 α (HIF-1α)/vascular endothelial growth factor (VEGF) expression of glioma and growth of endothelial cells. *J Cancer Res Clin Oncol* 2005;131:205–13.
34. Okabe T, Okamoto I, Tsukioka S, Uchida J, Iwasa T, Yoshida T, Hatashita E, Yamada Y, Satoh T, Tamura K, Fukuoka M, Nakagawa K. Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase. *Mol Cancer Ther* 2008;7:599–606.
35. Brun Y, Wang XP, Willemot J, Sevenet T, Demenge P. Experimental study of antidiarrheal activity of Salicairine. *Fundam Clin Pharmacol* 1998;12:30–6.
36. Yokoyama Y, Dhanabal M, Griffioen AW, Sukhatme VP, Ramakrishnan S. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. *Cancer Res* 2000;60:2190–6.
37. Uchino H, Matsumura Y, Negishi T, Koizumi F, Hayashi T, Honda T, Nishiyama N, Kataoka K, Naito S, Kakizoe T. Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats. *Br J Cancer* 2005;93: 678–87.
38. Okamoto I, Nishimura T, Miyazaki M, Yoshioka H, Kubo A, Takeda K, Ebi N, Sugawara S, Katakami N, Fukuoka M, Nakagawa K. Phase II study of combination therapy with S-1 and irinotecan for advanced non-small cell lung cancer: west Japan thoracic oncology group 3505. *Clin Cancer Res* 2008;14: 5250–4.
39. Inokuchi M, Yamashita T, Yamada H, Kojima K, Ichikawa W, Nihei Z, Kawano T, Sugihara K. Phase I/II study of S-1 combined with irinotecan for metastatic advanced gastric cancer. *Br J Cancer* 2006; 94:1130–5.
40. Takechi T, Okabe H, Ikeda K, Fujioka A, Nakagawa F, Ohshimo H, Kitazato K, Fukushima M. Correlations between antitumor activities of fluoropyrimidines and DPD activity in lung tumor xenografts. *Oncol Rep* 2005;14:33–9.
41. Ohe Y, Sasaki Y, Shinkai T, Eguchi K, Tamura T, Kojima A, Kunikane H, Okamoto H, Karato A, Ohmatsu H, Kanzawa F, Saijo N. Phase I study and pharmacokinetics of CPT-11 with 5-day continuous infusion. *J Natl Cancer Inst* 1992;84:972–4.
42. Masuda N, Fukuoka M, Kusunoki Y, Matsui K, Takifuji N, Kudoh S, Negoro S, Nishioka M, Nakagawa K, Takada M. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1992; 10:1225–9.
43. Ohno R, Okada K, Masaoka T, Kuramoto A, Arima T, Yoshida Y, Ariyoshi H, Ichimaru M, Sakai Y, Oguro M, Ito Y, Morishima Y, et al. An early phase II study of CPT-11: a new derivative of camptothecin, for the treatment of leukemia and lymphoma. *J Clin Oncol* 1990;8:1907–12.
44. Sakuramoto S, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Imamura H, Higashino M, Yamamura Y, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007;357:1810–20.
45. Nagano T, Yasunaga M, Goto K, Koga Y, Kuroda J-i, Nishimura Y, Sugino T, Nishiwaki Y, Matsumura Y. Antitumor activity of NK012 combined with cisplatin against small-cell lung cancer and intestinal mucosal changes in tumor-bearing mouse after treatment. *Clin Cancer Res* 2009;15: 4348–55.

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Clinical Cancer Research

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Cancer Therapy: Preclinical

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

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Abstract

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

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

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

Conclusion: This study suggests that NK012 is strongly effective against liver metastases and does not damage the liver despite the long retention time of NK012 in Kupffer cells. *Clin Cancer Res; 16(19); 4822–31.* ©*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 tomoisomerase I. Although SN-38 has 1,000-fold more potent cytotoxic activity than CPT-11 (13), it has been clinically unavailable due to its water-insoluble nature. Additionally, its metabolic conversion rate is only <10% of the original volume of CPT-11

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Translational Relevance

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

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

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

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

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

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

Materials and Methods

Drugs

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

Cell culture

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

Establishment of HT-29 cell line stably expressing firefly luciferase

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

Liver metastasis model

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