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Fast Track

Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma

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glioma • drug delivery system • blood brain barrier (BBB) • SN-38 • micelles

ABSTRACT



Recent published reports on clinical trials of CPT-11 indicate the effectiveness of this compound, a prodrug of SN-38, against malignant glioma in combination with anti-vascular endothelial growth factor antibody. Here, we determined if NK012, and SN-38 incorporating micelle, can be an appropriate formulation for glioblastoma treatment compared with CPT-11. *In vitro* cytotoxicity was evaluated against several glioma lines with NK012, CPT-11, SN-38, ACNU, CDDP and etoposide. For the *in vivo* test, a human glioma line (U87MG) transfected with the *luciferase* gene was inoculated into nude mice brain for pharmacokinetic analysis by fluorescence microscopy and high-performance liquid chromatography after intravenous injection of NK012 and CPT-11. *In vivo* antitumor activity of NK012 and CPT-11 was evaluated by bioluminescence image and Kaplan-Meier analyses. The growth-inhibitory effects of NK012 were 34- to 444-fold more potent than those of CPT-11. Markedly enhanced and prolonged distribution of free SN-38 in the xenografts was observed after NK012 injection compared with CPT-11. NK012 showed significantly potent antitumor activity against an orthotopic glioblastoma multiforme xenograft and significantly longer survival rate than CPT-11 ($p = 0.0014$). This implies that NK012 can pass through the blood brain tumor barrier effectively. NK012, which combines enhanced distribution with prolonged sustained release, may be ideal for glioma treatment. Currently, a phase I study of NK012 is almost complete in Japan and the US. The present translational study warrants the clinical phase II study of NK012 in patients with malignant glioma. © 2008 Wiley-Liss, Inc.

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DIGITAL OBJECT IDENTIFIER (DOI)10.1002/jjc.24171 [About DOI](#)**ARTICLE TEXT**

Malignant astrocytomas, such as anaplastic astrocytoma and glioblastoma multiforme (GBM), are the most common and highly vascularized glial tumors of the brain. At least 80 percent of malignant gliomas are categorized as GBM.[1] Currently, GBM patients have a mean survival of only 50 weeks following the standard treatment consisting of surgical and adjuvant therapies.[2] A recent phase III randomized trial for newly diagnosed GBM demonstrated that radiation therapy with concurrent temozolomide treatment, followed by 6 months of temozolomide treatment was superior to radiation therapy alone in terms of overall survival.[3] In addition, several clinical trials have demonstrated that the median survival times of patients with recurrence were only 3-6 months.[4]

The anticancer plant alkaloid 7-ethyl-10-hydroxy-camptothecin (SN-38) is a broad spectrum anticancer agent targeting DNA topoisomerase I with a different mechanism of action compared with alkylating agents such as temozolomide. Although SN-38 has shown promising anticancer activity *in vitro* and *in vivo*, its clinical application has remained dormant because of its low therapeutic efficacy and severe toxic effects.[5][6] Irinotecan hydrochloride (CPT-11), a prodrug of SN-38, shows some antitumor activities in patient with recurrent GBM, with response rates of 0 to 17% in several trials.[7-10] CPT-11 activity is thus similar to that of other agents used for recurrent GBM.[9] A recent phase II trial for recurrent GBM demonstrated that the combination of CPT-11 and bevacizumab, an antivascular endothelial growth factor (VEGF) monoclonal antibody, is an effective treatment against the neoplasia with a 6-month progression-free survival rate of 46% and a 6-month overall survival rate of 77%.[11][12] However, there is an increased risk of developing venous thromboembolic disease and intracranial hemorrhage with this combination therapy. Therefore, there is an urgent need to develop treatment modalities by which cytotoxic drugs can exert more potent antitumor activity to their full potential with modest adverse effects and thereby reasonably prolong the overall survival in GBM patients.

The purpose of the drug delivery system (DDS) is to achieve selective delivery of antitumor agents to tumor tissue at an effective concentration for the appropriate duration of time in order to reduce the adverse effects of the administered drug and simultaneously enhance its antitumor effect. There are 2 main concepts in DDS, active targeting and passive targeting. Active targeting involves a monoclonal antibody and a ligand to a tumor-related receptor. Doxil, a doxorubicine incorporated polyethylene glycol conjugated liposome, is categorized under passive targeting agents and are already in clinical use.[13][14] NK012, a novel SN-38-incorporating polymeric micelle, is a prodrug of SN-38 similar to CPT-11 and categorized under passive targeting agent as well. Although CPT-11 is converted to SN-38 in tumors by carboxylesterase (CE), the metabolic conversion rate is within 2-8% of the original volume of CPT-11.[15][16] In contrast, the release rate of SN-38 from NK012 is 74% under physiologic pH condition even without CEs.[17] Recently, we have demonstrated that NK012 exerted significantly more potent antitumor activity against various human tumor xenografts than CPT-11.[17-20] However, there is a fundamental question whether such nanoparticles can reach brain tumors across the tumor microvessels. In the present study, therefore, we established an orthotopic glioma model in this experiment and then evaluated whether NK012 can pass through the BTB and exert its antitumor effect on orthotopic human glioma xenografts in comparison with CPT-11.

Material and methods**Drugs**

NK012 and SN-38 were donated by Nippon Kayaku Co., Ltd. (Tokyo, Japan). The size of NK012 was \approx 20 nm in diameter with a narrow size distribution.[17] ACNU [1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea, nimstine] was purchased from DAIICHI SANKYO Co., Ltd. (Tokyo, Japan). CDDP (cis-diamminedichloroplatinum) and CPT-11 were purchased from Yakult Co., Ltd. (Tokyo, Japan). Etoposide [4-demethlepipodophyllotoxin-9-(4, 6-O-ethylidene- β -D-glucopyranoside)] was purchased from BIOMOL (Plymouth Meeting, PA).

Cells and animals

Five human glioma cell lines, namely U87MG, U251MG, U118MG, LN18 and LN229, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenu-Hoerden, Germany), penicillin, streptomycin and amphotericin B (100 units/ml, 100 μ g/ml and 25 μ g/ml, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO₂ at 37°C. Six- to eight-week-old athymic nude mice (nu/nu; Charles River Japan,

Kanagawa, Japan) were used for this study. U87MG cells (1×10^5) were injected into the cerebral hemisphere using a Hamilton syringe through an entry point 1 mm anterior and 1.8 mm lateral to the bregma to an intraparenchymal depth of 2.5 mm. The rate of injection was 0.5 μ l/min, and the needle was left in place for 5 min after completion of the injection. 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, Japan; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Establishment of U87MG cell line stably expressing firefly luciferase and YFP mutant Venus

For the *in vivo* bioluminescence imaging of orthotopic brain tumors, the U87MG cell line stably expressing firefly luciferase

and the YFP mutant Venus was established. Briefly, the coding sequence for firefly luciferase and Venus was subcloned into the pIRES Vector (Clontech Laboratories, Mountain View, CA). The fragment consists of Luciferase-IRES-Venus generated from the plasmid with the restriction enzymes Nhe I and Not I. This fragment was subcloned into the pEF6/V5-His Vector (Invitrogen, Carlsbad, CA) to generate plasmids of pEF6-Luciferase IRES Venus. U87MG cells (2×10^6) were seeded onto 10-cm dishes 24 hr before transfection. The cells were transfected with 10 μ g of pEF6-Luciferase IRES Venus using FuGENE HD Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions, and then incubated for 48 hr at 37°C. The cells were then passaged in medium containing Blasticidin (10 μ g/ml; InvivoGen, San Diego, CA) to select for the Blasticidin resistance gene integrated in the pEF6/V5-His plasmids. Venus expression was used as a surrogate marker of luciferase-positive cells. Venus-expressing U87MG cells (U87MG/Luc) were sorted using the BD FACS Aria cell sorter (BD Biosciences, San Jose, CA), and expanded in selection medium. The accuracy of a quantitative bioluminescence image as an indicator of U87MG/Luc cell number was analyzed using the Photon Imager animal imaging system *in vitro*, as described under *in vivo* growth inhibition assay. This analysis demonstrated clear correlation between a quantitative bioluminescence image and cell number ($R^2 = 0.99$). The sensitivity of U87MG/Luc cells to each drug (NK012, CPT-11, SN-38, ACNU, CDDP and etoposide) was almost similar to that of parental U87MG cells (data not shown).

In vitro growth inhibition assay

Cell growth inhibition was measured by the tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). Briefly, cells (5×10^3 cells/well) in 96-well plates were incubated overnight. Then, growth medium was changed to new medium with various concentrations of SN-38, NK012, CPT-11, ACNU, etoposide and CDDP. After 72 hr of incubation, medium was changed to new medium containing 10% WST-8 reagents. After 1 hr of incubation, the absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). Cell viability was measured and compared with that of the control cells. Each experiment was carried out in triplicates and was repeated at least 3 times. Data were averaged and normalized against the nontreated controls to generate dose-response curves. The number of living cells (% Control) was calculated using the following formula: % Control = (each absorbance - absorbance of blank well)/absorbance of control well $\times 100$.

Evaluation of NK012 and CPT-11 distribution in tumor tissue by fluorescence microscopy

The U87MG orthotopic xenograft model described earlier was used for the analysis of the biodistribution of NK012 and CPT-11. Twenty days after U87MG/Luc inoculation, the maximum tolerated dose (MTD) of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was injected intravenously into the tail vein of mice. At this point, tumor size reached to about 3 mm in diameter according to the preliminary experiment (data not shown). Two, 12 or 24 hr after NK012 or CPT-11 injection, mice were also administered with fluorescein *Lycopersicon esculentum* lectin (100 μ l/mouse) (Vector Laboratories, Burlingame, CA) to visualize tumor blood vessels. Tumors were then excised and embedded in optimal cutting temperature compound and frozen at -80°C until use. Tissue sections (6 μ m thick) were prepared using Tissue-Tek Cryo3 (Sakura Finetek USA, Inc., Torrance, CA), and frozen sections were examined under a fluorescence microscope, BIOREVO BZ9000 (Keyence, Osaka, Japan), at an excitation wavelength of 377 nm and an emission wavelength 447 nm to evaluate the distribution of CPT-11 and NK012 within the tumor tissues. Because formulations containing SN-38 bound *via* ester bonds possess a particular fluorescence, both CPT-11 and NK012 were detected under the same fluorescence conditions. Image data were recorded using BZ-II Analyzer 1.10 software (Keyence, Osaka, Japan).

Pharmacokinetics study of NK012 and CPT-11

Female BALB/c nude mice bearing U87MG/Luc tumor ($n = 3$) were used for the analysis of the biodistribution of NK012 and CPT-11. Twenty days after the intracranial injection of U87MG/Luc cells, NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was intravenously administered to the mice. Under anesthesia, blood, normal brain tissues and tumor tissues were obtained 2, 12, 24 and 72 hr after NK012 or CPT-11 administration. Blood samples were collected in microtubes and immediately centrifuged at 1,600g for 15 min at 4°C. All samples were stored at -80°C until use.

The normal brain and tumor samples were rinsed with physiologic 0.9% NaCl solution, mixed with 0.1 M glycine-HCl buffer (pH 3.0)/methanol at 5 w/w%, and then homogenized. To analyze the concentration of free SN-38 and CPT-11, 100 μ l of the tumor homogenates was mixed with 20 μ l of 1 mM phosphoric acid/methanol (1:1), 40 μ l of ultrapure water and 60 μ l of camptothecin solution (10 ng/ml for SN-38 and 15 ng/ml for CPT-11) as an internal standard. To quantify free SN-38 and CPT-11 in plasma, 25 μ l of plasma was mixed with 25 μ l of 0.1 M HCl, and then added with 20 μ l of 1 mM phosphoric acid/methanol (1:1) and 100 μ l of CPT solution (10 ng/ml both for SN-38 and CPT-11). The samples were vortexed vigorously for 10 sec, and then filtered through Ultrafree-MC centrifugal filter devices with a cut-off molecular diameter of 0.45 μ m (Millipore Co., Bedford, MA). Reversed-phase HPLC was performed at 35°C on a Mightysil RP-18 GP column 150 \times 4.6 mm² (Kanto Chemical Co., Inc., Tokyo, Japan). Fifty microliters of a sample was injected into an Alliance Waters 2795 HPLC system (Waters, Milford, MA) 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 and that originating from CPT-11 was detected at 430 nm with an excitation wavelength of 365 nm. The mobile phase was a mixture of 100 nmol/l ammonium acetate (pH 4.2) and methanol (11:9 (v/v)). 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).

For polymer-bound SN-38 detection, SN-38 was released from the conjugate. Briefly, 20 μ l of plasma and 100 μ l of tissue samples were diluted with 20 μ l of methanol (50%, v/v) and 20 μ l of NaOH (0.3 mol/l for plasma and 0.7 mol/l for tissue). The samples were incubated for 15 min at 25°C. After incubation, 20 μ l of HCl (0.3 mol/l for plasma and 0.7 mol/l

for tissue) and 60 μl of CPT solution (10 ng/ml for SN-38 and 15 ng/ml for CPT-11) were added to the samples, and then the hydrolysate was filtered through a MultiScreen Solvint. Fifty microliters of the filtrate was applied to the same HPLC system as described earlier.

In vivo growth inhibition assay

Experiment 1

Six-week-old mice were subcutaneously inoculated with 1×10^7 U87MG/Luc cells in the flank region. When tumor volume reached $\approx 605 \text{ mm}^3$, mice were randomly divided into test groups consisting of 3 mice per group (day 0). Drug was intravenously administered on days 0, 4 and 8 into the tail vein. NK012 was administered at its MTD of 30 mg/kg/day. The reference drug CPT-11 was given at its MTD of 66.7 mg/kg/day in the optimal schedule reported.[17][21] The length (*i*) and width (*ii*) of tumor masses were measured twice a week, and tumor volume (TV) was calculated as follows: $\text{TV} = (a \times b^2)/2$. Relative tumor volumes (RTVs) at day *n* were calculated according to the following formula: $\text{RTV} = \text{TV}_n / \text{TV}_0$, where TV_n is the tumor volume at day *n*, and TV_0 is the tumor volume at day 0.

Experiment 2

To assess the antitumor effect of NK012 and CPT-11, *in vivo* bioluminescence imaging studies were performed using the Photon Imager animal imaging system (Biospace, Paris, France). For imaging, mice with intracranial U87MG/Luc tumor were simultaneously anesthetized with isoflurane and D-luciferine potassium salt (Synchem, Germany), and normal 0.9% NaCl solution was intraperitoneally administered at a dose of 125 mg/kg body weight, and images were obtained 5 min after the injection. For bioluminescence image analysis, regions of interest encompassing the intracranial area of a signal were defined using Photo Vision software (Biospace, Paris, France), and total numbers of photons per minute (cpm) were recorded. The pseudo-color luminescent image from violet (least intense) to red (most intense) represented the spatial distribution of detected photon counts emerging from active luciferase within the animal. Twenty days after U87MG inoculation, treatment was started (day 0). Normal 0.9% NaCl solution (*n* = 4), NK012 (30 mg/kg, *n* = 4), or CPT-11 (66.7 mg/kg, *n* = 4) was intravenously administered to mice on days 0, 4 and 8. *In vivo* bioluminescence imaging studies were performed on days 0, 14, 21 and 28 from the day of treatment initiation. To determine the effect of treatment on the time to change of intensity, Student's *t* test was carried out using the StatView 5.0 software package. *p* < 0.05 was regarded as significant.

Experiment 3

Mice with intracranial U87MG/Luc tumor was randomly divided into 3 groups consisting of 6 mice per group. NK012 (30 mg/kg/day) and CPT-11 (66.7 mg/kg/day) were intravenously given on days 0 (20 days after tumor inoculation), 4 and 8. After treatment, mice were maintained until each animal showed signs of morbidity (*i.e.*, 10% weight loss and neurological deficit), at which point they were sacrificed. Kaplan-Meier analysis was performed to determine the effect of drugs on time to morbidity, and statistical differences were ranked according to the Mantel-Cox log-rank test using StatView 5.0.

Statistical analysis

Data were expressed as mean \pm SD. Significance of differences was calculated using the unpaired *t* test with repeated measures of StatView 5.0. *p* < 0.05 was regarded as significant.

Results



Cellular sensitivity of glioblastoma cells to SN-38, NK012 and CPT-11

The IC_{50} values of NK012 for the cell lines ranged from 0.0087 $\mu\text{mol/l}$ (U251MG cells) to 0.36 $\mu\text{mol/l}$ (LN229 cells). The growth inhibitory effects of NK012 were 34- to 444-fold more potent than those of CPT-11, 400- to 12818-fold more potent than those of ACNU, 52- to 523-fold more potent than those of CDDP, and 3- to 278-fold more potent than those of etoposide. On the other hand, the IC_{50} values of NK012 were almost similar to those of SN-38 (Table. I).

Table I. IC_{50} Values of SN-38, NK012, CPT-11, ACNU, CDDP and VP-16 in Various Human Glioblastoma Cell Lines

Cell line	$\text{IC}_{50}(\mu\text{mol/l})$					
	SN38	NK012	CPT-11	ACNU	CDDP	Etoposide
LN18	0.052 \pm 0.0034	0.069 \pm 0.0242	13.0 \pm 0.88	729 \pm 30	3.57 \pm 0.08	3.84 \pm 0.14
LN229	0.28 \pm 0.1094	0.36 \pm 0.0489	12.2 \pm 1.23	144 \pm 23	21.4 \pm 0.62	0.945 \pm 0.025
U87MG	0.18 \pm 0.0216	0.093 \pm 0.0038	18.1 \pm 3.06	865 \pm 86	9.06 \pm 0.57	20.8 \pm 5.23
U118MG	0.0089 \pm 0.0003	0.022 \pm 0.0017	4.85 \pm 0.14	282 \pm 22	3.35 \pm 0.35	4.05 \pm 0.18
U251MG	0.0076 \pm 0.0001	0.0087 \pm 0.0002	3.86 \pm 0.04	51.6 \pm 3.7	4.55 \pm 0.03	2.42 \pm 0.13

Each cell line was treated in triplicate for 72 hr.
WST-8 assay was used for obtaining IC₅₀ value.

Antitumor activity of NK012 and CPT-11 on subcutaneous U87MG/Luc xenografts

Potent antitumor activity was observed in mice treated with NK012 at 30 mg/kg *in vivo* (Fig. 1a). In mice treated with NK012, tumor volume started to decrease on day 5, and the tumor completely disappeared by day 23, with no relapse observed until 80 days after treatment. Although CPT-11 at 66.7 mg/kg/day exerted antitumor activity compared with the control group, tumor volume continued to increase consistently. Comparison of the relative tumor volume at day 8 revealed significant differences between the NK012-treated and CPT-11-treated groups ($p = 0.0095$). Although treatment-related body weight loss was observed in mice treated with each drug, body weight recovery was observed by day 19 (Fig. 1b). These results clearly show the significant *in vivo* activity of NK012 against the U87MG/Luc tumor xenograft.

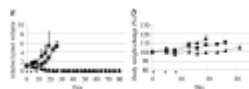


Figure 1. Effects of NK012 and CPT-11 on U87MG/Luc tumor xenograft. (a) Tumor volume in mice treated with CPT-11 or NK012. U87MG/Luc tumor was subcutaneously inoculated into the flank of mice, as described in the Materials and methods section. NaCl (0.9%) solution (●), CPT-11 at 66.7 mg/kg (■) and NK012 at 30 mg/kg (▲) were intravenously administered on days 0, 4 and 8 (arrows). Points, mean; bars, SD. * $p < 0.05$. (b) Treatment-related body weight loss occurred in mice treated with CPT-11 and NK012. Points, mean; bars, SD. [Normal View 22K | Magnified View 59K]

Studies on distribution of NK012 and CPT-11 in orthotopic U87MG/Luc tumor tissues

Both NK012 and CPT-11 formulations accumulated in the tumor tissue but not in the normal brain tissue (Fig. 2). However, the drug distribution pattern was clearly different between NK012 and CPT-11. In sections of the U87MG/Luc tumor treated with CPT-11, maximum drug accumulation was observed within 2 hr of CPT-11 injection. Twelve hours after the injection, fluorescence originating from CPT-11 had almost disappeared. Subsequently, no accumulation of CPT-11 was observed within the tumor tissues. However, in sections of the U87MG/Luc tumor treated with NK012, fluorescence from NK012 started appearing around tumor blood vessels 2 hr after intravenous injection and lasted until 24 hr. After 12 hr, the fluorescent area began to increase and the maximum fluorescence area was observed 24 hr after the injection.

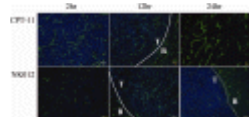
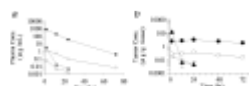


Figure 2. Distribution of NK012 or CPT-11 in U87MG/Luc glioma xenografts. Mice bearing U87MG/Luc tumor were injected with NK012 (30 mg/kg/day) or CPT-11 (66.7 mg/kg/day). Tumor tissues were excised 2, 12 and 24 hr after the intravenous injection of NK012 or CPT-11. Each mouse was administered fluorescein-labeled *Lycopersicon esculentum* lectin 5 min before sacrifice 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 477 nm. The same fluorescence conditions can be applied for visualizing NK012 and CPT-11 fluorescence. Free SN-38 could not be detected under these fluorescence conditions. The white lines indicate the border between the tumor and the brain tissue. T, U87MG/Luc tumor; B, normal brain tissue. (Scale bars: 20 μ m). [Normal View 52K | Magnified View 283K]

Pharmacokinetics analysis of NK012 and CPT-11 in mice bearing orthotopic U87MG/Luc xenografts

Microscopic observations were confirmed quantitatively by measuring the amount of SN-38 extracted from each solid tumor by reversed-phase HPLC. After CPT-11 injection, the concentrations of CPT-11 and free SN-38 in plasma decreased rapidly with time in a log-linear fashion. On the other hand, the plasma concentration of NK012 (polymer-bound SN-38) showed slower clearance than that of CPT-11. Free SN-38 released from NK012 also showed slow clearance than that of SN-38 converted from CPT-11 (Fig. 3a). Meanwhile, there was a significant difference in drug accumulation in the tumor between CPT-11 and NK012, that is, the accumulation of NK012 in the U87MG/Luc tumor was significantly higher than that of CPT-11 (Fig. 3b) and that the concentration of free SN-38 originating from NK012 was maintained at 24.6 ng/g even 72 hr after injection (Table II). On the other hand, only slight conversion from CPT-11 to SN-38 was observed from 2 to 24 hr in the U87MG/Luc tumor, and no SN-38 was detected thereafter (Fig. 3b). This result suggests that the BTB of the tumor was partially destroyed in the tumor vasculature and both drugs extravasated from the tumor blood vessels. In addition, these results indicate that NK012 can remain in the tumor tissue for a longer period and continue to release free SN-38.

Figure 3. Plasma, brain tissue and orthotopic tumor concentrations of respective analytes after intravenous administration of NK012 (30



mg/kg/day) and CPT-11 (66.7 mg/kg/day) to U87MG/Luc-bearing nude mice. (a) plasma; (b) tumor. ■, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); ▲, CPT-11; ▲, free SN-38 converted from CPT-11.

[Normal View 22K | Magnified View 59K]

Table II. Tumor and Plasma Concentration of SN38 After an I.V. Administration of NK012 (30 mg/kg) and CPT-11 (66.7mg/kg) to Nude Mice Bearing U87MG/Luc Brain Tumor

Formulation tested	Analyte	Time after administration (hr)			
		2	12	24	72
NK012	Free SN-38 Plasma (ng/ml)	1113	511	90.0	6.88
	Tumor (ng/g)	67.7	84.1	137	24.6
CPT-11	Free SN-38 5 Plasma (ng/ml)	62.0	4.74	1.97	ND
	Tumor (ng/g)	31.8	7.41	2.14	ND

Data were expressed as means of three mice.

Free SN-38; SN-38 released from NK012 or converted from CPT-11.

ND, not detectable.

Antitumor activity of NK012 and CPT-11 against orthotopic U87MG/Luc glioma xenografts

Antitumor activity was observed in mice treated with NK012 at 30 mg/kg/day and CPT-11 at 66.7 mg/kg/day *in vivo* (Fig. 4a). ANOVA analysis revealed a significant difference between the control group and the NK012-treated group ($p = 0.02$). However there was no significant difference between the control group and the CPT-11-treated group ($p = 0.23$) and between NK012 and CPT-11 ($p = 0.21$) (Fig. 4b). Comparison of the relative tumor volume at day 14 revealed significant differences between NK012 (30 mg/kg/day) and CPT-11 (66.7 mg/kg/day) ($p = 0.049$). Kaplan-Meier analysis showed that a significant improvement in survival rate was observed in the NK012 treatment group compared with the control ($p = 0.001$) and CPT-11 treatment groups ($p = 0.0014$) (Fig. 4c).

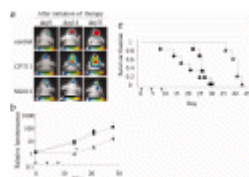


Figure 4. Antitumor effect of NK012 or CPT-11 on orthotopic xenograft and survival. Mice receiving intracranial injections of U87MG/Luc were assigned into groups 20 days after tumor inoculation. Mice were intravenously administered with 0.9% NaCl solution (■), NK012 (30 mg/kg/day, ▲) or CPT-11 (66.7 mg/kg/day, ■) on days 0 (20 days after tumor inoculation), 4 and 8 (arrows). (a) Representative luminescence intensity images obtained in individual control and treatment-group mice on the days indicated. (b) Antitumor effect of NK012 or CPT-11 on days 14, 21 and 28. Each group consisted of 4 mice. Points, mean; bars, SD. * $p < 0.05$. (c) Treatment effects of NK012 on survival. Survival was assessed by Kaplan-Meier analysis. Each group consisted of 6 mice. Experiments were repeated twice with similar results.

[Normal View 39K | Magnified View 114K]

Discussion



The diameter of a micelle carrier is approximately in the range of 10-100 nm, which is smaller than that of a liposome. Although this size is small, it is still sufficiently large to prevent renal secretion of the carrier. The micelle systems can evade nonspecific capture by the reticuloendothelial system in various organs because the outer shell of the micelle is covered with polyethyleneglycol. Therefore, drug-incorporating micelles can be expected to have a long plasma half-life, which permits a large amount of the micelles to reach tumor tissues, extravasate from tumor capillaries, and then be retained in tumor tissues for a long time by utilizing the enhanced permeability and retention (EPR) effect.[22]

Several factors are reportedly involved in vascular permeability in the body. Among them, bradykinin is the most potent vascular permeability factor. We succeeded in purifying 2 types of kinin from the ascitic fluid of a patient with gastric cancer.[23] We also clarified that this kinin generation system was triggered by the activated Hageman factor, an intrinsic coagulation factor XII.[24] Meanwhile, Dvorak et al. discovered that the vascular permeability factor (VPF) is involved in tumor vascular permeability.[25] Later, it was found that VPF was identical to VEGF.[26] Recently, an extrinsic coagulation factor, namely, a tissue factor, has been shown to activate VEGF production.[27][28] Thus, both intrinsic and extrinsic coagulation factors may be involved in tumor vascular permeability. Furthermore, there have been several reports to date indicating the increasing expression of a tissue factor in human glioma.[29][30] Also, it is well known that glioma is a typical

hypervascular tumor with an irregular vascular architecture and a high expression level of VEGF.[31] Therefore, it may be speculated that nanoparticles extravasate from tumor capillaries and accumulate more preferentially in brain glioma.

NK012, an SN-38-incorporating polymeric micelle, is a novel type of micellar formulation with long-time accumulation in tumors, and shows prolonged sustained release of SN-38 within the tumor.[17][20] We have thus far reported that NK012 shows significantly higher antitumor activity against various human tumor xenografts including small cell lung cancer,[17] colorectal cancer,[19] renal cancer,[18] and pancreatic cancer[20] compared with CPT-11. In addition, we have recently seen an increasing number of reports of clinical trials indicating the effectiveness of CPT-11 against brain glioma in combination with anti-VEGF antibody[11][12][32][33]

Under these circumstances, it may be reasonable to conduct an investigation into the advantages of administering NK012 over CPT-11 for treatment against human glioma tumor xenografts. In the present study, we showed that NK012 exerted a significant antitumor activity in U87MG/Luc subcutaneous xenografts (Fig. 1). In the tumor intravenously administered with NK012 (30 mg/kg), NK012 accumulated within and around tumor blood vessels in the orthotopic xenografts 2 hr after the injection. Thereafter, NK012 started to spread from the blood vessel within the tumor tissue of the xenografts. Fluorescence originating from NK012 then increased up to the maximum in the entire tumor by 24 hr after NK012 injection. On the other hand, fluorescence originating from CPT-11 increased up to the maximum 2 hr after its injection, indicating that the maximum distribution of CPT-11 was achieved within 2 hr of injection. Twelve hours after intravenous injection, fluorescence from CPT-11 had almost disappeared, and subsequently, no accumulation of CPT-11 was observed within the tumor tissues. The therapeutic effect of NK012 was superior to that of CPT-11 in terms of antitumor effect and survival. Because the antitumor activity of SN-38 is time-dependent, the superiority of NK012 over CPT-11 may be due to the enhanced accumulation of NK012 and the prolonged sustained release of SN-38 from NK012 within the tumor tissues. Nevertheless, free SN-38 was not detected in the normal brain tissues at any measurement time after intravenous injection of NK012 or CPT-11 (data not shown). It is thus speculated that both NK012 and CPT-11 are unable to cross the BBB in the normal brain, but can pass through tumor vessels effectively. In clinical brain glioma, however, when the tumor recurs, it would most likely recur in adjacent regions of the brain with an intact blood brain barrier. Namely, at the border between the brain tumor and normal brain tissue, malignant glioma cells and normal brain tissues intermix in the gradient and angiogenesis occurs at sites where there are large accumulation of tumor cells under local hypoxia.[34] In addition, there is no clear evidence if tumor vessels of orthotopic brain tumor xenografts are identical to those of real human brain tumors in terms of their structure and function. Therefore, it may be better to consider to conduct an investigation of the advantages of administering some anti-angiogenic inhibitors in combination with NK012 against highly invasive tumor models established by several methods such as direct implantation of patient surgical specimens into the brains of nude mice,[35] transplantation of patient surgical material s.c. in nude mice followed by dissociation and orthotopic reinjection of these xenotransplants,[36] engraftment of glioblastoma-derived spheroids after short-term culture into rat brain,[37] and engraftment of glioblastomastem cell-enriched cultures into mouse brain.[38][39]

The dose-limiting toxicities of CPT-11 appeared to be neutropenia and diarrhea. However, in our previous data, there was no significant difference in the level of SN-38 in the small intestine between NK012-treated and CPT-11-treated mice.[17] It was also reported that NK012 showed significant antitumor effect with diminishing incidence of diarrhea compared with CPT-11.[40] In 2 individual phase 1 trials in Japan and the US, no serious diarrhea has been reported.[41][42] In addition, one confirmed partial response (PR) was obtained in a patient with metastatic esophageal cancer in a Japanese trial,[41] and 3 PRs in a patient with breast cancer and 1 PR in a patient with small cell lung cancer in a US phase 1 trial.[42]

In conclusion, we demonstrated not only the enhanced accumulation, distribution, and retention of NK012 within glioma xenografts but also the superiority of the antitumor activity of NK012 compared with CPT-11. Taking the present data together with very recent clinical data from phase 1 trials, a phase 2 trial in patients with recurrent glioma may be warranted.

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REFERENCES



- 1 DeAngelis LM. Brain tumors. *N Engl J Med* 2001; **344**: 114-23. Links
- 2 Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK. The WHO classification of tumors of the nervous system. *J Neuropathol Exp Neurol* 2002; **61**: 215-25 discussion 26-9. Links
- 3 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005; **352**: 987-96. Links
- 4 Wong ET, Hess KR, Gleason MJ, Jaeckle KA, Kyritsis AP, Prados MD, Levin VA, Yung WK. Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials. *J Clin Oncol* 1999; **17**: 2572-8. Links
- 5 Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. *Cancer Res* 1972; **32**: 2643-50. Links
- 6 Gallo RC, Whang-Peng J, Adamson RH. Studies on the antitumor activity, mechanism of action, and cell cycle effects

- of camptothecin. *J Natl Cancer Inst* 1971; **46**: 789-95. Links
- 7 Friedman HS, Petros WP, Friedman AH, Schaaf LJ, Kerby T, Lawyer J, Parry M, Houghton PJ, Lovell S, Rasheed K, Cloughesy T, Stewart ES, et al. Irinotecan therapy in adults with recurrent or progressive malignant glioma. *J Clin Oncol* 1999; **17**: 1516-25. Links
 - 8 Cloughesy TF, Filka E, Kuhn J, Nelson G, Kabbinavar F, Friedman H, Miller LL, Elfring GL. Two studies evaluating irinotecan treatment for recurrent malignant glioma using an every-3-week regimen. *Cancer* 2003; **97**: 2381-6. Links
 - 9 Chamberlain MC. Salvage chemotherapy with CPT-11 for recurrent glioblastoma multiforme. *J Neurooncol* 2002; **56**: 183-8. Links
 - 10 Prados MD, Lamborn K, Yung WK, Jaeckle K, Robins HI, Mehta M, Fine HA, Wen PY, Cloughesy T, Chang S, Nicholas MK, Schiff D, et al. A phase 2 trial of irinotecan (CPT-11) in patients with recurrent malignant glioma: a North American brain tumor consortium study. *Neuro Oncol* 2006; **8**: 189-93. Links
 - 11 Vredenburgh JJ, Desjardins A, Herndon JE, II, Dowell JM, Reardon DA, Quinn JA, Rich JN, Sathornsumetee S, Gururangan S, Wagner M, Bigner DD, Friedman AH, et al. Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. *Clin Cancer Res* 2007; **13**: 1253-9. Links
 - 12 Vredenburgh JJ, Desjardins A, Herndon JE, II, Marcello J, Reardon DA, Quinn JA, Rich JN, Sathornsumetee S, Gururangan S, Sampson J, Wagner M, Bailey L, et al. Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. *J Clin Oncol* 2007; **25**: 4722-9. Links
 - 13 Gradishar WJ. Albumin-bound nanoparticle paclitaxel. *Clin Adv Hematol Oncol* 2005; **3**: 348-9. Links
 - 14 Muggia FM. Liposomal encapsulated anthracyclines: new therapeutic horizons. *Curr Oncol Rep* 2001; **3**: 156-62. Links
 - 15 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. Links
 - 16 Rothenberg ML, Kuhn JG, Burris HA, III, Nelson J, Eckardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI, et al. Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993; **11**: 2194-204. Links
 - 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. Links
 - 18 Sumitomo M, Koizumi F, Asano T, Horiguchi A, Ito K, Kakizoe T, Hayakawa M, Matsumura Y. Novel SN-38-incorporated polymeric micelle, NK012, strongly suppresses renal cancer progression. *Cancer Res* 2008; **68**: 1631-5. Links
 - 19 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. Links
 - 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. Links
 - 21 Kawato Y, Furuta T, Aonuma M, Yasuoka M, Yokokura T, Matsumoto K. Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother Pharmacol* 1991; **28**: 192-8. Links
 - 22 Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumor-tropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986; **46**: 6387-92. Links
 - 23 Maeda H, Matsumura Y, Kato H. Purification and identification of [hydroxypropyl]3]bradykinin in ascitic fluid from a patient with gastric cancer. *J Biol Chem* 1988; **263**: 16051-4. Links
 - 24 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. Links
 - 25 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. Links
 - 26 Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; **246**: 1306-9. Links
 - 27 Abe K, Shoji M, Chen J, Bierhaus A, Danave I, Micko C, Casper K, Dillehay DL, Nawroth PP, Rickles FR. Regulation of vascular endothelial growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. *Proc Natl Acad Sci USA* 1999; **96**: 8663-8. Links
 - 28 Belting M, Dorrell MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, Carmeliet P, Mueller BM, Friedlander M, Ruf W. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 2004; **10**: 502-9. Links
 - 29 Rong Y, Post DE, Pieper RO, Durden DL, Van Meir EG, Brat DJ. PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. *Cancer Res* 2005; **65**: 1406-13. Links
 - 30 Hamada K, Kuratsu J, Saitoh Y, Takeshima H, Nishi T, Ushio Y. Expression of tissue factor correlates with grade of malignancy in human glioma. *Cancer* 1996; **77**: 1877-83. Links
 - 31 Takano S, Yoshii Y, Kondo S, Suzuki H, Maruno T, Shirai S, Nose T. Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. *Cancer Res* 1996; **56**: 2185-90. Links
 - 32 Chowdhary S, Wong ET. Bevacizumab combined with irinotecan for recurrent glioblastoma multiforme-improvement over available therapy? *Nat Clin Pract Neurol* 2008; **4**: 242-3. Links

- 33 Chamberlain MC Bevacizumab plus irinotecan in recurrent glioblastoma. *J Clin Oncol* 2008; **26**: 1012-3. Links
- 34 Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 1992; **359**: 845-8. Links
- 35 Horten BC, Basler GA, Shapiro WR. Xenograft of human malignant glial tumors into brains of nude mice. A histopathological study. *J Neuropathol Exp Neurol* 1981; **40**: 493-511. Links
- 36 Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, Schroeder MA, James CD. Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. *Neuro Oncol* 2005; **7**: 164-76. Links
- 37 Engebraaten O, Hjortland GO, Hirschberg H, Fodstad O. Growth of precultured human glioma specimens in nude rat brain. *J Neurosurg* 1999; **90**: 125-32. Links
- 38 Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, Fiocco R, Foroni C, Dimeco F, Vescovi A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004; **64**: 7011-21. Links
- 39 Gunther HS, Schmidt NO, Phillips HS, Kemming D, Kharbanda S, Soriano R, Modrusan Z, Meissner H, Westphal M, Lamszus K. Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. *Oncogene* 2008; **27**: 2897-909. Links
- 40 Onda T, Nakamura I, Seno C, Matsumoto S, Kitagawa M, Okamoto K, Nishikawa K, Suzuki M. Superior antitumor activity of NK012, 7-ethyl-10-hydroxycamptothecin-incorporating micellar nanoparticle, to irinotecan [abstract 3062]. *Proc Am Assoc Cancer Res* 2006; **47**: 720s. Links
- 41 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 GI* 2008 (Abstract no 485). Links
- 42 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). Links