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74. Characterization of a novel anti-insoluble-fibrin chimeric antibody (AFCA) for cancer stromal target (CAST) therapy and diagnosis. Yohei Hisada, Masahiro Yasunaga, Yasuhiro Matsumura 2013 AACR Annual Meeting 2013/4/5 Washington DC
75. Development of CAST (cancer stromal targeting) therapy Masahiro Yasunaga, Takashi Sugino, Atsushi Tsuji, Tsuneo Saga, Shino Manabe, Yasuhiro Matsumura 2013 AACR Annual Meeting 2013/4/5 Washington DC
76. Development of CAST (Cancer stromal targeting) therapy. Masahiro Yasunaga, Shino Manabe, David Tarin, Yasuhiro Matsumura 4th International Conference on Tumor Progression and Therapeutic Resistance 2014/3/9 Boston

constituent factor of cancer stroma and anti-tumor compound. 発明人：松村保広 安永正浩 眞鍋史乃
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2. 発明の名称：不溶性フィブリンと結合しかつ可溶性フィブリン、フィブリン分解産物及びフィブリノーゲンと結合しない抗体を取得するための抗原及び取得されたモノクローナル抗体 発明人：松村保広 安永正浩、久田洋平 出願人：独立行政法人国立がん研究センター 特願2013-39625 (平成25年2月28日)
3. 発明の名称：抗 Tissue Factor モノクローナル抗体 発明人：松村保広、安永正浩、古賀宣勝、山本祥之、佐藤隆太、津村遼、片岡一則、西山伸宏、三浦裕、眞鍋史乃、加藤泰己、出願人：独立行政法人国立がん研究センター、国立大学法人東京大学、独立行政法人理化学研究所、ナノキャリア株式会社 特願2014-18586 (平成26年2月3日)

H. 知的財産権の出願・登録状況

1. 発明の名称：Novel cancer targeting therapy using complex of substance capable of binding specifically to

Clinical Cancer Research



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

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Cancer Therapy: Preclinical**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**Jun-ichiro Kuroda^{1,2}, Jun-ichi Kuratsu², Masahiro Yasunaga¹, Yoshikatsu Koga¹, Hirotsugu Kenmotsu¹, Takashi Sugino³, and Yasuhiro Matsumura¹**Abstract**

Purpose: To clarify the effect of bevacizumab on NK012 therapy in mice bearing U87MG glioblastoma orthotopic xenografts in comparison with the combination therapy of irinotecan hydrochloride (CPT-11) with bevacizumab.

Experimental Design: NK012 at 7-ethyl-10-hydroxycamptothecin (SN-38) equivalent dose of 30 mg/kg was administered intravenously three times every 4 days with or without bevacizumab. CPT-11 at 66.7 mg/kg was administered intravenously three times every 4 days or CPT-11 at 40 mg/kg/d over 5 consecutive days with or without bevacizumab. Bevacizumab was administered intraperitoneally six times every 4 days in each experiment. *In vivo* antitumor effects were evaluated by bioluminescence imaging, histopathologic evaluation, and immunohistochemistry. To evaluate interaction with bevacizumab, free SN-38 concentration in tumor tissues was examined by high-performance liquid chromatography.

Results: CPT-11 in combination with bevacizumab showed significantly more potent antitumor activity and longer survival than CPT-11 monotherapy ($P < 0.05$). However, there was no difference between NK012 monotherapy and NK012 in combination with bevacizumab. Concentration of free SN-38 released from NK012 in tumor tissue decreased in combination with bevacizumab ($P = 0.027$). NK012 monotherapy or NK012 with bevacizumab showed potent antitumor activity and longer survival than any dosing method of CPT-11 in combination with bevacizumab ($P < 0.05$). Orthotopic tumors treated with NK012 showed decreased tumor cellularity and lower Ki-67 index ($P < 0.001$) relative to those treated with CPT-11/bevacizumab.

Conclusions: The present study using orthotopic glioblastoma model in mice may warrant further preclinical evaluation of NK012 before conducting the clinical trial of the drug, because the antitumor activity of NK012 monotherapy was superior to the combination therapy of CPT-11 with bevacizumab. *Clin Cancer Res*; 16(2); 521–9. ©2010 AACR.

Malignant glioma, such as glioblastoma multiforme and anaplastic astrocytoma, are the most commonly occurring primary malignant brain tumors, and glioblastoma multiforme is well known as a typical hypervascular tumor with a high expression level of vascular endothelial growth factor (VEGF; ref. 1). Currently, glioblastoma multiforme patients have a mean survival time of only 50 weeks

following the standard treatment consisting of surgical and adjuvant therapies (2). However, a recent phase III randomized trial for newly diagnosed glioblastoma multiforme showed 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). Furthermore, several clinical trials have shown that the median survival time of patients with recurrence was only 30 weeks (4). Therefore, a novel antitumor agent based on a new approach for the recurrent malignant glioma is eagerly awaited.

7-Ethyl-10-hydroxycamptothecin (SN-38) is a broad-spectrum anticancer agent targeting DNA topoisomerase I. Irinotecan hydrochloride (CPT-11), a prodrug of SN-38, shows some antitumor activity in patients with recurrent glioblastoma multiforme, with response rates of 0 to 17% in several trials (5–8). CPT-11 single-agent chemotherapy activity is thus similar to that of other agents used for

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Translational Relevance

A recent phase II trial for recurrent glioblastoma multiforme showed that irinotecan hydrochloride (CPT-11) combined with bevacizumab is a promising and unprecedentedly effective treatment against the recurrent glioblastoma multiforme. However, there may be an increasing risk of developing venous thrombotic disease and intracranial hemorrhage with this combination therapy. The 7-ethyl-10-hydroxycamptothecin-incorporating polymeric micelle NK012 has been shown to have significant antitumor activity against several cancer mouse models compared with CPT-11. Two phase I trials in Japan and the United States showed that patients treated with NK012 did not develop grade 3/4 diarrhea, one of the major adverse effects of CPT-11. Here, NK012 showed potent antitumor activity and longer survival than CPT-11 in combination with bevacizumab in glioblastoma multiforme orthotopic tumor in mice. These results warrant clinical evaluation in patients with malignant glioma.

recurrent glioblastoma multiforme (7). Meanwhile, glioblastoma cells express high levels of VEGF *in situ*. Accordingly, antiangiogenic strategies may be a promising approach for malignant gliomas hypervascular in nature. A recent phase II trial for recurrent glioblastoma multiforme showed that CPT-11 combined with bevacizumab, an anti-VEGF monoclonal IgG1 antibody, is a promising and unprecedentedly effective treatment against the recurrent malignant glioma with a 6-month progression-free survival rate of 46% and a 6-month overall survival rate of 77% (9, 10). On the other hand, there may be an increased risk of developing venous thromboembolic disease and intracranial hemorrhage with this combination therapy. Therefore, it is reasonable to develop other available 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 of recurrent glioblastoma multiforme patients.

NK012, a SN-38-incorporating polymeric micelle, is a prodrug of SN-38 similar to CPT-11. Polymer-conjugated drugs categorized under drug delivery system agents are favorably extravasated from tumor vessels into the interstitium of tumors due to the enhanced permeability and retention effect (11, 12). The enhanced permeability and retention effect is based on the following pathophysiologic characteristics of solid tumor tissues: hypervascularity; incomplete vascular architecture; secretion of vascular permeability factors stimulating extravasation within cancer tissue; and absence of effective lymphatic drainage from the tumors, which impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Moreover, macromolecules cannot freely leak out from normal vessels; thus, the adverse effect of an anticancer

agent can be reduced. Very recently, we showed that NK012 exerted significantly more potent antitumor activity against several kinds of tumors including human glioma in xenograft models (13). In the present study, we report the antitumor activity of NK012 compared with CPT-11 combined with bevacizumab against orthotopic U87MG glioblastoma in nude mice.

Materials and Methods

Drugs. NK012 was supplied by Nippon Kayaku. Lyophilized NK012 was dissolved in sterile distilled water at a concentration of 5 mg/mL (SN-38 equivalent dose) just before administration to mice. The size of NK012 was ~20 nm in diameter with a narrow size distribution (12). CPT-11 was purchased from Yakult Honsha. Bevacizumab was purchased from Chugai Pharmaceutical.

Cell cultures. The human glioblastoma cell line U87MG was obtained directly from the American Type Culture Collection. A U87MG cell line clone stably expressing firefly luciferase named U87MG/Luc was established from polyclonal U87MG/Luc reported previously (13). The sensitivity of U87MG/Luc cells to NK012 and CPT-11 was almost similar to that of parental U87MG cells (data not shown). U87MG/Luc cells were maintained in DMEM supplemented with 10% fetal bovine serum (Cell Culture Technologies), penicillin, streptomycin, and amphotericin B (100 units/mL, 100 µg/mL, and 25 µg/mL, respectively; Sigma) in a humidified atmosphere containing 5% CO₂ at 37°C.

In vivo orthotopic model and imaging. Six- to 8-week-old female athymic BALB/c *nu/nu* mice (Charles River Japan) were used for this study. U87MG/Luc cells (5×10^5) suspended in 5 µL PBS were injected into the right frontal lobe of each mouse as described previously (13). *In vivo* bioluminescence imaging studies were done using the Photon Imager animal imaging system (BioSpace). For imaging, mice with intracranial U87MG/Luc tumor were simultaneously anesthetized with isoflurane, and D-luciferin potassium salt (Synchem) was intraperitoneally administered at a dose of 2.5 mg/mouse. For bioluminescence image analysis, regions of interest encompassing the intracranial area of a signal were defined using Photo Vision software (BioSpace), and the total number of photons per minute [counts/min (cpm)] was recorded. The pseudo-color luminescent image represented the spatial distribution of detected photon counts emerging from active luciferase within the animal. All animal procedures were done 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.

In vivo tumor growth inhibition assay. Eight days after inoculation of U87MG/Luc cells into the right hemisphere of the brain, mice with cpm >2,000 were randomly assigned into eight test groups of five mice

(2,296-15,624 cpm). After the randomization of mice based on cpm, we confirmed that the mean value of cpm was statistically identical between groups.

Treatment was started (day 0; Table 1). *In vivo* bioluminescence imaging was done by Photon Imager and luciferase activity was measured once a week (days 0, 7, 14, 21, 28, and 35). Body weight of each mouse of the treatment experiment was also measured once a week (days 0, 7, 14, 21, 28, and 35), and mortality and morbidity were checked daily from the day of treatment initiation. Simultaneously, to assess the survival of mice with intracranial U87MG/Luc tumor, mice were maintained until each animal showed signs of morbidity (20% weight loss and neurologic deficit), at which point they were sacrificed.

In all groups, the total dose of NK012 and CPT-11 was 90 mg/kg (SN-38 equivalent dose) and 200 mg/kg, respectively. A total dose of 200 mg/kg was shown previously to be the maximum tolerated dose (MTD) of CPT-11 in nude mice (14, 15). As CPT-11 is a schedule-dependent anticancer agent, it was administered under two different treatment regimens (16). Each drug was administered according to the mouse body weight: (a) normal 0.9% NaCl solution (q4d × 4, intravenously), (b) NK012 (30 mg/kg, q4d × 3, intravenously), (c) CPT-11 (66.7 mg/kg, q4d × 3, intravenously), (d) CPT-11 (40 mg/kg, qd × 5, intravenously), (e) bevacizumab (5 mg/kg, q4d × 6, intraperitoneally), (f) NK012 (30 mg/kg, q4d × 3, intravenously) with bevacizumab (5 mg/kg, q4d × 6, intraperitoneally), (g) CPT-11 (66.7 mg/kg, q4d × 3, intravenously) with bevacizumab (5 mg/kg, q4d × 6, intraperitoneally), or (h) CPT-11 (40 mg/kg, qd × 5, intravenously) with bevacizumab (5 mg/kg, q4d × 6, intraperitoneally) was administered to the mice (Table 1). In the case of combination therapy, drugs were administered concomitantly.

Evaluation of antitumor activity. The antitumor activity of each treatment was evaluated according to three criteria: (a) number of tumor regressions, (b) tumor growth delay, and (c) Kaplan-Meier analysis to determine the effect on the time to morbidity. Decrease >50% of the initial pho-

ton count (cpm) was defined as a tumor regression. It had to be observed for at least two consecutive photon-counting events to be retained. Tumor growth delay was defined as the difference in the median time to reach a photon count of 5-fold compared with that of day 0 between the treated group and the control group (14). To evaluate the change in photon count of each treatment group, repeated-measures ANOVA was carried out using the StatView 5.0 software package. $P < 0.05$ was regarded as significant. Statistical differences in the Kaplan-Meier curve of each group were ranked according to the Breslow-Gehan-Wilcoxon test using StatView 5.0.

Immunohistochemistry. Histologic sections were taken from U87MG/Luc orthotopic tumor tissues at day 15 from the initiation of each therapy. The time points for analysis were chosen according to when the best antitumor activity was obtained. The brain was removed from the skull, fixed in buffered 4% paraformaldehyde, embedded in paraffin, and then cut into 3- μ m-thick sections. Conventional H&E-stained sections were prepared for general histopathologic evaluation. Immunohistochemistry was done using antibodies to human Ki-67 (BD Pharmingen), human VEGF (Santa Cruz Biotechnology), and mouse CD34 (MEC 14.7; Abcam). For antigen retrieval, sections were autoclaved in Dako REAL Target Retrieval Solution (Dako Denmark). Detection was done by Vectastain Elite avidin-biotin complex kit (Vector Laboratories) for CD34 and EnVision⁺ system labeled polymer-horseradish peroxidase anti-mouse (DakoCytomation) for Ki-67 and anti-rabbit for VEGF. The proliferation index was evaluated by counting Ki-67⁺ cells per 1,000 tumor cells using ImagePro Plus analysis software. VEGF immunoreactivity area was quantified using the analysis software BZ Analyzer (Keyence) with a constant color threshold in 10 high-power fields per slide (×400) and is given in percent of positive area in field of view. Tumor vascularity was assessed by counting CD34⁺ microvessels in 10 high-power fields per slide (×400). The small intestine was sampled at 5 cm from the pyloric part for the jejunum and 5 cm

Table 1. Activity of three anticancer drugs against orthotopic U87MG xenografts

Treatment course	Dose (mg/kg/d)	Schedule	Total dose (mg/kg)	No. animals/group	Maximum body weight loss (%)	Toxic death	Tumor regression	Tumor growth delay (d)	Median overall survival (d)
Control	—	—	—	5	—	—	—	—	29
NK012	30	q4d × 3	90	5	3.8	0	3	>18	48
CPT-11	66.7	q4d × 3	200	5	6.6	0	0	8	36
CPT-11	40	qd × 5	200	5	2	0	1	8	34
Bevacizumab	5	q4d × 6	30	5	1.9	0	0	4	40
NK012/bevacizumab	30/5	q4d × 3/q4d × 6	90/30	5	0.9	0	1	12	46
CPT-11/bevacizumab	66.7/5	q4d × 3/q4d × 6	200/30	5	0	0	2	7	40
CPT-11/bevacizumab	40/5	qd × 5/q4d × 6	200/30	5	0.7	0	2	8	41

NOTE: Maximum body weight loss (days 0-28).

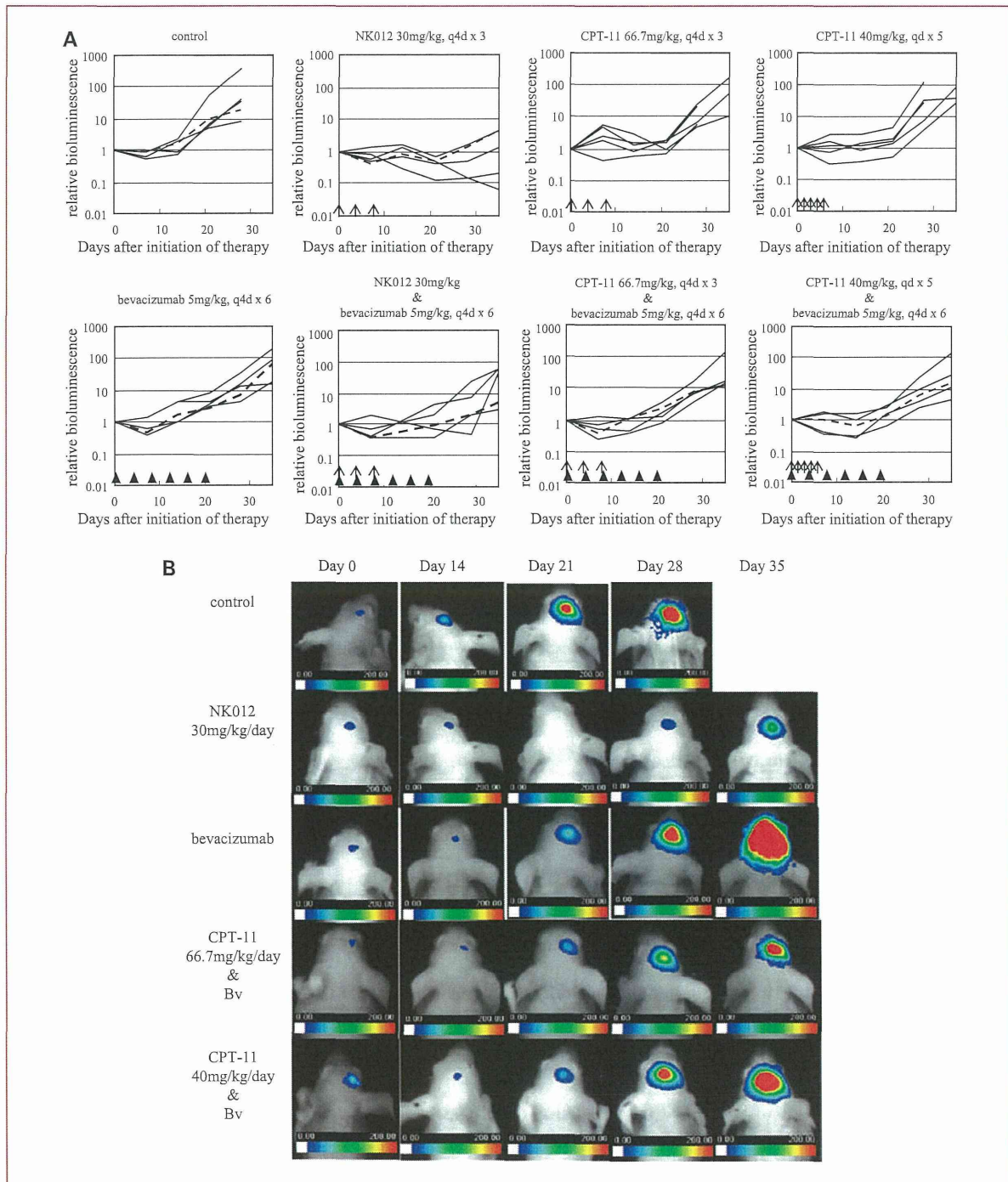


Fig. 1. Effects of NK012 and CPT-11 in U87MG/Luc mouse models. Cells were injected intracranially in athymic mice. Treatments were initiated 8 d after tumor inoculation with NK012, 30 mg/kg intravenously, thrice every 4 d; CPT-11, 67 mg/kg intravenously, thrice every 4 d; CPT-11, 40 mg/kg intravenously, daily over 5 consecutive d; bevacizumab, 5 mg/kg intraperitoneally, six times every 4 d; or both modalities and 0.9% NaCl solution control. †, NK012 or CPT-11 intravenous administration; ▲, bevacizumab intraperitoneal administration. *A*, antitumor activity of NK012 or CPT-11 was evaluated by counting the number of photons using the Photon Imager system. The discontinued lines in some of the graphs represent individuals that died during the experimental course and the subsequent assay was not conducted. The dashed lines correspond to the mice of each therapy expressed in *B*. *B*, images of U87MG/Luc mouse model treated with each regimen taken using the Photon Imager system on days 0, 14, 21, 28, and 35 after the initiation of therapy. Data derived from the same mice are expressed as dashed lines in *A*.

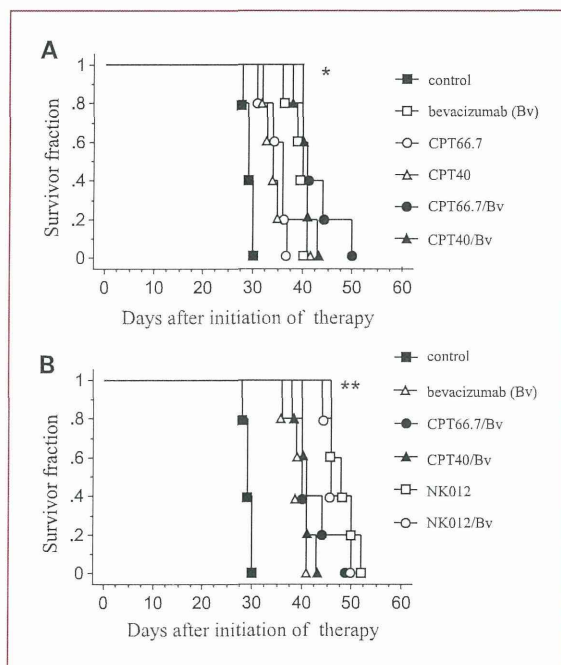


Fig. 2. Survival curves of U87MG/Luc mouse models in each regimen. A, ■, 0.9% NaCl solution; □, bevacizumab, 5 mg/kg, q4d × 6; ●, CPT-11, 66.7 mg/kg, q4d × 3 with bevacizumab, 5 mg/kg, q4d × 6; ○, CPT-11, 66.7 mg/kg, q4d × 3; △, CPT-11, 40 mg/kg, qd × 5. CPT-11/bevacizumab is significantly superior to CPT-11 monotherapy [CPT66.7 versus CPT66.7/bevacizumab ($P < 0.01$) and CPT40 versus CPT40/bevacizumab ($P < 0.05$)]. B, □, NK012, 30 mg/kg/d, q4d × 3; ○, NK012, 30 mg/kg/d with bevacizumab, 5 mg/kg, q4d × 6; △, bevacizumab, 5 mg/kg, q4d × 6; ■, 0.9% NaCl solution; ●, CPT-11, 66.7 mg/kg, q4d × 3 with bevacizumab, 5 mg/kg, q4d × 6; ▲, CPT-11, 40 mg/kg, qd × 5 with bevacizumab, 5 mg/kg, q4d × 6. NK012 monotherapy is significantly superior to CPT66.7/bevacizumab ($P < 0.01$) and CPT40/bevacizumab ($P < 0.05$).

from the ileocecal junction for the ileum. Samples were fixed in 10 formalin, embedded in paraffin, sectioned, and stained with H&E. Inflammation was scored by using an inflammation scale from - to ++, with - indicating absent inflammation, + indicating mild inflammation predominantly infiltrated with lymphocytes, and ++ indicating active inflammation infiltrated with lymphocytes and neutrophils. All histopathologic and immunohistologic analysis and interpretation were done directly by an experienced pathologist.

Pharmacokinetics study of NK012 and CPT-11 combined with bevacizumab. Four mice bearing U87MG/Luc tumor per group were used for the biodistribution analysis of NK012 and CPT-11. Twenty-eight days after the intracranial injection of U87MG/Luc cells, NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was intravenously administered with or without simultaneous intraperitoneal administration of bevacizumab (5 mg/kg) to the mice. After euthanasia, tumor tissues were obtained at T_{max} of each drug, 12 h after NK012 and 3 h after CPT-11 administration, respectively (12, 13). Each tumor was excised without the adjacent

normal brain tissue. The size of tumor was ~5 mm in diameter. The tumor samples were rinsed with 0.9% NaCl solution, mixed with 0.1 mol/L glycine-HCl buffer (pH 3.0)/methanol at 5% (w/w), and then homogenized using Precellys 24 (Bertin Technologies). The samples were vortexed vigorously for 10 s and then filtered through an Ultrafree-MC centrifugal filter device with a cutoff molecular diameter of 0.45 μ m (Millipore). We had confirmed that the filtered solution contained only free SN-38. Reverse-phase high-performance liquid chromatography was done at 35°C on a Mightysil RP-18 GP column 150 × 4.6 mm (Kanto Chemical). Fifty microliters of a sample were injected into an Alliance 2795 high-performance liquid chromatography system (Waters Associates) equipped with a Waters 2475 multi λ fluorescence detector. 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 for calibration against the corresponding peak area derived from the CPT internal standard. Peak data were recorded using a chromatography management system (MassLynx version 4.0; Waters Associates).

Statistical analysis. Data were expressed as mean \pm SD. Significance of differences was calculated using the unpaired two-tailed t test with StatView 5.0. $P < 0.05$ was regarded as statistically significant. Kaplan-Meier analysis was done to determine the antitumor activity of each treatment on the time to morbidity, and statistical differences were ranked according to the Breslow-Gehan-Wilcoxon test using StatView 5.0. To evaluate the change in photon count of each treatment group, repeated-measures ANOVA was done.

Results

Antitumor response of U87MG xenograft. NK012 at the MTD (30 mg/kg/d) administered thrice every 4 days proved to be the most active against xenografts, with three tumor regressions and tumor growth delay of >18 days (Table 1). In combination with bevacizumab, CPT-11 at the MTD (66.7 mg/kg/d) administered thrice every 4 days and at the MTD (40 mg/kg/d) administered daily over 5 consecutive days induced two tumor regressions and tumor growth delay of 7 and 8 days, respectively. Without bevacizumab, CPT-11 at the MTD (40 mg/kg) administered daily over 5 consecutive days induced one tumor regression (Table 1). Comparison of the relative photon counts on repeated-measures ANOVA (days 0-28) revealed significant differences in photon counts between mice treated with NK012 and those treated with CPT-11 (66.7 mg/kg/d, q4d × 3) in combination with bevacizumab ($P = 0.02$; Fig. 1A and B). Tendency of differences between mice treated with NK012 and those treated with CPT-11 (40 mg/kg/d, qd × 5) in combination with bevacizumab ($P = 0.14$) was observed (Fig. 1A and B).

The median overall survival time was most prolonged in the NK012 (30 mg/kg/d, q4d × 3) group for 1 week

compared with the CPT-11/bevacizumab group (Table 1). The CPT-11 group with bevacizumab showed longer median overall survival time compared with the CPT-11 monotherapy group with two schedules: 66.7 mg/kg/d, q4d \times 3, and 40 mg/kg/d, qd \times 5, respectively (Table 1). Kaplan-Meier analysis showed a significant survival benefit of the CPT-11/bevacizumab group compared with the CPT-11 monotherapy (66.7 mg/kg/d, q4d \times 3; $P = 0.004$) and CPT-11 (40 mg/kg/d, qd \times 5; $P = 0.036$), respectively (Fig. 2A). Furthermore, Kaplan-Meier analysis showed a significant survival benefit in the NK012 group compared with the CPT-11 (66.7 mg/kg/d, q4d \times 3)/bevacizumab group ($P = 0.046$) and CPT-11 (40 mg/kg/d, qd \times 5)/bevacizumab group ($P = 0.0041$), respectively (Fig. 2B). However, there was no significant difference between NK012/bevacizumab group and NK012 monotherapy group ($P = 0.45$; Fig. 2B). There was no severe

body weight loss or toxic death according to treatment (Table 1).

Histologic results. Histologic examinations revealed that decreased cellularity, increased tumor stroma, and inflammatory cell infiltration were observed in the tumors treated with NK012. Tumors treated with other regimens showed no apparent morphologic differences from the control tumors (Fig. 3A). Concordant with morphologic changes, the number of Ki-67⁺ tumor cells decreased in tumors treated with NK012 compared with CPT-11/bevacizumab (Fig. 3A and B; $P < 0.001$). Quantification of VEGF-positive area and microvessel density decreased in tumors treated with NK012 compared with other treatment regimens (Fig. 3A, C, and D; $P < 0.05$). Microvessel density dramatically decreased in tumors treated with bevacizumab in combination with any formulation of the anticancer drug (Fig. 3A and C). The small intestinal mucosa of mice treated

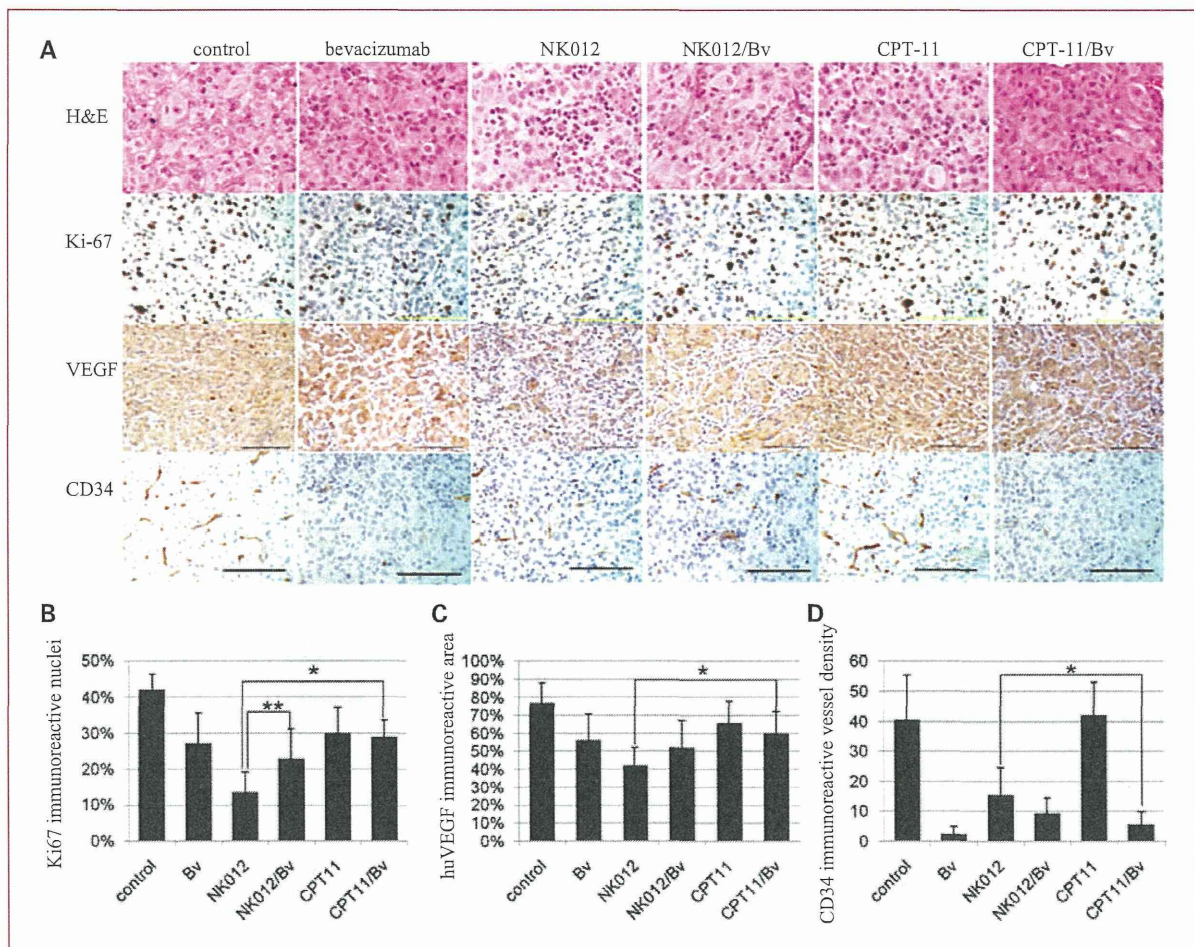


Fig. 3. Tissue-based studies of U87MG/Luc orthotopic xenografts of nude mice treated with NK012, CPT-11, bevacizumab, NK012/bevacizumab, or CPT-11/bevacizumab. *A*, H&E staining of representative xenograft regions (magnification, $\times 400$). Immunohistochemical analysis of tumor cells stained with anti-Ki-67 nuclear antigen, anti-VEGF, and angiogenesis with anti-CD34 antibody (bar, 100 μ m). Comparison between xenografts treated with each regimen. *B*, proliferation index by Ki-67. *C*, human VEGF immunoreactive area (%). *D*, angiogenesis by vessel density. *, $P < 0.01$; **, $P < 0.05$ (two-tailed Student's *t* test).

with NK012 or NK012/bevacizumab showed no histologic changes including fibrosis, active inflammation, or shortening and decrease in number of villi in the small intestinal mucosa as reported previously (ref. 17; data not shown).

Tissue concentration of free SN-38 after administration of NK012 and CPT-11 in combination with bevacizumab. The concentration of free SN-38 in orthotopic glioblastoma tissue after the administration of NK012 and CPT-11 in combination with bevacizumab was examined to evaluate the interaction between these anticancer agents. In the case of NK012 administration in combination with bevacizumab, free SN-38 concentration in tumor tissue was significantly decreased compared with SN-38 concentration when NK012 was administered alone (Fig. 4A; $P = 0.027$). On the other hand, in the case of CPT-11 administration in combination with bevacizumab, free SN-38 concentration in tumor tissue was almost similar to SN-38 concentration when CPT-11 was administered alone (Fig. 4B; $P = 0.66$).

Discussion

Concomitant chemoradiotherapy with surgery followed by single-agent adjuvant treatment with the alkylating agent temozolomide is the current standard of care for the patients with glioblastoma multiforme (3). However, tumor recurrence is experienced by almost all glioblastoma multiforme patients after the first-line therapy. Combination therapy of CPT-11 with bevacizumab is now a recognized second-line therapy in recurrent glioblastoma multiforme.

The main purpose of this study was to clarify the advantage of combination therapy of NK012, a SN-38-incorporating polymeric micelle, with bevacizumab against orthotopic U87MG glioblastoma multiforme tumor in mice. Single use of NK012 exerted superior antitumor activity in the orthotopic tumors compared with CPT-11 combined with bevacizumab. The NK012 single-agent treatment group showed the most prolonged survival of all treatment groups, and a statistically significant difference was revealed by the Kaplan-Meier analysis compared with the CPT-11/bevacizumab group (66.7 mg/kg/d, q4d \times 3; $P = 0.046$ and 40 mg/kg/d, qd \times 5; $P = 0.0041$).

The present study showed that the addition of the anti-VEGF monoclonal antibody bevacizumab to the CPT-11 therapy resulted in markedly increased activity, the same as reported clinically (9, 10). Although the mechanisms underlying the activities of bevacizumab remain unclear, the following factors are considered to be important: direct antiangiogenic effects and cytotoxics against vascular endothelial cells and other stromal elements, direct effects on tumor cells expressing VEGF receptors and stem cell-like glioma cells (18), and improvement of the delivery of anticancer drug by forced normalization of tumor vasculature (19). Bevacizumab is a humanized monoclonal antibody that does not cross-react with mouse VEGF, and the efficacy and toxicity in combination with cytotoxic drugs are not the same in mouse and human. In mice,

however, bevacizumab can react with VEGF secreted from human tumor xenograft and leads to tumor vessel decrease, reduction in vessel permeability and diameter (20), and decrease in interstitial fluid pressure in xenografts (21).

NK012, a novel SN-38-incorporating polymeric micelle, is a prodrug of SN-38 similar to CPT-11. Although CPT-11 is converted to SN-38 in tumors by carboxylesterase, the metabolic conversion rate is within 2% to 8% of the original volume of CPT-11 (22, 23). In contrast, the release rate of SN-38 from NK012 is 74% under physiologic pH conditions even without carboxylesterases (12). Recently, we showed that NK012 exerted significantly more potent antitumor activity against various human tumor xenografts compared with CPT-11 (12, 24–26). The diameter of a micelle carrier is in the approximate range of 10 to 100 nm. Although this size is small, it is still sufficiently large to prevent renal secretion of the carrier. The micelle system can evade nonspecific capture by the

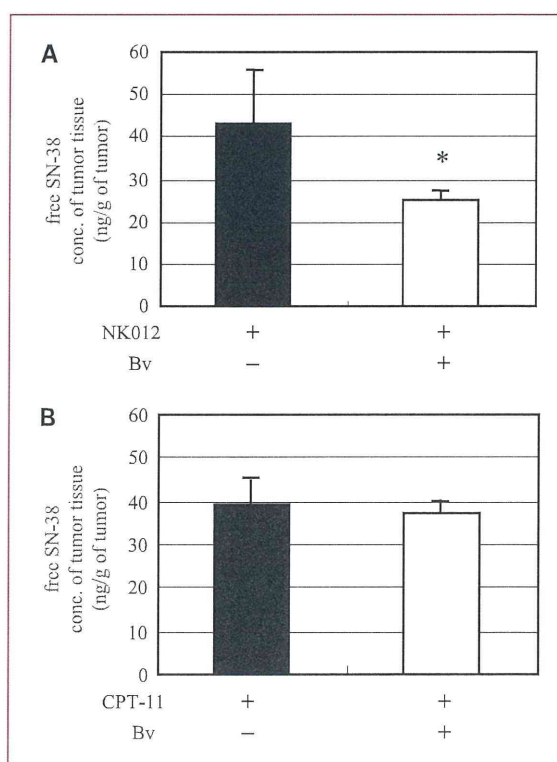


Fig. 4. Concentration of free SN-38 of tumor tissue at T_{max} . NK012 (30 mg/kg intravenously), CPT-11 (66.7 mg/kg intravenously), or bevacizumab (5 mg/kg intraperitoneally) was administered 28 d after intracranial injection of U87MG/Luc (columns, mean; bars, SD). **A**, concentration (conc.) of free SN-38 in glioma tissue of U87MG/Luc mouse model after administration of NK012 alone (black column) and NK012 with bevacizumab (white column). **B**, concentration of free SN-38 in glioma tissue of U87MG/Luc mouse model after administration of CPT-11 alone (black column) and CPT-11 with bevacizumab (white column). *, $P < 0.05$, significant to NK012 alone (two-tailed Student's t test).

reticuloendothelial system in various organs because the outer shell of the micelle is covered with polyethylene glycol. Therefore, a drug-incorporating micelle 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 using the enhanced permeability and retention effect (11). This prolonged retention of NK012 in the tumor and sustained release of free SN-38 from NK012 may be responsible for its more potent antitumor activity observed in the present study (27).

In this study, the antitumor effect was observed by means of bioluminescence imaging. Bioluminescence imaging revealed antitumor activity compared with CPT-11 in combination with bevacizumab. However, antiangiogenic agents can suppress the extravasation of contrast agents such as gadolinium, and gadolinium-contrast magnetic resonance imaging may give a false response (28). This means that bevacizumab is suspected of having a negative effect on bioluminescence imaging by reducing the permeability of tumor vessels. Therefore, we also evaluated the antitumor activity pathologically and immunohistochemically. Consequently, the NK012 group showed a high therapeutic advantage in Ki-67 index in a pathology analysis compared with the other treatment groups.

Interestingly, bevacizumab could not potentiate the antitumor activity of NK012 and elongate the survival of NK012. In the present pharmacologic study, the free SN-38 concentration in the tumor tissue decreased significantly when NK012 was administered in combination with bevacizumab compared with NK012 monotherapy. Results available to date and the results from the present study lead to the consideration that the reduced accumulation of NK012 by bevacizumab may cancel the direct effect of bevacizumab and NK012 against orthotopic glioma in mice. The influence of bevacizumab might be different in macromolecule such as NK012 and small compound such as CPT-11. Enhanced vascular permeability might be more important for macromolecule than small mole-

cule. A further study is necessary to clarify the phenomenon. Dose-limiting toxicities of CPT-11 are neutropenia and diarrhea. Diarrhea was not observed in the NK012 treatment group, and the intestinal toxicity was not observed by the pathology examination as reported previously (17). As for this, it is understood that serious diarrhea has not been reported in MTD of NK012 in two phase I clinical trials against advanced solid tumors in Japan and the United States (29, 30).

In conclusion, NK012 showed a higher therapeutic index in U87MG glioblastoma in mice compared with CPT-11/bevacizumab. Therefore, data from the present study may warrant further preclinical evaluation of NK012 before conducting the clinical trial of the drug.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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