

- polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res* 1991; 51: 3229-36.
- 6 Khayat D, Antoine EC, Coeffic D. Taxol in the management of cancers of the breast and the ovary. *Cancer Invest* 2000; 18: 242-60.
 - 7 Carney DN. Chemotherapy in the management of patients with inoperable non-small cell lung cancer. *Semin Oncol* 1996; 23: 71-5.
 - 8 Weiss RB, Donehower RC, Wiernik PH *et al.* Hypersensitivity reactions from taxol. *J Clin Oncol* 1990; 8: 1263-8.
 - 9 Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med* 1995; 332: 1004-14.
 - 10 Rowinsky EK, Chaudhry V, Forastiere AA *et al.* Phase I and pharmacologic study of paclitaxel and cisplatin with granulocyte colony-stimulating factor: neuromuscular toxicity is dose-limiting. *J Clin Oncol* 1993; 11: 2010-20.
 - 11 Wasserheit C, Frazein A, Oratz R *et al.* Phase II trial of paclitaxel and cisplatin in women with advanced breast cancer: an active regimen with limiting neurotoxicity. *J Clin Oncol* 1996; 14: 1993-9.
 - 12 Hamaguchi T, Matsumura Y, Suzuki M *et al.* NK105, a paclitaxel-incorporating micellar nanoparticle formulation, can extend in vivo antitumor activity and reduce the neurotoxicity of paclitaxel. *Br J Cancer* 2005; 92: 1240-6.
 - 13 Hamaguchi T, Kato K, Matsumura Y *et al.* A Phase I and Pharmacokinetic Study of NK105, a Paclitaxel-incorporating Micellar Nanoparticle Formulation. *Br J Cancer* 2007; 97: 170-6.
 - 14 Horwich A, Sleijfer DT, Fossa SD *et al.* Randomized trial of bleomycin, etoposide, and cisplatin compared with bleomycin, etoposide, and carboplatin in good-prognosis metastatic nonseminomatous germ cell cancer: a Multiinstitutional Medical Research Council/European Organization for Research and Treatment of Cancer Trial. *J Clin Oncol* 1997; 15: 1844-52.
 - 15 Roth BJ. Chemotherapy for advanced bladder cancer. *Semin Oncol* 1996; 23: 633-44.
 - 16 Pinzani V, Bressolle F, Haug JJ, Galtier M, Blayac JP, Balmes P. Cisplatin-induced renal toxicity and toxicity-modulating strategies: a review. *Cancer Chemother Pharmacol* 1994; 35: 1-9.
 - 17 Cleare MJ, Hydes PC, Malerbi BW, Watkins DM. Anti-tumor platinum complexes. relationships between chemical properties and activity. *Biochimie* 1978; 60: 835-50.
 - 18 du Bois A, Luck HJ, Meier W *et al.* A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. *J Natl Cancer Inst* 2003; 95: 1320-9.
 - 19 Cassidy J, Taberner J, Twelves C *et al.* XELOX (capecitabine plus oxaliplatin): active first-line therapy for patients with metastatic colorectal cancer. *J Clin Oncol* 2004; 22: 2084-91.
 - 20 Horwich A, Sleijfer DT, Fossa SD *et al.* Randomized trial of bleomycin, etoposide, and cisplatin compared with bleomycin, etoposide, and carboplatin in good-prognosis metastatic nonseminomatous germ cell cancer: a Multi-institutional Medical Research Council/European Organization for Research and Treatment of Cancer Trial. *J Clin Oncol* 1997; 15: 1844-52.
 - 21 Bellmunt J, Ribas A, Eres N *et al.* Carboplatin-based versus cisplatin-based chemotherapy in the treatment of surgically incurable advanced bladder carcinoma. *Cancer* 1997; 80: 1966-72.
 - 22 Nishiyama N, Okazaki S, Matsumura Y *et al.* Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice. *Cancer Res* 2003; 63: 8977-83.
 - 23 Uchino H, Matsumura Y, Negishi T *et al.* Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats. *Br J Cancer* 2005; 93: 678-87.
 - 24 Wilson RH, Plummer R, Adam J *et al.* Phase I and pharmacokinetic study of NC-6004, a new platinum entity of cisplatin-conjugated polymer forming micelles. *Am Soc Clin Oncol* 2008; (Abstract# 2573).
 - 25 Saltz LB, Cox JV, Blanke C *et al.* Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. *Irinotecan Study Group N Engl J Med* 2000; 343: 905-14.
 - 26 Douillard JY, Cunningham D, Roth AD *et al.* Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 2000; 355: 1041-7.
 - 27 Noda K, Nishiwaki Y, Kawahara M *et al.* Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002; 346: 85-91.
 - 28 Negoro S, Masuda N, Takada Y *et al.* CPT-11 Lung Cancer Study Group West. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. *Br J Cancer* 2003; 88: 335-41.
 - 29 Bodurka DC, Levenback C, Wolf JK *et al.* Phase II trial of irinotecan in patients with metastatic epithelial ovarian cancer or peritoneal cancer. *J Clin Oncol* 2003; 21: 291-7.
 - 30 Takimoto CH, Arbuck SG. Topoisomerase I targeting agents: the camptothecins. In: Chabner BA, Lango DL, eds. *Cancer Chemotherapy and Biotherapy: Principal and Practice*, 3rd edn. Philadelphia (PA): Lippincott Williams & Wilkins, 2001: 579-646.
 - 31 Slatter JG, Schaaf LJ, Sams JP *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.
 - 32 Rothenberg ML, Kuhn JG, Burris HA 3rd *et al.* Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993; 11: 2194-204.
 - 33 Guichard S, Terret C, Hennebelle I *et al.* CPT-11 converting carboxylesterase and topoisomerase activities in tumor and normal colon and liver tissues. *Br J Cancer* 1999; 80: 364-70.
 - 34 Koizumi F, Kitagawa M, Negishi T *et al.* Novel SN-38-incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor-secreting bulky tumors. *Cancer Res* 2006; 66: 10048-56.
 - 35 Saito Y, Yasunaga M, Kuroda J, Koga Y, Matsumura Y. Enhanced distribution of NK012 and prolonged sustained-release of SN-38 within tumors are the key strategic point for a hypovascular tumor. *Cancer Sci* 2008; 99: 1258-64.
 - 36 Nakajima T, Yasunaga M, Matsumura Y *et al.* 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: 22148-53.
 - 37 Sumitomo M, Koizumi F, Asano T *et al.* Novel SN-38-incorporated polymeric micelles, NK012, strongly suppress renal cancer progression. *Cancer Res* 2008; 2008; 122: 2148-53.
 - 38 Kuroda J, Kuratsu J, Yasunaga M, Koga Y, Matsumura Y. Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. *Int J Cancer* 2009; in press.
 - 39 Nakajima-Eguchi T, Yanagihara K *et al.* 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; in press.
 - 40 Kato K, Hamaguchi T, Shirao K *et al.* Phase I study of NK012, polymer micelle SN-38, in patients with advanced cancer. *Proc Am Soc Clin Oncol GI* 2008; (Abstract#485).
 - 41 Burris III HA, Infante JR, Spigel DR *et al.* A phase I dose-escalation study of NK012. *Proc Am Soc Clin Oncol* 2008; (Abstract#2538).
 - 42 Nishiyama N, Kataoka K. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol Ther* 2006; 112 (3): 630-48.
 - 43 Cabral H, Nishiyama N, Okazaki S, Koyama H, Kataoka K. Preparation and biological properties of dichloro (1,2-diaminocyclohexane) platinum (II) (DACHPt)-loaded polymeric micelles. *J Control Release* 2005; 101 (1-3): 223-32.
 - 44 Cabral H, Nishiyama N, Kataoka K. Optimization of (1,2-diaminocyclohexane) platinum (II)-loaded polymeric micelles directed to improved tumor targeting and enhanced antitumor activity. *J Control Release* 2007; 121 (3): 146-55.
 - 45 Bae Y, Fukushima S, Harada A, Kataoka K. Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: polymeric micelles that are responsive to intracellular pH change. *Angew Chem Int Ed* 2003; 42 (38): 4640-3.
 - 46 Kano MR, Bae Y, Iwata C *et al.* Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-beta signaling. *P Natl Acad Sci USA* 2007; 104 (9): 3460-5.
 - 47 Stapert HR, Nishiyama N, Jiang D-L, Aida T, Kataoka K. Polyion complex micelles encapsulating light-harvesting ionic dendrimer zinc porphyrins. *Langmuir* 2000; 16 (21): 8182-8.
 - 48 Kataoka K, Togawa H, Harada A, Yasugi K, Matsumoto T, Katayose S. Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. *Macromolecules* 1996; 29 (26): 8556-7.
 - 49 Matsumoto S, Christie RJ, Nishiyama N *et al.* Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules* in press.
 - 50 Miyata K, Oba M, Kano MR *et al.* Polyplex micelles from triblock copolymers composed of tandemly aligned segments with biocompatible, endosomal escaping, and DNA-condensing functions for systemic gene delivery to pancreatic tumor. *Pharm. Res* in press.

NK012

Apoptosis Inducer
Oncolytic

Nanosized-micellar particle containing 7-ethyl-10-hydroxycamptothecin (EHC), consisting of poly(ethylene glycol)-poly(glutamic acid) block copolymer chemically bound to EHC

EN: 417411

ABSTRACT

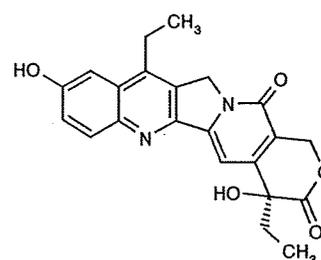
Selective tumor targeting by therapeutic agents is a long-standing pharmacological goal to improve selectivity and therapeutic indices. Most scientists have sought to use active receptor-mediated tumor-targeting systems, although the passive targeting afforded by the enhanced permeability and retention (EPR) effect provides a versatile and nonsaturable opportunity for tumor-selective delivery. Polymeric micelles are ideally suited to exploit the EPR effect, and they have been used for the delivery of a range of anticancer drugs in preclinical and clinical studies. NK012 is an SN-38-loaded polymeric micelle constructed in an aqueous milieu by the self-assembly of an amphiphilic block copolymer, PEG-PGlu(SN-38). Recently, we have demonstrated that NK012 exerts significantly more potent antitumor activity against various human tumor xenografts than irinotecan (CPT-11). Preclinical and clinical studies of NK012 up to the present are reviewed here.

BACKGROUND

Nanotechnology is one of the fastest moving technologies and is presently contributing significantly to the progress of medical science. Drugs categorized under the drug delivery system (DDS) are prepared primarily by utilizing nanotechnology. In the field of oncology, DDS drugs have been prepared and evaluated in preclinical and/or clinical trials, with some already approved for clinical use (Table I). More specifically, DDS can be used for active or passive targeting of tumor tissues. Active targeting refers to the development of monoclonal antibodies directed against tumor-related molecules, allowing targeting of a tumor from the specific binding of antibodies with respective antigens. However, the application of DDS using monoclonal antibodies is restricted to tumors expressing high levels of related antigens. Passive targeting can be achieved by utilizing the enhanced permeability and retention (EPR) effect (1, 2). This effect is based on the pathophysiological characteristics of solid tumor tissues, namely, hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues (Fig. 1A, B).

Several techniques have been developed to maximally utilize the EPR effect, including modification of drug structures and the development of drug carriers. Polymeric micelle-based anticancer drugs were originally developed by Kataoka et al. in the late 1980s and early 1990s (3-5). Polymeric micelles were expected to increase the accumulation of drugs in tumor tissues by utilizing the EPR effect, as well as to incorporate various kinds of drugs into their inner core with relatively high stability by chemical conjugation or physical entrapment. Also, the size of micelles can be controlled within the diameter range of 20-100 nm to ensure that they do not penetrate normal vessel walls. With this development, it is expected that the incidence of drug-induced side effects may be decreased owing to reduced drug distribution in normal tissues.

Irinotecan hydrochloride (CPT-11) has been demonstrated to be active against colorectal, lung and ovarian cancers (6-10). CPT-11 is a prodrug that is converted to 7-ethyl-10-hydroxycamptothecin (SN-38, **1**), a biologically active metabolite of CPT-11, by carboxylesterases. SN-38 is an analogue of the plant alkaloid camptothecin, which targets DNA topoisomerase I. SN-38 exhibits up to 1,000-fold more potent cytotoxic activity against various cancer cells in vitro than CPT-11 (11). Although CPT-11 is converted to SN-38 in the liver and tumors, the metabolic conversion rate is less than 10% of the original volume of CPT-11 (12, 13). Moreover, the conversion of CPT-11 to SN-38 depends on the genetic interindividual variability of car-



SN-38 (1)

Y. Matsumura.

Investigative Treatment Division, Research Centre for Innovative Oncology, National Cancer Center Hospital, Kashiwa, Japan. Correspondence: ymatsum@east.ncc.go.jp

Table 1. Examples of DDS in oncology and their stage of development.

Name	Platform	Compound	Clinical stage
<i>Passive targeting</i>			
NK-105	Micelles	Paclitaxel	II
NC-6004	Micelles	Cisplatin	I/II
NK012	Micelles	SN-38	II
Zinostatin stimalamer (SMANCS)	Polymer conjugate	Neocarzinostatin	Launched
Doxil	Liposome	Doxorubicin	Launched
Abraxane	Albumin-coated nanoparticle	Paclitaxel	Launched
Opaxio	Polymer conjugate	Paclitaxel	Prereg.
CT-2106	Polymer conjugate	Camptothecin	II
EndoTAG	Cationic liposome	Paclitaxel	II
Mureletecan	Polymer conjugate	Camptothecin	I
LE-SN-38	Liposome	SN-38	II
PK1	Polymer conjugate	Doxorubicin	II
IT-101	Polymer conjugate	Camptothecin	II
SP-1049C	Micelles	Doxorubicin	II
CPX-1	Liposome	CPT-11, floxuridine	II
<i>Active targeting</i>			
Mylotarg	Anti-CD33 antibody	Calicheamicin	Launched
Zevalin	Anti-CD20 antibody	90Y	Launched
Bexxar	Anti-CD20 antibody	¹³¹ I	Launched
PK2	Galactose-polymer	Doxorubicin	I
MCC-465	Antibody liposome	Doxorubicin	I
MBP-426	Transferrin-liposome	Oxaliplatin	I
CALAA-01	Transferrin-polymer	siRNA	I
Trastuzumab-DM1	Anti-HER2 antibody	DM1	III

boxylesterase activity (14). Thus, further efficient use of SN-38 might be of great advantage and may be attractive for cancer treatment. The progress in the manufacturing technology of "micellar nanoparticles" may make it possible to use SN-38 for in vivo experiments and further clinical use.

NK012 is an SN-38-incorporating polymeric micelle that can accumulate selectively in solid tumor tissues utilizing the EPR effect. The micelle is constructed in an aqueous milieu by the self-assembly of an amphiphilic block copolymer, PEG-PGLu(SN-38) (15). NK012 was obtained as a freeze-dried formulation and contained about 20% (w/w) of SN-38. The mean particle size of NK012 is 20 nm in diameter, with a relatively narrow range (Fig. 2A). The release rates of SN-38 from NK012 in phosphate-buffered saline (PBS) at 37 °C were 57% and 74%, respectively, at 24 and 48 h, and in 5% glucose solution at the same temperature and times 1% and 3%, respectively (Fig. 2B). These results indicate that NK012 can release SN-38 under neutral conditions even without a hydrolytic enzyme, and is stable in 5% glucose solution. Thus, NK012 is suggested to be stable before administration and starts to release SN-38 gradually under physiological conditions following administration.

PRECLINICAL PHARMACOLOGY

Following CPT-11 injection, the plasma concentrations of CPT-11 and SN-38 rapidly decrease with time in a log-linear fashion. On the other hand, NK012 (polymer-bound SN-38) exhibited slower clearance. In tumor xenografts, NK012 clearance was significantly slower and the free SN-38 concentration was maintained for a long time following injection (15, 16). Interestingly, there was no significant dif-

ference in the kinetic characteristics of free SN-38 in the small intestine between mice treated with NK012 and CPT-11.

Deviating from the usual experimental tumor models, tumors were allowed to grow until they became very large (around 1.5 cm) and treatment was then initiated. NK012 showed potent antitumor activity against bulky small cell lung cancer SBC-3/Neo tumors compared with CPT-11. Striking antitumor activity was observed in mice treated with NK012 when its antitumor activity was compared with CPT-11 using SBC-3/VEGF cells. In the clinical setting, CPT-11/5-fluorouracil (5-FU) combination therapy is now a standard regimen for colorectal cancer (6, 7). It was therefore speculated that the use of NK012 in place of CPT-11 in combination with 5-FU might yield superior results. As expected, the therapeutic effect of NK012/5-FU was significantly superior to that of CPT-11/5-FU against human colon adenocarcinoma HT-29 xenografts ($P = 0.0004$) (17). In other tumors, such as renal cancer (18), glioma (19), gastric cancer (20) and pancreatic cancer (16), NK012 exerted significantly superior antitumor activity and was associated with longer survival compared with CPT-11. In an orthotopic glioma xenograft model, both NK012 and CPT-11 appeared to be able to effectively extravasate from the blood-brain tumor barrier but not from normal brain vessels (Fig. 2C) (19). In human pancreatic adenocarcinoma Capan-1 tumor xenografts, a hypovascular tumor model, it was also demonstrated that NK012 showed significantly more potent antitumor activity than CPT-11. Pharmacological examination revealed that only a slight conversion of SN-38 from CPT-11 was observed from 1 h to 24 h, and no SN-38 was detected thereafter. On the other hand, SN-38 released from NK012 continued to be detected from 1 h to 96 h fol-

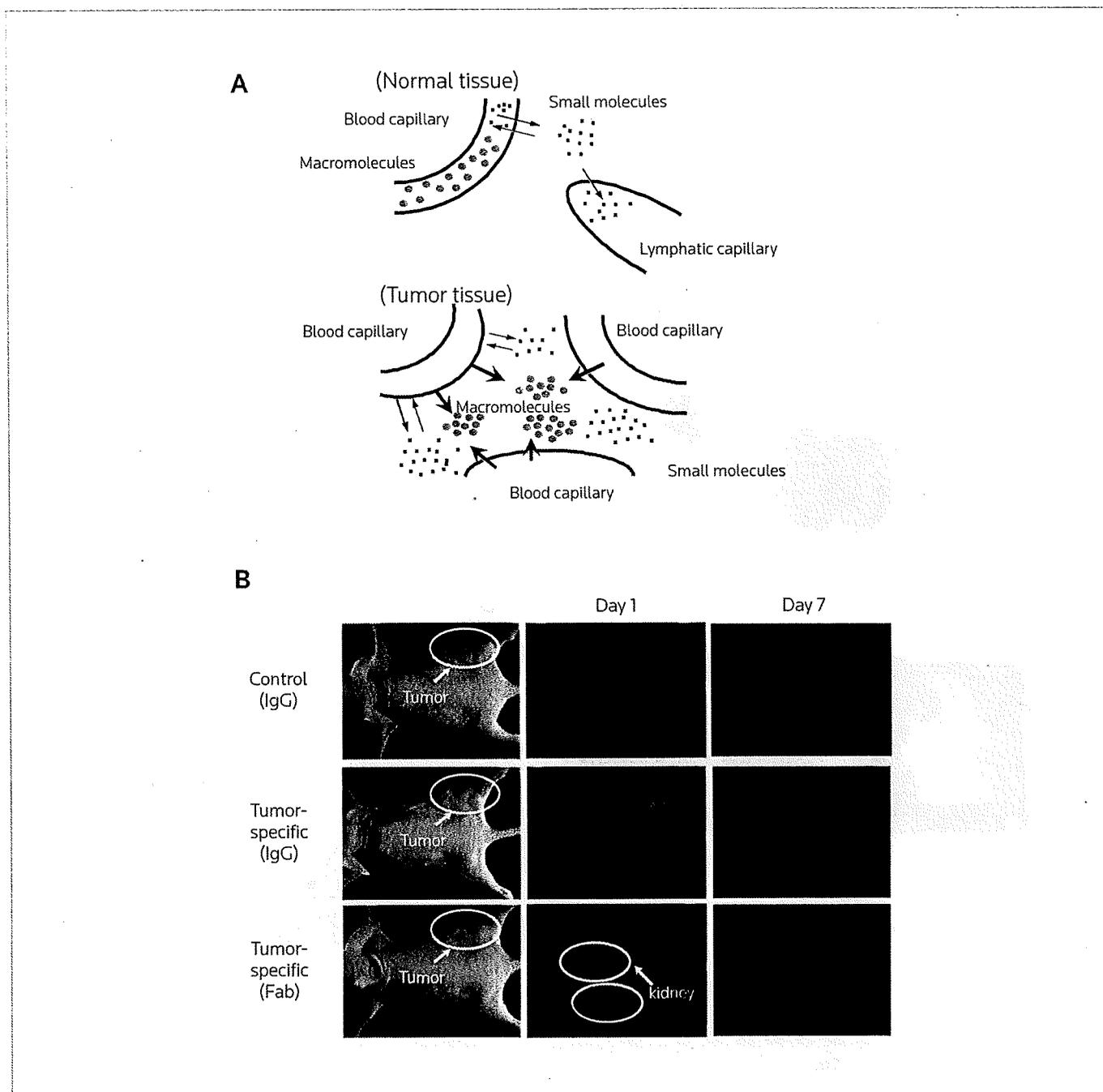


Figure 1. A. A diagram of normal and tumor tissue demonstrating the presence of a lymphatic duct in normal tissue (upper) but the absence of any lymphatic duct in tumor tissue (lower). Small molecules easily leak from normal vessels in the body, which endows them with a short plasma half-life. On the other hand, macromolecules have a long plasma half-life because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system in various organs. In the solid tumor tissues shown in the lower panel, it was found that solid tumors generally possess several pathophysiological characteristics: hypervascularity, secretion of vascular permeability factors stimulating extravasation of macromolecules within the cancer, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. These characteristics of solid tumors are the basis of the enhanced permeability and retention effect, or the EPR effect. **B.** In vivo imaging demonstrated that both control whole IgG and specific whole monoclonal antibody (mAb) accumulated selectively in the tumor tissue on day 1 after i.v. injection. On day 7, a greater degree of retention of the specific whole mAb as compared to the control IgG was noted. On the other hand, the F(ab) region of the specific mAb with a molecular weight of 50,000 accumulated in the tumor to the same extent as the control whole IgG. Interestingly, fluorescence of the F(ab) could also be detected in both kidneys, which implied that the F(ab) could easily pass through the kidney glomerulus. This accumulation of the control IgG in the tumor represents the EPR effect. The findings suggest that not only the specific affinity of the mAb, but also the size of the molecules and the stability of the molecules in blood, are important for tumor-selective targeting.

lowing NK012 injection (16) (Fig. 2D). Thus, NK012, which combines enhanced distribution with sustained release of SN-38 within tumors, is ideal for the treatment of hypovascular tumors since the antitumor activity of SN-38 is time-dependent.

CLINICAL STUDIES

Two independent phase I clinical trials have been conducted at the National Cancer Center in Japan (21) and the Sarah Cannon Cancer Center in the U.S. (22) in patients with advanced solid tumors to

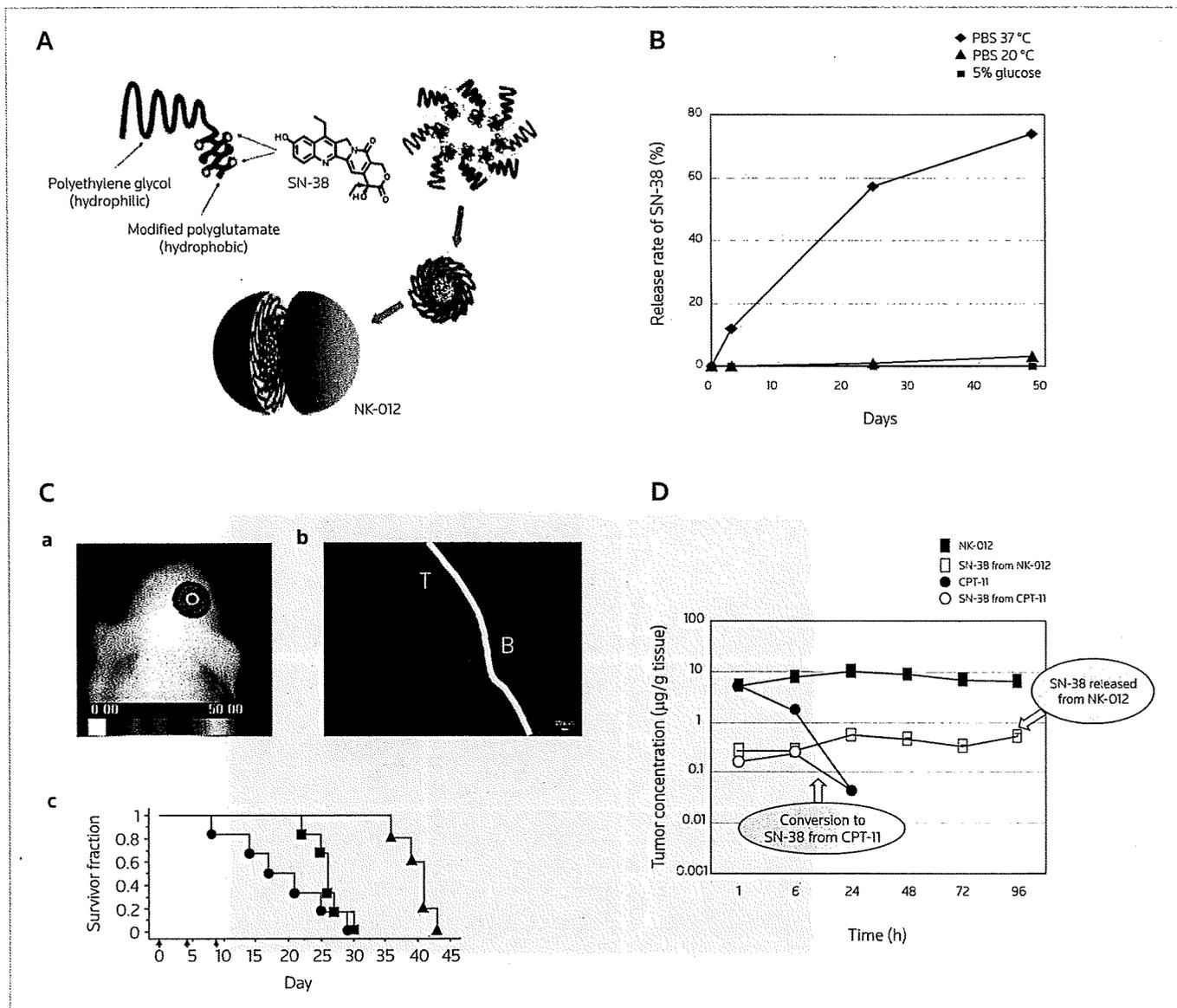


Figure 2. A. Schematic structure of NK012. A polymeric micelle carrier of NK012 consists of a block copolymer of polyethylene glycol (PEG; molecular weight of about 12,000) and partially modified polyglutamate (about 20 units). PEG (hydrophilic) is believed to be the outer shell and SN-38 is incorporated into the inner core of the micelle. B. The release rates of SN-38 from NK012 in phosphate-buffered saline (PBS) were 74% at 48 h but only 3% at 48 h in 5% glucose solution. It is therefore suggested that NK012 is stable in 5% glucose solution before administration and starts to release SN-38 gradually under physiological conditions after administration. C. a) An orthotopic glioma model. Twenty days after U-87 MG/Luc inoculation, the maximum tolerated dose (MTD) of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was injected i.v. into the tail vein of mice. b) 24 h after NK012 (blue) injection, mice were also administered fluorescein *Lycopersicon esculentum* lectin (100 μL/mouse) to visualize tumor blood vessels. T, tumor; B, normal brain. c) NK012 (30 mg/kg/day), CPT-11 (66.7 mg/kg/day) (■) and saline (●) were given i.v. on days 0 (20 days after tumor inoculation), 4 and 8 (▼). 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. D. Tumor distribution of CPT-11, NK012 (or polymer-bound SN-38) and free SN-38 after administration of NK012 and CPT-11 to mice bearing human pancreatic adenocarcinoma Capan-1 (a) or PSN-1 (b) xenografts. The time profiles of polymer-bound SN-38 (■), free SN-38 released from NK012 (□), CPT-11 (●) and free SN-38 converted from CPT-11 (○) were obtained by high-performance liquid chromatographic (HPLC) analysis. The time points examined were 1, 6, 24, 48, 72 and 96 h after administration of CPT-11 or NK012.

define the maximum tolerated dose (MTD), dose-limiting toxicity (DLT) and recommended phase II dose. NK012 was infused i.v. over 30 min every 21 days until disease progression or unacceptable toxicity. The MTD was 37 mg/m² in the U.S. and 28 mg/m² in Japan. The recommended dose was the same (28 mg/m²) in both countries. DLT was mostly neutropenia or related events, and diarrhea was mild. The pharmacokinetic profile in the U.S. study was similar to that in the Japanese study. Antitumor activity was also promising. Partial responses were obtained in three patients with triple-negative breast cancer, one patient with esophageal cancer, one patient with small cell lung cancer and one patient with lung carcinoid. Phase II studies in patients with triple-negative breast and colorectal cancers are being planned in the U.S. and Japan, respectively.

DISCLOSURE

This work was supported by Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare of Japan, a Grant-in-Aid for Scientific Research or Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, and Japanese Foundation for Multidisciplinary Treatment of Cancer and the Princess Takamatsu Cancer Research Fund (07-23908). The author declares no potential conflict of interest.

REFERENCES

1. Matsumura, Y., Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumorotropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Res* 1986, 46(12, Pt. 1): 6387-92.
2. Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. *J Control Release* 2000, 65(1-2): 271-84.
3. Kataoka, K., Kwon, G.S., Yokoyama, M., Okano, T., Sakurai, Y. Block copolymer micelles as vehicles for drug delivery. *J Control Release* 1993, 24: 119-32.
4. Yokoyama, M., Miyauchi, M., Yamada, N. et al. Polymer micelles as novel drug carrier: Adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *J Control Release* 1990, 11: 269-78.
5. Yokoyama, M., Okano, T., Sakurai, Y., Ekimoto, H., Shibazaki, C., Kataoka, K. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res* 1991, 51(12): 3229-36.
6. Saltz, L.B., Cox, J.V., Blanke, C. et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. *Irinotecan Study Group. N Engl J Med* 2000, 343(13): 905-14.
7. Douillard, J.Y., Cunningham, D., Roth, A.D. et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: A multicentre randomised trial. *Lancet* 2000, 355(9209): 1041-7.
8. Noda, K., Nishiwaki, Y., Kawahara, M. et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002, 346(2): 85-91.
9. Negoro, S., Masuda, N., Takada, Y. et al. CPT-11 Lung Cancer Study Group West. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. *Br J Cancer* 2003, 88(3): 335-41.
10. Bodurka, D.C., Levenback, C., Wolf, J.K. et al. Phase II trial of irinotecan in patients with metastatic epithelial ovarian cancer or peritoneal cancer. *J Clin Oncol* 2003, 21(2): 291-7.
11. Takimoto, C.H., Arbuck, S.G. Topoisomerase I targeting agents: The camptothecins. In: *Cancer Chemotherapy and Biotherapy: Principal and Practice*, 3rd Ed. Chabner, B.A., Lango, D.L. (Eds.). Lippincott Williams & Wilkins: Philadelphia, 2001, 579-646.
12. Slatter, J.G., Schaaf, L.J., Sams, J.P. 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(4): 423-33.
13. Rothenberg, M.L., Kuhn, J.G., Burris, H.A. 3rd et al. Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993, 11(11): 2194-204.
14. Guichard, S., Terret, C., Hennebelle, I. et al. CPT-11 converting carboxylesterase and topoisomerase activities in tumor and normal colon and liver tissues. *Br J Cancer* 1999, 80(3-4): 364-70.
15. Koizumi, F., Kitagawa, M., Negishi, T. et al. Novel SN-38-incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor-secreting bulky tumors. *Cancer Res* 2006, 66(20): 10048-56.
16. Saito, Y., Yasunaga, M., Kuroda, J., Koga, Y., Matsumura, Y. Enhanced distribution of NK012 and prolonged sustained-release of SN-38 within tumors are the key strategic point for a hypovascular tumor. *Cancer Sci* 2008, 99(6): 1258-64.
17. Nakajima, T.E., Yasunaga, M., Kano, Y. et al. 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(9): 2148-53.
18. Sumitomo, M., Koizumi, F., Asano, T. et al. Novel SN-38-incorporated polymeric micelle, NK012, strongly suppresses renal cancer progression. *Cancer Res* 2008, 68(6): 1631-5.
19. Kuroda, J., Kuratsu, J., Yasunaga, M., Koga, Y., Matsumura, Y. Potent anti-tumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. *Int J Cancer* 2009, 124(11): 2505-11.
20. Nakajima, T.E., Yanagihara, K., Takigahira, M. et al. 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(22): 9318-22.
21. Toshihiko, D., Fuse, N., Ohtsu, A. et al. A phase I dose escalation study of NK012, polymer micelle of irinotecan metabolite SN-38, in patients with advanced cancer. *Eur J Cancer Suppl [20th EORTC-NCI-AACR Symp Mol Targets Cancer Ther (Oct 21-24, Geneva) 2008]* 2008, 6(12): Abst 423.
22. Burris, H.A. III, Infante, J.R., Spigel, D.R. et al. A phase I dose-escalation study of NK012. *Proc Am Soc Clin Oncol [44th Annu Meet Am Soc Clin Oncol (ASCO) (May 30-June 3, Chicago) 2008]* 2008, 26(15, Suppl.): Abst 2538.

ADDITIONAL REFERENCES

- Onda, T., Mashiba, H., Seno, C. et al. Sequence-dependent combination effect of NK012, a novel micellar nanoparticle of SN-38, with 5-fluorouracil in mouse colon cancer model. *Proc Am Assoc Cancer Res (AACR) 2008*, 49: Abst 5626.
- Matsumoto, S.-I., Goda, R., Onda, T. et al. NK012, SN-38-incorporating micellar nanoparticle, improves tumor disposition of SN-38 and elicits superior anti-tumor activity to irinotecan. *Proc Am Assoc Cancer Res (AACR) 2008*, 49: Abst 5627.
- Nakajima, T.E., Yanagihara, K., Takigahira, M. et al. Antitumor activity of polymeric micelles incorporating SN-38 (NK012) in mouse models of orthotopically planted gastric carcinoma progressing peritoneal dissemination. *Proc Am Assoc Cancer Res (AACR) 2008*, 49: Abst LB-109.
- Kato, K., Hamaguchi, T., Shirao, K. et al. Interim analysis of phase I study of NK012, polymer micelle SN-38, in patients with advanced cancer. *Gastrointest Cancers Symp (Jan 25-27, Orlando) 2008*, Abst 485.

- Onda, T., Ichimura, E., Matsumoto, S.-I., Okamoto, K., Nishikawa, K. *Marked anti-tumor activity of NK012, 7-ethyl-10-hydroxycamptothecin-incorporating micellar nanoparticle, in liver metastatic tumor model of colorectal cancer.* Proc Am Assoc Cancer Res (AACR) 2007, 48: Abst 1495.
- Nakajima, T., Matsumura, Y., Koizumi, F. et al. *Synergistic anti-tumor activity of novel polymeric micelles incorporating SN-38 (NK012) in combination with 5-fluorouracil (5-FU) in mouse model of colon cancer.* Proc Am Assoc Cancer Res (AACR) 2007, 48: Abst 4729.
- Sumitomo, M., Kuroda, K., Koizumi, F. et al. *Novel SN-38-incorporating polymeric micelles, NK012, eradicate bulky renal tumors.* J Urol 2007, 177(4, Suppl.): Abst 312.
- Saito, Y., Yasunaga, M., Matsumura, Y. *NK012, SN-38-incorporating micelles can eradicate pancreatic solid tumor with abundant stroma.* 66th Annu Meet Jpn Cancer Assoc (Oct 3-5, Yokohama) 2007, Abst O-447.
- Onda, T., Nakamura, I., Ichimura, E. et al. *Micellar NK012 drastically improves intratumoral SN-38-delivery and elicits superior anti-tumor activity to irinotecan.* 66th Annu Meet Jpn Cancer Assoc (Oct 3-5, Yokohama) 2007, Abst P-1275.
- Okamoto, K., Seno, C., Nakamura, I., Onda, T., Nishikawa, K. *Synergistic anti-tumor activity of NK012, SN-38-incorporating micellar nanoparticles, in combination with bevacizumab.* 66th Annu Meet Jpn Cancer Assoc (Oct 3-5, Yokohama) 2007, Abst P-1277.
- Onda, T., Nakamura, I., Seno, C. et al. *Superior antitumor activity of NK012, 7-ethyl-10-hydroxycamptothecin-incorporating micellar nanoparticle, to irinotecan.* Proc Am Assoc Cancer Res (AACR) 2006, 47: Abst 3062.
- Koizumi, F., Matsumura, Y. *Novel SN-38-incorporating polymeric micelles, NK012 eradicate vascular endothelial growth factor-secreting bulky tumors.* Proc Am Assoc Cancer Res (AACR) 2006, 47: Abst 3063.

FAST TRACK

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

Jun-ichiro Kuroda^{1,2}, Jun-ichi Kuratsu², Masahiro Yasunaga¹, Yoshikatsu Koga¹, Yohei Saito¹ and Yasuhiro Matsumura^{1*}

¹Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Japan

²Department of Neurosurgery, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

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.

Key words: glioma; drug delivery system; blood brain barrier (BBB); SN-38; micelles

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 doxorubicin 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 ~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'-demethylepipodophyllotoxin-9-(4, 6-O-ethylidene-β-D-glucopyranoside)] was purchased from BIOMOL (Plymouth Meeting, PA).

Grant sponsors: Ministry of Health, Labour and Welfare of Japan (Third Term Comprehensive Control Research for Cancer), Core Research for Evolutional Science and Technology, Ministry of Education, Culture, Sports, Science and Technology.

*Correspondence to: Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa 277-8577, Japan. Fax: +81-4-7134-6857. E-mail: yhmatsum@east.ncc.go.jp

Received 10 September 2008; Accepted after revision 28 October 2008
DOI 10.1002/ijc.24171

Published online 24 November 2008 in Wiley InterScience (www.interscience.wiley.com).

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 µg/ml and 25 µg/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 µl/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 µg 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 µg/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 µl/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 µm 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 µl of the tumor homogenates was mixed with 20 µl of 1 mM phosphoric acid/methanol (1:1), 40 µl of ultrapure water and 60 µl 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 µl of plasma was mixed with 25 µl of 0.1 M HCl, and then added with 20 µl of 1 mM phosphoric acid/methanol (1:1) and 100 µl 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 µm (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 µl of plasma and 100 µl of tissue

TABLE 1 - IC₅₀ VALUES OF SN-38, NK012, CPT-11, ACNU, CDDP AND VP-16 IN VARIOUS HUMAN GLIOBLASTOMA CELL LINES

Cell line	IC ₅₀ (μ 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.

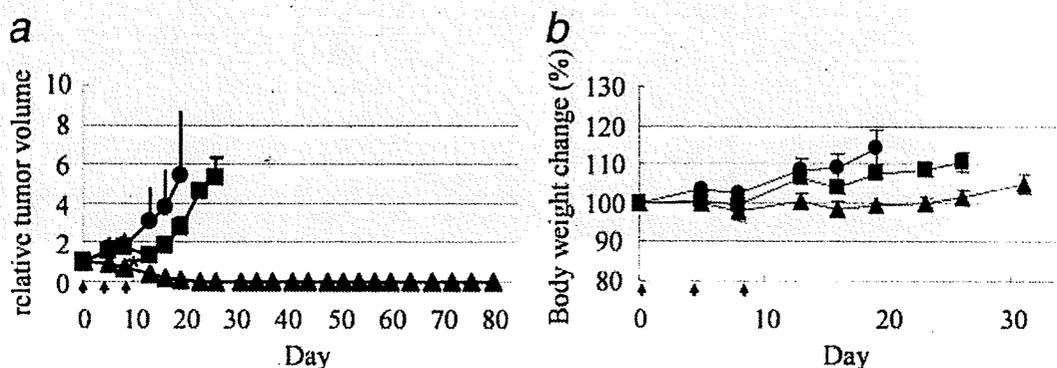


FIGURE 1 - Effects of NK012 and CPT-11 on U87MG/Luc tumor xenograft. (a) Tumor volume in mice treated with CPT-11 or NK012. U87MG/Luc tumor was subcutaneously inoculated into the flank of mice, as described in the Materials and methods section. NaCl (0.9%) solution (●), CPT-11 at 66.7 mg/kg (■) and NK012 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.

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 μ l 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 ~ 605 mm³, 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: $TV = (a \times b^2)/2$. Relative tumor volumes (RTVs) at day *n* were calculated according to the following formula: $RTV = TV_n / TV_0$, 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 α -luciferine potassium salt (Synchem, Germany), and normal 0.9% NaCl solution was intraperitoneally administrated 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 were 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 IC₅₀ values of NK012 for the cell lines ranged from 0.0087 μ mol/l (U251MG cells) to 0.36 μ 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

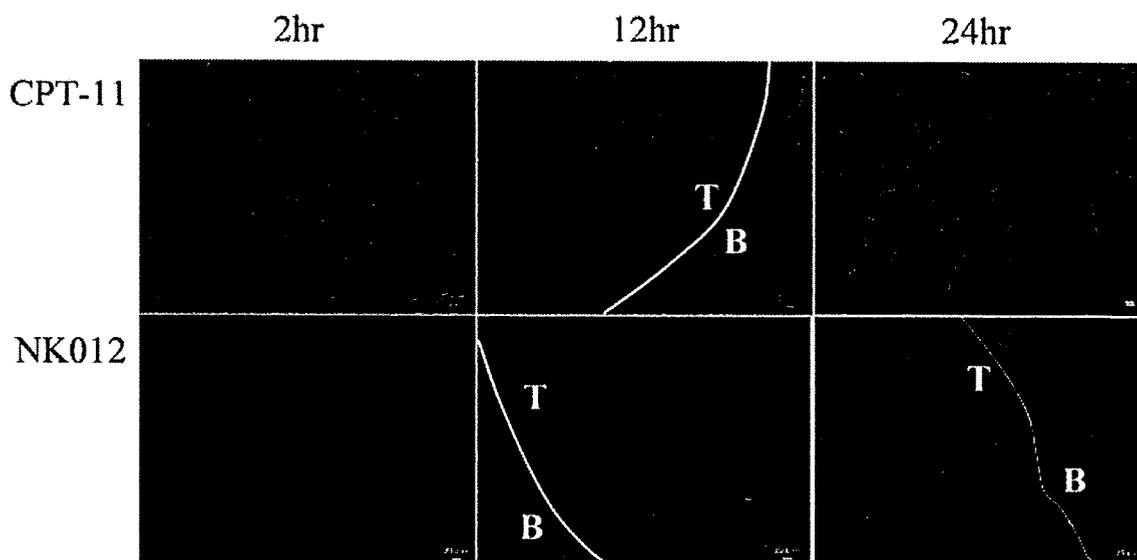


FIGURE 2 – Distribution of NK012 or CPT-11 in U87MG/Luc glioma xenografts. Mice bearing U87MG/Luc tumor were injected with NK012 (30 mg/kg/day) or CPT-11 (66.7 mg/kg/day). Tumor tissues were excised 2, 12 and 24 hr after the intravenous injection of NK012 or CPT-11. Each mouse was administered fluorescein-labeled *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).

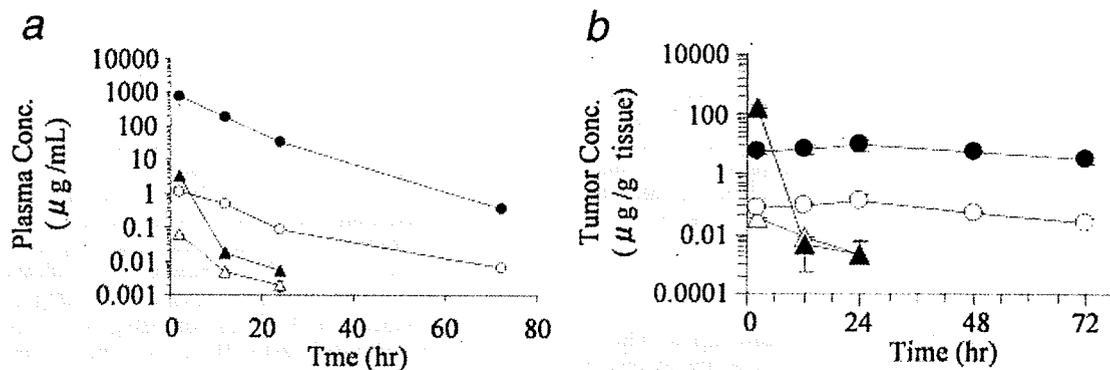


FIGURE 3 – Plasma, brain tissue and orthotopic tumor concentrations of respective analytes after intravenous administration of NK012 (30 mg/kg/day) and CPT-11 (66.7 mg/kg/day) to U87MG/Luc-bearing nude mice. (a) plasma; (b) tumor. ●, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); ▲, CPT-11; △, free SN-38 converted from CPT-11.

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).

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.

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

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.

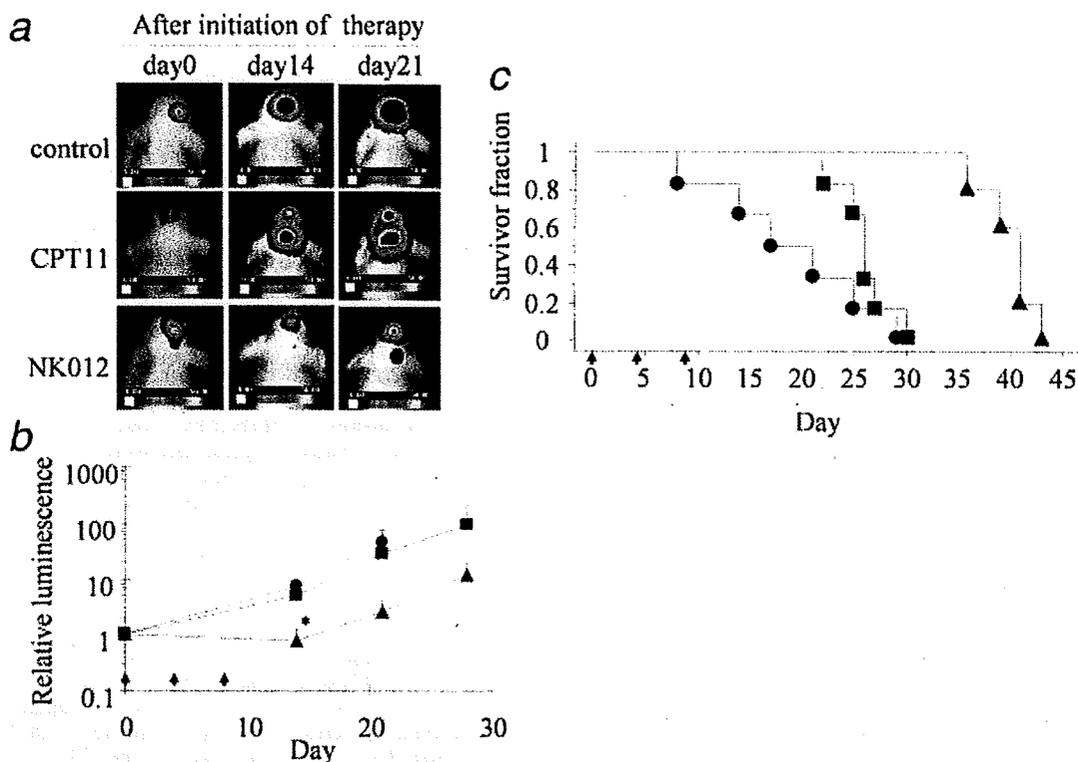


FIGURE 4 - Antitumor effect of NK012 or CPT-11 on orthotopic xenograft and survival. Mice receiving intracranial injections of U87MG/Luc were assigned into groups 20 days after tumor inoculation. Mice were intravenously administered with 0.9% NaCl solution (●), NK012 (30 mg/kg/day, ▲) or CPT-11 (66.7 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.

hr. After 12 hr, the fluorescent area began to increase and the maximum fluorescence area was observed 24 hr after the injection.

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 concentra-

tion 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.

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).

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 hyper-vascular 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 cancer²⁰ 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 glioblastoma stem 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.

Acknowledgements

The authors thank Ms. N. Mie, Ms. H. Miyatake and Ms. M. Ohtsu for their technical assistance and Ms. K. Shiina for her secretarial assistance.

References

- DeAngelis LM. Brain tumors. *N Engl J Med* 2001;344:114–23.
- 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.
- 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.
- 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.

5. Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. *Cancer Res* 1972;32:2643-50.
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.
7. Friedman HS, Petros WP, Friedman AH, Schaaf LJ, Kerby T, Lawyer J, Parry M, Houghton PJ, Lovell S, Rasheed K, Cloughsey T, Stewart ES, et al. Irinotecan therapy in adults with recurrent or progressive malignant glioma. *J Clin Oncol* 1999;17:1516-25.
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.
9. Chamberlain MC. Salvage chemotherapy with CPT-11 for recurrent glioblastoma multiforme. *J Neurooncol* 2002;56:183-8.
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.
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.
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.
13. Gradishar WJ. Albumin-bound nanoparticle paclitaxel. *Clin Adv Hematol Oncol* 2005;3:348-9.
14. Muggia FM. Liposomal encapsulated anthracyclines: new therapeutic horizons. *Curr Oncol Rep* 2001;3:156-62.
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.
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.
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. 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.
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.
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. 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.
22. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986; 46:6387-92.
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.
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.
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.
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.
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.
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.
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.
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.
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.
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.
33. Chamberlain MC. Bevacizumab plus irinotecan in recurrent glioblastoma. *J Clin Oncol* 2008;26:1012-3.
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.
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.
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.
37. Enggebraaten O, Hjortland GO, Hirschberg H, Fodstad O. Growth of precultured human glioma specimens in nude rat brain. *J Neurosurg* 1999;90:125-32.
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.
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.
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.
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).
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).

Antitumor Activity of NK012 Combined with Cisplatin against Small Cell Lung Cancer and Intestinal Mucosal Changes in Tumor-Bearing Mouse after Treatment

Tatsuya Nagano,^{1,2,3} Masahiro Yasunaga,¹ Koichi Goto,² Hirotsugu Kenmotsu,² Yoshikatsu Koga,¹ Jun-ichiro Kuroda,¹ Yoshihiro Nishimura,³ Takashi Sugino,⁴ Yutaka Nishiwaki,² and Yasuhiro Matsumura¹

Abstract Purpose: To investigate the advantages of treatment with the SN-38–incorporating polymeric micelles NK012 over CPT-11 in combination with cisplatin [*cis*-dichlorodiammineplatinum (II) (CDDP)] in mice bearing a small cell lung cancer xenograft in terms of antitumor activity and toxicity, particularly intestinal toxicity.

Experimental Design: Cytotoxic effects were evaluated in human small cell lung cancer cell lines [H69, H82, and vascular endothelial growth factor (VEGF)–secreting cells (SBC-3/VEGF and its mock transfectant SBC-3/Neo)]. *In vivo* antitumor effects were evaluated in SBC-3/Neo–bearing and SBC-3/VEGF–bearing mice after NK012/CDDP or CPT-11/CDDP administration on days 0, 7, and 14. Drug distribution was analyzed by high-performance liquid chromatography or fluorescence microscopy, and the small intestine was pathologically examined.

Results: The *in vitro* growth-inhibitory effects of NK012 were 198- to 532-fold more potent than those of CPT-11. A significant difference in the relative tumor volume on day 30 was found between NK012/CDDP and CPT-11/CDDP treatments ($P = 0.0058$). Inflammatory changes in the small intestinal mucosa were rare in all NK012-treated mice but were commonly observed in CPT-11–treated mice. Moreover, a large amount of CPT-11 was excreted into the feces and high CPT-11 concentration was detected in the small intestinal epithelium. On the other hand, a small amount of NK012 was found in the feces and NK012 was weakly and uniformly distributed in the mucosal interstitium.

Conclusions: NK012/CDDP combination may be a promising candidate regimen against lung cancer without severe diarrhea toxicity and therefore warrants further clinical evaluation.

SN-38 or 7-ethyl-10-hydroxy-camptothecin is a biologically active metabolite of irinotecan hydrochloride (CPT-11) and is formed through CPT-11 conversion by carboxylesterases. SN-38 is active against various human cancers, such as colorectal, lung, and ovarian cancer (1–4). Although SN-38 shows up to 1,000-fold more potent cytotoxic activity against various cancer

cell lines than CPT-11 *in vitro* (5), it has been clinically unavailable because of its water-insoluble nature, and the conversion rate from CPT-11 to SN-38 is <10% of the original CPT-11 volume in the body (6, 7).

The SN-38–incorporating polymeric micelles NK012 seem to have the advantage of passive targeting of the drug delivery system. In this passive targeting of drug delivery system, the drug accumulates in tumor tissue by using the enhanced permeability and retention effect (8–11). This enhanced permeability and retention effect is based on several pathologic mechanisms, which include hypervascularity, secretion of tumor vascular permeability factors stimulating extravasation of macromolecules including nanoparticles such as liposomes and micelles, and the absence of an effective lymphatic drainage of macromolecules accumulated in solid tumor tissue. Recent studies showed that NK012 has a significantly more potent antitumor activity than CPT-11 against small cell lung cancer (SCLC; ref. 12), colorectal cancer (13), renal cancer (14), pancreatic cancer (15), stomach cancer (16), and glioma (17).

It was previously reported that the SN-38/*cis*-dichlorodiammineplatinum (II) (CDDP) combination showed synergistic effects (18). The median survival of SCLC patients treated with the CPT-11/cisplatin (CDDP) combination was significantly longer than that of SCLC patients treated with the etoposide/CDDP combination in a randomized phase III study ($P = 0.002$) conducted by the Japanese Cooperative Oncology

Authors' Affiliations: ¹Investigative Treatment Division, Research Center for Innovative Oncology and ²Thoracic Oncology Division, National Cancer Center Hospital East, Chiba, Japan; ³Division of Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; and ⁴Department of Pathology, Fukushima Medical University School of Medicine, Fukushima, Japan

Received 12/25/08; revised 3/3/09; accepted 3/9/09; published OnlineFirst 6/9/09.

Grant support: Grant-in-Aid from the Third Term Comprehensive Control Research for Cancer; Ministry of Health, Labor and Welfare grant H19-025 (K. Goto, Y. Nishiwaki, and Y. Matsumura); Ministry of Education, Culture, Sports, Science and Technology Scientific Research on Priority Areas grant 17016087 (Y. Matsumura); and Japanese Foundation for Multidisciplinary Treatment of Cancer (Y. Matsumura), and the Princess Takamatsu Cancer Research Fund (07-23908).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yasuhiro Matsumura, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. Phone: 81-4-7133-1111, ext. 5400; Fax: 81-4-7134-6866; E-mail: yhmatsum@east.ncc.go.jp.

©2009 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-08-3334

Translational Relevance

The SN-38-incorporating polymeric micelles NK012 has been shown to have significant antitumor activity against several cancer mouse models compared with CPT-11. The phase I study showed that patients treated with NK012 did not develop grade 3/4 diarrhea, one of the major adverse effects of CPT-11. Here, the antitumor activity of NK012/cisplatin combination was compared with that of CPT-11/cisplatin combination, one of the most active regimens against SCLC and NSCLC in the clinic. We also evaluated the pharmacologic and toxic profiles of the drug combinations, particularly in terms of diarrhea. NK012/cisplatin showed a significant potent antitumor activity against an SBC-3 xenograft compared with CPT-11/cisplatin. Moreover, inflammatory pathologic changes were rarely observed in the small intestinal mucosa of the NK012-treated mouse but were commonly observed in the CPT-11-treated mouse. NK012/cisplatin combination chemotherapy is thus a promising regimen against lung cancer without severe diarrhea toxicity and therefore warrants further clinical evaluation.

Group (19). Therefore, CPT-11/CDDP is considered to be one of the most active regimens against SCLC in Japan. A recent randomized phase III study showed that CPT-11/CDDP was equal to other platinum-based regimens, such as carboplatin plus paclitaxel, CDDP plus gemcitabine, and CDDP plus vinorelbine, in terms of response rate and overall survival in non-SCLC (NSCLC) patients (20).

One of the major clinically important toxic effects or dose-limiting factors of CPT-11 is severe late-onset diarrhea (21–23). We previously showed that there was no significant difference in the kinetic character of free SN-38 in the small intestine of mice bearing the SCLC cell line SBC-3 and treated with NK012 and CPT-11 (12). Furthermore, in two independent phase I clinical trials in Japan (24) and the United States (25), nonhematologic toxicities were minimal and grade 3/4 diarrhea was absent.

In this context, we conducted this study to investigate the advantages of NK012/CDDP over CPT-11/CDDP in mice bearing a SCLC xenograft in terms of antitumor activity and toxic effects, particularly intestinal toxicity.

Materials and Methods

Drugs and cells. SN-38 and NK012 were prepared by Nippon Kayaku Co. Ltd. CPT-11 was purchased from Yakult Honsha Co. Ltd. CDDP was obtained from WC Heraeus GmbH & Co. KG.

Among the SCLC cell lines used, SBC-3 was kindly provided by Dr. I. Kimura (Okayama University, Okayama, Japan), and H69 and H82 were purchased from the American Type Culture Collection. SBC-3, H69, and H82 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies) and 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. Vascular endothelial growth factor (VEGF)-secreting cells, SBC-3/VEGF and its mock transfectant SBC-3/Neo, were generated

from SBC-3 cells transfected with BMG-Neo-VEGF and BMG-Neo, as described (26).

In vitro study. The growth-inhibitory effects of NK012, CPT-11, SN-38, and CDDP were examined by tetrazolium salt-based proliferation assay (WST-8 assay; Wako Chemicals). One hundred microliters of a suspension of exponentially growing cells (1×10^5 /mL of SBC-3/Neo and SBC-3/VEGF or 1×10^6 /mL of H69 and H82) were placed into the wells of a 96-well plate and incubated for 24 h at 37°C. Then, after medium removal, 100 µL of medium containing various concentrations of each drug were added to the wells and then incubated for 72 h 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 h at 37°C. The growth-inhibitory effects of each drug were assessed spectrophotometrically (SpectraMax 190, Molecular Devices Corp.). The IC₅₀ value was determined on the dose-response curves. The nature of interaction between NK012 and CDDP against SCLC cell lines, SBC-3/Neo, SBC-3/VEGF, H69, and H82, was evaluated by median-effect plot analyses and the combination index method of Chou and Talalay (27).

Experimental mice model. Female BALB/c nude mice (6 wk old) were purchased from SLC Japan. Mice were inoculated s.c. in the flank with 1×10^7 cells/150 µL cell suspension of SBC-3/Neo and SBC-3/VEGF cell lines.

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; 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 growth inhibition assay. When the tumor volume (TV) reached 1,500 mm³, mice were randomly divided into test groups consisting of five mice per group (day 0). Drugs were i.v. administered into the tail vein on days 0, 7, and 14. NK012 was given at SN-38 equivalent doses of 10 and 5 mg/kg/d, which are one third and one sixth of the maximum tolerated dose, respectively. The reference drug, CPT-11, was given at 22 and 10 mg/kg/d, which are one third and one sixth of the maximum tolerated dose, respectively. CDDP was simultaneously given on the same day at 2.5 mg/kg/d based on a previous report (28). In preliminary experiment, NK012 (5 mg/kg) plus CDDP (2.5 mg/kg) seemed to be superior to NK012 (5 mg/kg) alone in these tumors. 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 TV (RTV) on day *n* was calculated using $RTV = TV_n / TV_0$, where *TV_n* is the TV on day *n* and *TV₀* is the TV on day 0. Relative BW (RBW) was calculated using $RBW = BW_n / BW_0$. Differences in RTV and RBW between the treatment groups on day 30 were analyzed using the unpaired *t* test.

Pharmacokinetic analysis by high-performance liquid chromatography. Female BALB/c nude mice (*n* = 3) bearing SBC-3/Neo and SBC-3/VEGF tumors (1,500 mm³) were used for drug pharmacokinetic analysis. NK012 or CPT-11 was administered at an equimolar dose of 20 or 30 mg/kg on day 0, respectively, as reported (12). CDDP was simultaneously given at 2.5 mg/kg. Mice were sacrificed 1, 6, 24, and 72 h (day 3) after administration. Plasma samples, tumors, upper small intestine, and feces were obtained and stored at -80°C until analysis.

SN-38 was extracted for each sample and reversed-phase high-performance liquid chromatography was done as reported (12).

Pathologic studies of small intestinal mucosa. CPT-11 and NK012 were injected to female BALB/c nude mice (*n* = 3) at the same dose schedules as those used in the treatment experiment. On day 14 after the last dosing, mice were sacrificed and parts of the small intestine were sampled at 5 cm from the pyloric part for the jejunum and 5 cm from the ileocecal junction for the ileum. Samples were fixed in 10% formalin, paraffin embedded, sectioned, and stained with H&E. Inflammation was scored by using an inflammation scale from - to ++, with - indicating absent inflammation, + showing mild

Table 1. *In vitro* growth-inhibitory activity of SN-38, NK012, CPT-11, and CDDP in human SCLC cells

Cell line	IC ₅₀ (μmol/L)			
	SN-38	NK012	CPT-11	CDDP
SBC-3/VEGF	0.00330 ± 0.00210	0.00365 ± 0.00005	1.11 ± 0.29	2.21 ± 0.36
SBC-3/Neo	0.00872 ± 0.00063	0.0101 ± 0.0006	5.05 ± 0.08	12.8 ± 1.5
H69	0.0205 ± 0.0195	0.0417 ± 0.0052	22.2 ± 5.9	6.23 ± 0.33
H82	0.00716 ± 0.00079	0.00998 ± 0.00328	1.98 ± 0.55	4.08 ± 3.79

inflammation predominantly infiltrated with lymphocytes, and ++ indicating active inflammation infiltrated with lymphocytes and neutrophils.

Distribution of NK012 or CPT-11 in small intestine by fluorescence microscopy. NK012 or CPT-11 was administered to female BALB/c nude mice at 20 or 30 mg/kg on day 0, respectively. Mice were sacrificed 1, 6, 24, and 72 h after drug injection, and the small intestine was excised at the middle portion and embedded in an OCT compound (Sakura Finetechnochemical Co. Ltd.) and frozen

at -80°C. Tissue sections (5 μm thick) were prepared using a cryostatic microtome (Tissue-Tek Cryo3, Sakura Finetechnochemical). Frozen sections were examined under a fluorescence microscope (Biorevo, Keyence) at a 358-nm excitation wavelength and a 461-nm emission wavelength to evaluate NK012 or CPT-11 distribution in the small intestine. Because formulations containing SN-38 bound via ester bonds possess a particular fluorescence, both NK012 and CPT-11 were detected under the same fluorescence conditions.

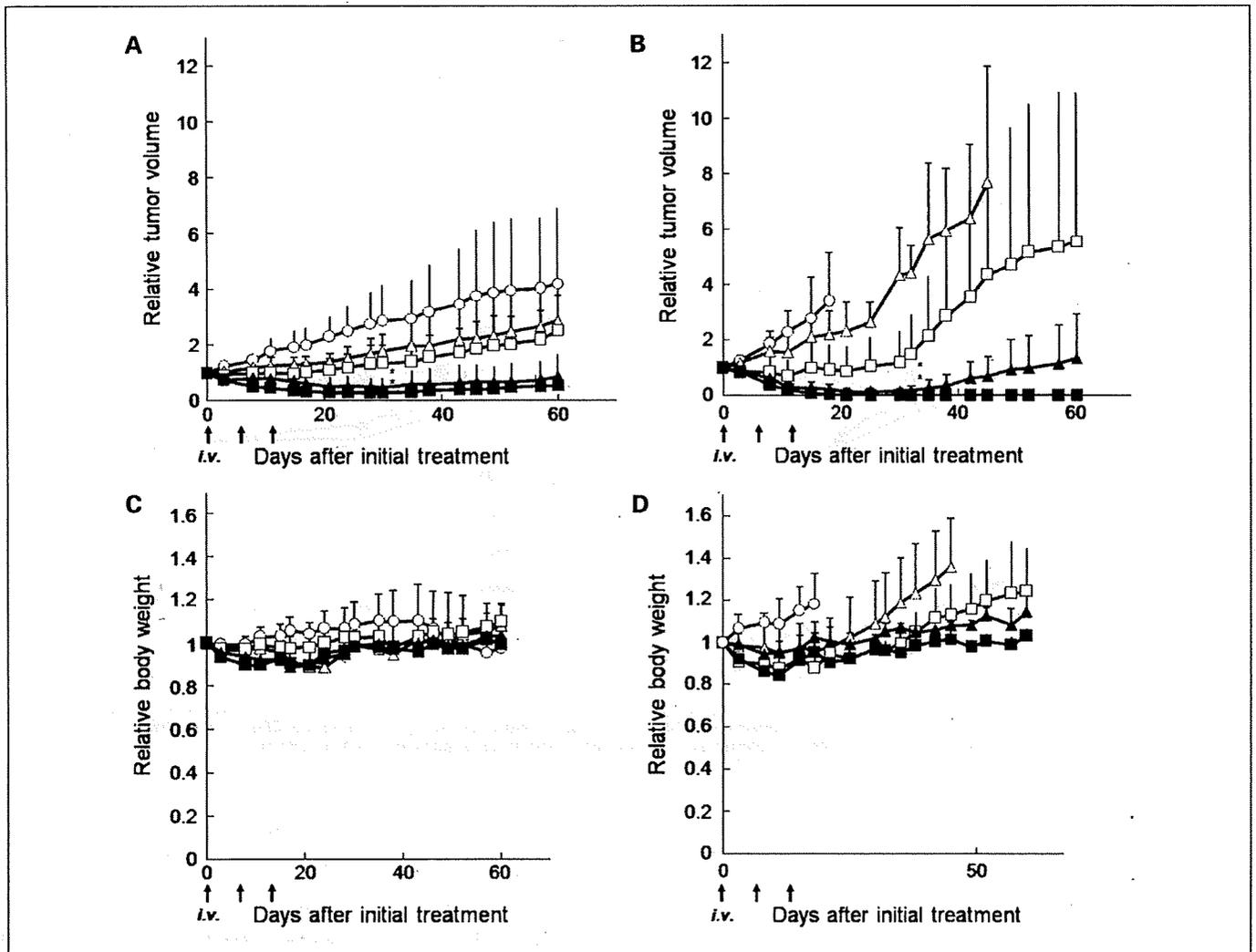


Fig. 1. Growth inhibitory effects of NK012/CDDP and CPT-11/CDDP on SBC-3/Neo and SBC-3/VEGF tumor xenografts. *A* and *B*, RTV in mice treated with NK012/CDDP or CPT-11/CDDP. SBC-3/Neo (*A* and *C*) and SBC-3/VEGF (*B* and *D*) tumors were inoculated s.c. into the flank of mice, as described in Materials and Methods. CPT-11 (10 mg/kg/d; Δ), CPT-11 (22 mg/kg/d; □), NK012 (5 mg/kg/d; ▲), or NK012 (10 mg/kg/d; ○) combined with CDDP (2.5 mg/kg/d) were i.v. administered on days 0, 7, and 14. ○, NaCl solution (0.9%) was i.v. administered as normal control. Points, mean; bars, SD. *, *P* < 0.05. *C* and *D*, treatment-related BW loss occurred in mice treated with NK012/CDDP and CPT-11/CDDP. Points, mean; bars, SD.

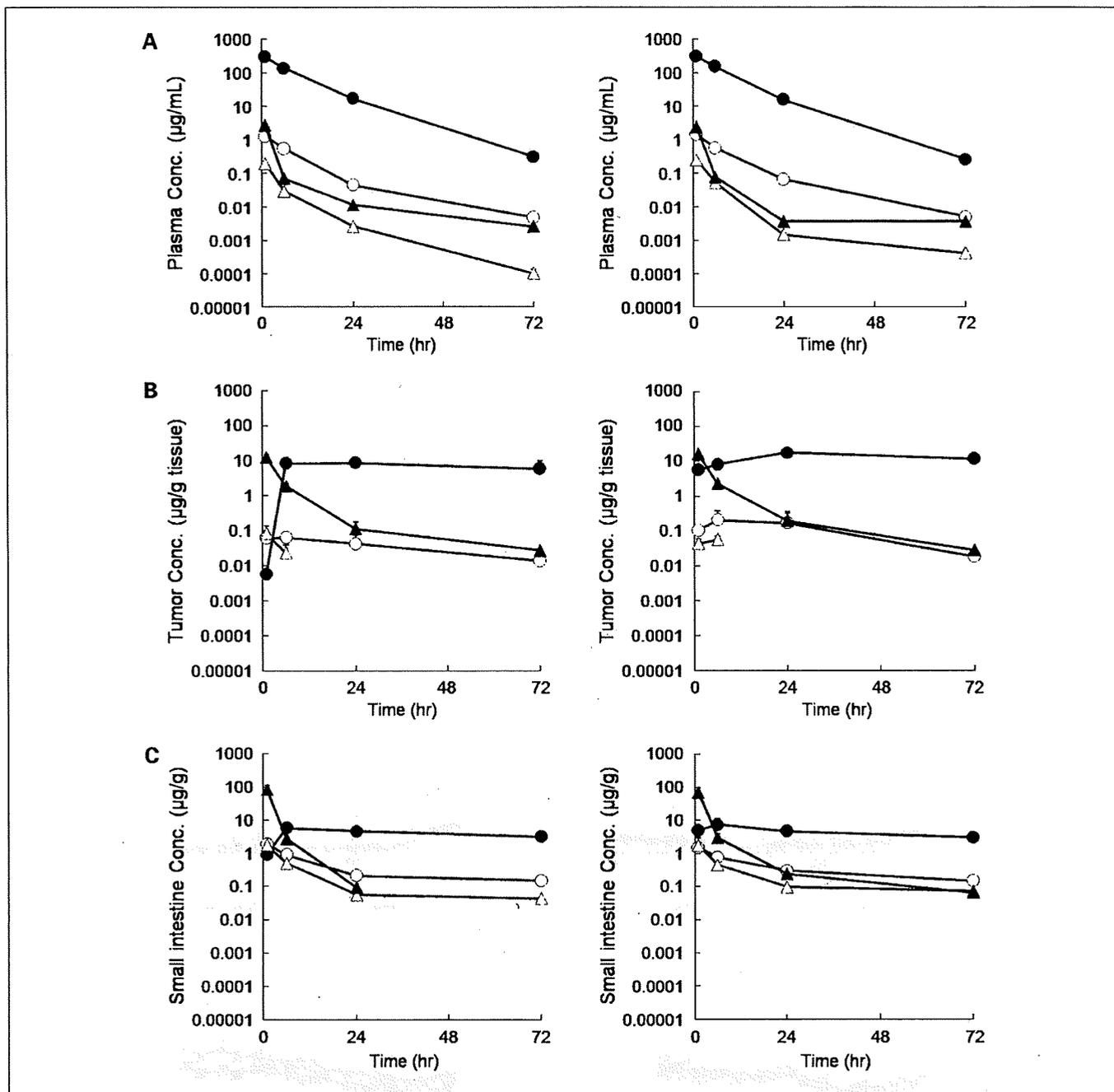


Fig. 2. Plasma, tumor, and small intestine concentrations of NK012, CPT-11, and free SN-38. Plasma (A), tumor (B), and small intestine (C) distribution of NK012, CPT-11, and free SN-38 after i.v. administration of CPT-11 (30 mg/kg) combined with CDDP (2.5 mg/kg) or NK012 (20 mg/kg) combined with CDDP (2.5 mg/kg). Left, SBC-3/Neo; right, SBC-3/VEGF. ●, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); △, SN-38 converted from CPT-11; ▲, CPT-11.

Statistical analysis. Data were analyzed with Student's *t* test when groups showed equal variances (*F* test) or with Welch's test when they showed unequal variances (*F* test). *P* < 0.05 was considered significant. All statistical tests were two sided, and data were expressed as mean ± SD.

Results

Cellular sensitivity of SCLC cells to NK012, CPT-11, SN-38, and CDDP. The IC₅₀ values of NK012 for the SCLC cell lines

ranged from 0.004 μmol/L (SBC-3/VEGF) to 0.041 μmol/L (H69; Table 1). The cytotoxic effects of NK012 were 198- to 532-fold higher than those of CPT-11, whereas those of NK012 were 1.10- to 2.00-fold lower than those of SN-38. These features were comparable with those reported previously (12, 13).

The molar ratios of NK012 to CDDP of 1:600 in SBC-3/VEGF, 1:120 in SBC-3/Neo, 1:150 in H69, and 1:400 in H82 were used for the drug combination studies based on the IC₅₀ values of NK012 and CDDP (Table 1). The synergic to additive

effect between NK012 and CDDP was observed in these SCLC cell lines (data not shown).

Antitumor activity of NK012/CDDP and CPT-11/CDDP against SBC-3/Neo and SBC-3/VEGF tumors. SBC-3/Neo and SBC-3/VEGF tumors treated with 5 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 10 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 ($P = 0.0024$, SBC-3/Neo; $P = 0.0437$, SBC-3/VEGF). Moreover, both tumors treated with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 22 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 ($P = 0.0058$, SBC-3/Neo; $P = 0.0478$, SBC-3/VEGF; Fig. 1A and B). 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 30 (Fig. 1C and D). A stronger antitumor activity against SBC-3/VEGF tumors was observed than against SBC-3/Neo tumors. The complete response rates achieved with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were 100% and 0% for SBC-3/VEGF and SBC-3/Neo, respectively. These results further confirm our previous findings that a more potent antitumor effect of NK012 is observed in highly vascularized tumors (12).

Pharmacokinetics of NK012 and CPT-11 after NK012/CDDP and CPT-11/CDDP administration in mice bearing SBC-3/Neo or SBC-3/VEGF tumors. After CPT-11/CDDP injection, the plasma concentrations of CPT-11 and SN-38 converted from CPT-11 decreased rapidly within 6 hours in a log-linear fashion (Fig. 2A). Those of NK012 (polymer-bound SN-38) and SN-38 released from NK012 decreased more gradually (Fig. 2A). As for the CPT-11 and free SN-38 concentrations in the SBC-3/Neo and SBC-3/VEGF tumors, they decreased rapidly within 6 hours, and almost no SN-38 converted from CPT-11 was detected at 24 hours in both tumors (Fig. 2B). In the case of NK012/CDDP administration, free SN-38 released from NK012 could be detected in the tumors even at 72 hours after administration (Fig. 2B). In contrast to the case of CPT-11/CDDP administration, the concentrations of free SN-38 released from NK012 were higher in the SBC-3/VEGF tumors than in the SBC-3/Neo tumors at any time point during the observation period (significant at 1 hour; $P = 0.013$).

Free SN-38 concentrations in the small intestine after NK012/CDDP or CPT-11/CDDP administration were still detectable up to 72 hours in a similar fashion. CPT-11 concentrations 1 hour after CPT-11/CDDP administration were significantly higher than NK012 concentrations after NK012/CDDP administration ($P = 0.0056$, SBC-3/Neo; $P = 0.017$, SBC-3/VEGF; Fig. 2C).

These kinetic profiles in liver, spleen, lung, and kidney of free SN-38 after NK012/CDDP or CPT-11/CDDP administration were almost similar to those of NK012 or CPT-11 when administered as a single agent, as described (data not shown; ref. 12).

Intestinal toxicity of NK012, NK012/CDDP, CPT-11, and CPT-11/CDDP. Pathologic findings and characteristic mucosal changes are shown in Table 2 and Fig. 3. The small intestinal mucosa of mice in the CPT-11 or CPT-11/CDDP treatment group showed fibrotic changes, and active inflammation with cellular invasion, healed erosion, deformed glandular alignment, and glandular duct disappearance were also found. On the other hand, the small intestinal mucosa of mice in the NK012/CDDP treatment group showed only mild shortening

and decreased number of villi or mild inflammatory cell invasion.

We next analyzed the concentrations of NK012, CPT-11, and free SN-38 in the feces. CPT-11 concentrations at 1 hour were significantly higher than NK012 concentrations ($P = 0.0021$) and decreased rapidly within 24 hours but remained detectable up to 72 hours. On the other hand, NK012 (polymer-bound SN-38) could be detected at a low concentration from 72 hours (Fig. 4A). To evaluate drug distribution over time, sections of the small intestine treated with NK012 or CPT-11 were examined by fluorescence microscopy. In the sections of CPT-11-treated small intestine, strong fluorescence originating from CPT-11 was detected in the epithelium of the small intestine, whereas weaker fluorescence originating from NK012 was distributed uniformly in the mucosal interstitium (Fig. 4B).

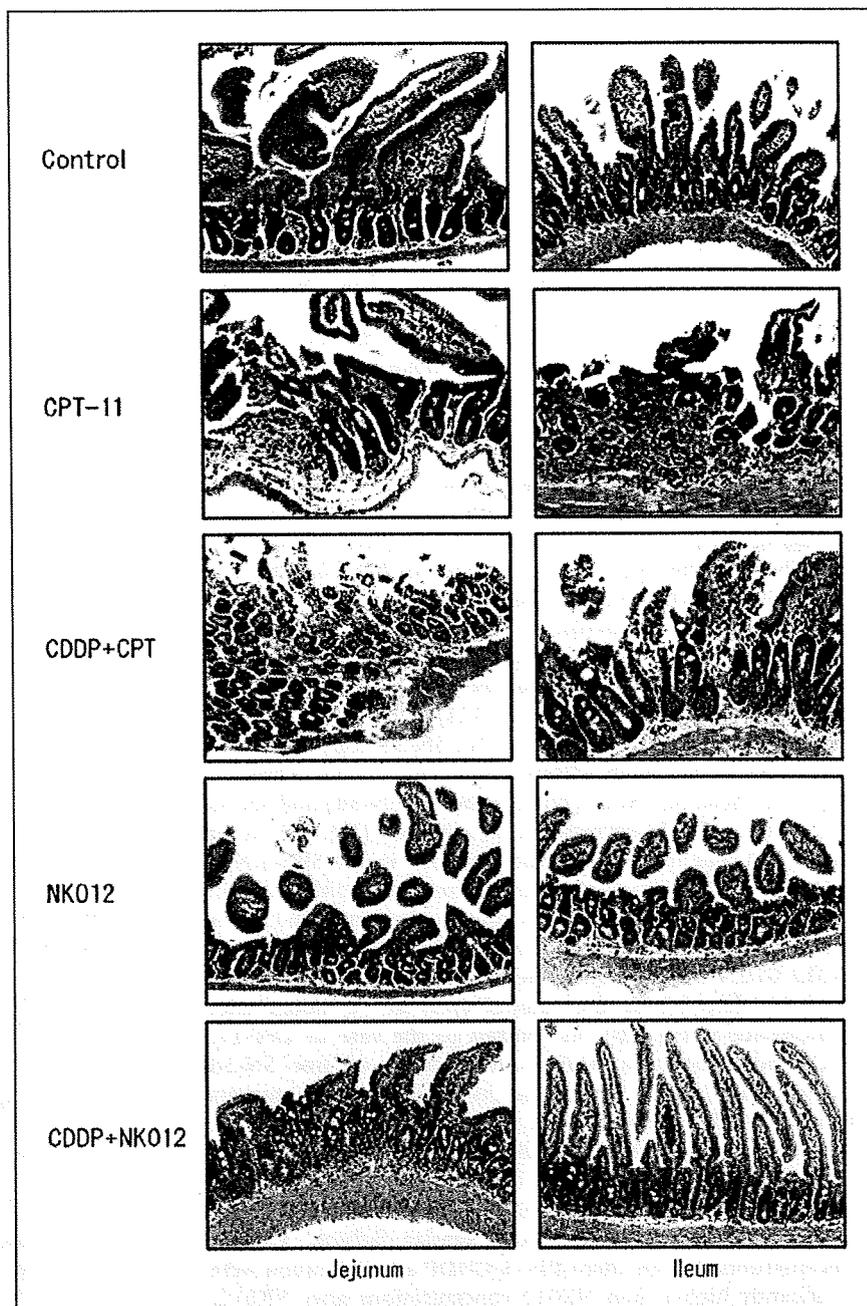
Discussion

Here, we compared the antitumor activity of NK012/CDDP with CPT-11/CDDP, the latter being one of the most active regimens against SCLC and NSCLC. The present data showed that when NK012/CDDP was administered, NK012 effectively accumulated in SBC-3/VEGF tumors and sufficiently exerted antitumor effects. This suggests that CDDP did not affect the permeability of tumor vessels and NK012 retention in the tumors. Hasegawa et al. (29) reported that 17 of 24 patients showed positive immunoreactivity for the VEGF protein in tumor specimens and that elevated serum VEGF levels were

Table 2. Pathologic analysis of small intestine after i.v. administration of drugs

Case no.	Treatment group	Site	Fibrosis	Inflammation	
1	Control	Jejunum	-	-	
		Ileum	-	-	
2	Control	Jejunum	-	-	
		Ileum	-	-	
3	Control	Jejunum	-	-	
		Ileum	-	-	
4	CPT-11	Jejunum	+	+	
		Ileum	+	++	Erosion
5	CPT-11	Jejunum	+	+	Edema
		Ileum	-	-	
6	CPT-11	Jejunum	-	-	
		Ileum	+	+	Erosion
7	CDDP + CPT	Jejunum	+	+	
		Ileum	-	-	
8	CDDP + CPT	Jejunum	+	+	
		Ileum	-	+	
9	CDDP + CPT	Jejunum	+	+	
		Ileum	-	-	
10	NK012	Jejunum	-	-	
		Ileum	-	-	
11	NK012	Jejunum	-	-	
		Ileum	-	-	
12	NK012	Jejunum	-	-	
		Ileum	-	+	
13	CDDP + NK012	Jejunum	-	+	
		Ileum	-	+	
14	CDDP + NK012	Jejunum	-	+	
		Ileum	-	-	
15	CDDP + NK012	Jejunum	-	+	
		Ileum	-	-	

Fig. 3. Pathologic findings and characteristic mucosal changes in mouse. Jejunal and ileal mucosae from mice treated with NaCl solution (0.9%) as control, CPT-11 (22 mg/kg), CPT-11 (22 mg/kg) combined with CDDP (2.5 mg/kg), NK012 (10 mg/kg), or NK012 (10 mg/kg) combined with CDDP (2.5 mg/kg) on days 0, 7, and 14 were examined on day 28 after drug injections. The jejunal mucosa of mice in the CPT-11 treatment group showed healed erosion with fibrotic changes and lymphocytic invasion. Glandular arrangement was severely altered. Active inflammation with inflammatory cell invasion and disappearance of gland ducts were observed on the ileal mucosa in the CPT-11 treatment group. In the CPT-11/CDDP treatment group, the jejunal mucosa also showed healed erosion with scar-like fibrotic growth and mild inflammatory cell invasion into the ileal mucosa. The jejunal and ileal mucosae in the NK012 treatment group and the ileal mucosa in the NK012/CDDP treatment group were almost the same as those in the control group, that is, without inflammatory changes. The jejunal mucosa in the NK012/CDDP treatment group showed mild shortening and decreased number of villi or mild inflammatory cell invasion.



associated with poor outcome in SCLC. As for NSCLC, it was reported that the percentage of VEGF-positive cells was $52 \pm 33\%$ (95% confidence interval, 41-64%; median, 70%), and this value showed a positive association with high vascular grade ($P = 0.008$) and poor survival ($P = 0.04$; ref. 30). Taking all data together, NK012/CDDP may therefore be clinically effective against lung cancers, particularly those with high VEGF production.

Pathologic examinations were also conducted to evaluate changes in the small intestinal mucosa on day 14 after treatment. This is because diarrhea is one of the clinical dose-limiting toxicities of CPT-11, and epithelial apoptosis was reported as a mucosal change induced by CPT-11 (31). This pathologic change was observed on day 6 after i.p. adminis-

tration of 100 mg/kg CPT-11 daily for 4 days. We found that the CPT-11-induced mucosal change was mainly fibrosis considered to be a form of recovery change from erosion. On the other hand, the small intestinal mucosa of the mice in the NK012/CDDP treatment group showed only mild shortening and decreased number of villi or mild inflammatory cell invasion. On comparison of these changes with those caused by CDDP (31), it was found that such alterations were mainly induced by CDDP rather than NK012.

A portion of SN-38 converted from CPT-11 undergoes subsequent conjugation as induced by UDP-glucuronyltransferase to form SN-38 β -glucuronide (SN-38-Glu; ref. 32). CPT-11, SN-38, and SN-38-Glu are excreted into the bile and then reach the small intestinal lumen (32, 33). SN-38-Glu is