available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Antitumour activity of NK012, SN-38-incorporating polymeric micelles, in hypovascular orthotopic pancreatic tumour

Yohei Saito [a,b], *Masahiro Yasunaga* [a], *Jun-ichiro Kuroda* [a], *Yoshikatsu Koga* [a], *Yasuhiro Matsumura* [a,*]

[a] *Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan*

[b] *Laboratory of Cancer Biology, Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan*

ARTICLE INFO

Article history:
Received 10 September 2009
Received in revised form 6 November 2009
Accepted 19 November 2009
Available online 16 December 2009

Keywords:
Pancreatic cancer
DDS (drug delivery system)
Polymer micelles
NK012

ABSTRACT

Human pancreatic cancer is refractory to chemotherapy partly because of blockage to penetration of anticancer agents. This issue must be taken into account particularly for the drug delivery system (DDS). The aim of the present study is to investigate how NK012 (SN-38-incorporating polymeric micelles) categorised as DDS exerts its antitumour effect in an orthotopic pancreatic tumour model compared with gemcitabine and irinotecan hydrochloride (CPT-11), a low-molecular-weight prodrug of a 7-ethyl-10-hydroxy-camptothecin (SN-38).

The maximum tolerated doses (MTDs) of NK012 (30 mg/kg/d), CPT-11 (66.7 mg/kg/d) and gemcitabine (16.5 mg/kg/d) were administered to mice bearing human pancreatic cancer cell (SUIT-2) xenografts implanted orthotopically. Antitumour effects of these compounds were evaluated. Drug distribution within the tumour was examined by fluorescence microscopy and high performance liquid chromatography (HPLC).

NK012 exerted potent antitumour effects compared with CPT-11 and gemcitabine. A high concentration of NK012 and SN-38 released from NK012 had been observed until 192 h. On the other hand, SN-38 converted from CPT-11 was detected only 1 h postinjection. Fluorescence from NK012 was detected up to 48 h, whereas that from CPT-11 almost disappeared by 24 h postinjection.

NK012 appeared to exert potent antitumour activity against intractable stroma-rich orthotopic pancreatic tumour xenografts due to its sufficient accumulation followed by the effective sustained release of SN-38 from NK012.

1. Introduction

Human pancreatic cancer is well known to have the worst prognosis.[1] At the time of diagnosis, the vast majority of the cancer extends beyond the pancreas. Direct invasion to nearby organs such as the stomach, duodenum, colon, spleen and kidney is common. Distant metastasis to the liver and peritoneal dissemination are also common.[2,3] Gemcitabine is a first-line therapy for patients with advanced pancreatic cancer; however, only a response rate within 6–11% was observed in pancreatic cancer patients treated with gemcitabine.[4,5] The recent success of molecular-targeting agents has some

* *Corresponding author:* Tel./fax: +81 4 7134 6857.
E-mail address: yhmatsum@east.ncc.go.jp (Y. Matsumura).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.11.014

impact on pancreatic cancer treatment. A recent phase III trial of gemcitabine alone versus gemcitabine and erlotinib (a tyrosine kinase inhibitor) in patients with advanced pancreatic cancer showed that overall survival was significantly improved with gemcitabine and erlotinib than with gemcitabine and placebo. However, the improvement in median overall survival with gemcitabine and erlotinib was modest (6.24 months versus 5.91 months).[6] Therefore, novel therapeutic approaches against invasive advanced pancreatic cancer are urgently needed.

There are several reasons why pancreatic cancer is intractable clinically. One is that anticancer drugs are not efficiently and sufficiently delivered to the cancer cells within pancreatic cancer tissues. This is because human pancreatic cancer is hypovascular[7,8] and is rich in interstitial tissue, which may hinder the efficient distribution of anticancer drugs to the entire pancreatic cancer tissue.

Passive targeting by the drug delivery system is based on the pathological features of many kinds of solid tumours. Solid tumours generally have the features of hypervascularity, irregular vascular architecture, enhanced vascular permeability and the absence of an effective lymphatic drainage that prevents efficient clearance of macromolecules. Using these characteristic tumour vasculatures, macro-molecular agents accumulate selectively in solid tumours compared with low-molecular agents, with less distribution to normal tissues. These vascular characteristics of solid tumours are the basis of the enhanced permeability and retention (EPR) effect.[9]

SN-38, a biologically active metabolite of CPT-11 has potent antitumour activity against several cancers.[10,11] However, it has not yet been used clinically because of its water insolubility and severe toxicity.[12,13] It has been recently shown that NK012, SN-38-incorporating polymeric micelles, can accumulate selectively in solid tumours by utilising the EPR effect and exerts significantly more potent activity against various human tumour xenografts than CPT-11.[14–18] In pancreatic cancer, we found that NK012 but not CPT-11 could eradicate subcutaneous pancreatic tumour xenografts[19] because of enhanced accumulation, distribution and retention within tumour tissues and the sustained release of SN-38 from NK012.

In the present study, we examined the antitumour effects and pharmaceutical features of NK012 compared with those of CPT-11 and gemcitabine using orthotopic human pancreatic cancer xenografts that are more similar to human pancreatic cancers in terms of tumour vascularity and interstitium.

2. Materials and methods

2.1. Drugs and cells

NK012 was prepared and supplied by Nippon Kayaku Co., Ltd. (Tokyo, Japan). CPT-11 was purchased from Yakult Co., Ltd. (Tokyo, Japan). SN-38 was supplied by Yakult Co., Ltd. Gemcitabine was purchased from Eli Lilly Japan K.K. (Kobe, Japan). The human pancreatic cancer cell line SUIT-2 was purchased from the Health Science Research Resources Bank (Osaka, Japan). SUIT-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), 100 units/ml streptomycin and 2 mmol/L L-glutamine (Sigma, St. Louis, MO, United States of America) in an atmosphere of 5% CO_2 at 37 °C.

2.2. Orthotopic pancreatic cancer mouse model

Four-weeks-old female BALB/c nude mice were purchased from CLEA Japan (Tokyo, Japan). SUIT-2 cells (5×10^6) were injected into the body of the pancreas of nude mouse after laparotomy under anaesthesia. All animal procedures were performed in compliance with the Guideline 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 for the use of experimental animals in Japan.

2.3. In vitro growth inhibition assay

Cell toxicity of NK012, SN-38, CPT-11 and gemcitabine was measured by tetrazolium salt-based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan), as described previously.[19] Data were averaged and normalised against a non-treated control 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 × 100.

2.4. Establishment of SUIT-2 cell lines stably expressing firefly luciferase and YFP mutant Venus

For the *in vivo* bioluminescence imaging of orthotopic pancreatic tumours, the SUIT-2 cell line stably expressing firefly luciferase and the yellow fluorescent protein (YFP) mutant Venus were established. The coding sequence for firefly luciferase and Venus was subcloned into the pIRES Vector (Clontech Laboratories, Mountain View, CA, United States of America). 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, United States of America) to generate plasmids of pEF6-Luciferase-IRES-Venus. SUIT-2 cells were transfected with these plasmids. Thereafter, we established SUIT-2 cell lines stably expressing firefly luciferase and the YFP mutant Venus.

2.5. Histological and immunohistochemical analyses

Tumour tissues were fixed in 10% formalin, and paraffin sections were prepared by the Tokyo Histopathologic Laboratory Co., Ltd. (Tokyo, Japan). For blood vessel staining, the sections were soaked 3 times for 5 min each in xylene, and then 3 times for 3 min each in ethanol to remove the paraffin. The sections were then rinsed with phosphate buffered saline (PBS), and endogeneous peroxidase was blocked with a 0.3% hydrogen peroxide solution in 100% methanol for 20 min, followed by 3 times of PBS rinses for 5 min. Then, Proteinase K (Dako, Glostrup, Denmark) was added. After the sections were rinsed 3 times for 5 min each with PBS, non-specific protein binding was blocked with 5% skim milk (BD, Franklin Lakes, NJ, United States of America) in PBS for 30 min at room

temperature. After 3 times of PBS rinses for 5 min, a polyclonal antibody against factor VIII (Invitrogen) was added at a dilution of 1:50, followed by incubation for 1 h and 3 times of PBS rinses for 5 min each. Biotinylated anti-rabbit IgG was added at a dilution of 1:50, followed by incubation for 1 h. The sections were rinsed 3 times with PBS, and Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, CA, United States of America) was used. The sections were rinsed again 3 times with PBS and incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB+) Liquid System (Dako) for 30 s. Finally, the sections were rinsed and counterstained with haematoxylin solution.

2.6. *In vivo growth inhibition assay*

2.6.1. *Experiment 1*

Orthotopic mice bearing a pancreatic tumour were randomly divided into four groups consisting of five mice per group. The maximum tolerated doses (MTDs) of NK012 (30 mg/kg at SN-38 equivalents dose, 0.076 mmol/kg) and CPT-11 (66.7 mg/kg; 0.098 mmol/kg) were intravenously injected on days 0 (21 d after tumour inoculation), 4 and 8. The MTD of gemcitabine (16.5 mg/kg) was administrated intraperitoneally on days 0, 3, 6 and 9 as described previously.[20] As a control, normal 0.9% NaCl solution was intravenously administered on days 0, 4 and 8. Kaplan–Meier analysis was performed to determine the effects of the drugs. Statistical differences were ranked according to the Mantel–Cox log-rank test using StatView 5.0. The percentage of Increase in Life Span (ILS%) was calculated as follows[21]: $ILS\% = (T/C - 1) \times 100$. T is the median survival days in drug treatment mouse group and C is the median survival days in control mouse group.

2.6.2. *Experiment 2*

To assess the antitumour effects of NK012, CPT-11 and gemcitabine, *in vivo* bioluminescence imaging studies were performed using the Photon Imager animal imaging system (Biospace, Paris, France). For imaging, mice bearing orthotopic pancreatic tumour were simultaneously anesthetised with isoflurane and D-luciferine potassium salt (Synchem, Germany), normal 0.9% NaCl solution was intraperitoneally administrated at 125 mg/kg, and images were taken 5 min postinjection. For bioluminescence image analysis, regions of interest encompassing the area of a signal were defined using Photo Vision software (Biospace), and total numbers of photons per minute (cpm) were recorded. The pseudo-colour luminescent image from violet (least intense) to red (most intense) represented the spatial distribution of detected photon counts emerging from active luciferase within an animal. Twenty-one days after SUIT-2/Luc inoculation, treatment was conducted as described in Experiment 1. *In vivo* bioluminescence imaging studies were performed on days 0, 3, 6, 9 and 12 from the day of treatment initiation. To determine the effects of treatment on the time to change luminescence intensity, ANOVA analysis was carried out using StatView 5.0 software. $P < 0.05$ was considered significant.

2.6.3. *Experiment 3*

Mice bearing orthotopic pancreatic tumour were treated as described in Experiment 1. Twelve days later from treatment initiation, tumours were excised from the pancreas. Thereafter, the length (a) and width (b) of tumour masses were measured; tumour volume was calculated as follows: tumour volume = $(a \times b^2) \times 0.5233$. At the same time, we measured tumour weight.

To evaluate the metastatic nodules of the implanted pancreatic cancer, we measured nodule area in the mesentery.

2.7. *Evaluation of NK012 and CPT-11 distribution in tumour tissue by fluorescence microscopy*

The SUIT-2 orthotopic pancreatic tumour tissues described above were used for the analysis of the biodistributions of NK012 and CPT-11. Twenty-one days after the SUIT-2 cell inoculation, the MTD of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was injected intravenously into the tail vein of mice. 1, 6, 24 and 48 h after NK012 or CPT-11 injection, the mice were administered with fluorescein *Lycopersicon esculentum* lectin (100 μl/mouse) (Vector Laboratories) to visualise tumour blood vessels. After sacrificing the mice under anaesthesia, tumours were then excised and embedded in an optimal cutting temperature compound and were frozen at –80 °C until use. Tissue sections (6 μm thick) were prepared using Tissue-Tek Cryo3 (Sakura Finetek United States of America, Inc., Torrance, CA, United States of America), and the 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 distributions of NK012 and CPT-11 within the tumour tissues. Because formulations containing SN-38 bound via ester bonds possess a particular fluorescence, both NK012 and CPT-11 could be detected under the same fluorescence conditions.

2.8. *Pharmacokinetics study of NK012 and CPT-11*

Mice bearing orthotopic SUIT-2 cells were used for the analysis of the biodistributions of NK012 and CPT-11. NK012 (30 mg/kg) and CPT-11 (66.7 mg/kg) were intravenously administered to mice bearing SUIT-2 cells or to normal mice. Mice were sacrificed under anaesthesia, and tumour and normal pancreatic tissues were obtained 1, 6, 24, 48, 72, 96, 120, 144, 168 and 192 h after NK012 or CPT-11 injection. Pharmacokinetics study was conducted as described previously.[14] Briefly, the tumour and normal pancreatic tissues were rinsed with physiological 0.9% NaCl solution, mixed with 0.1 M glycine–HCl buffer (pH 3.0)/methanol at w/w% and then homogenised. To analyse the concentration of free SN-38 and CPT-11, 100 μl of the tumour 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. The samples were vortexed vigorously for 10 s, and then filtered through Ultrafree-MC Centrifugal Filter Devices with a cut-off molecular diameter of 0.45 μm (Millipore Co., Bedford, MA, United States of America). Reversed-phase HPLC was performed at 35 °C on a Mightysil RP-18 GP column (150 × 4.6 mm; Kanto Chemical Co., Inc., Tokyo, Japan). A sample (50 μl) was injected into an Alliance Waters 2795 HPLC system (Waters, Milford, MA, United States of America) 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. SN-38 content was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the CPT-11 internal standard. Peak area was recorded using a chromatography management system (MassLynx v4.0, Waters). In these experiments, limit of the detection of CPT-11 or SN-38 was 0.002 μg/g tumour or0.018 μg /g tumour, respectively.

For polymer-bound SN-38 detection, SN-38 was released from the conjugate. Briefly, 100 μl of tissue samples was diluted with 20 μl of methanol (50 w/w%) and 20 μl of NaOH (0.7 mol/L). The samples were incubated for 15 min at 25 °C. After incubation, 20 μl of HCl (0.7 mol/L) and 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 Solvinert. The filtrate (15 μl) was applied to the same HPLC system described above.

2.9. Statistical analysis

Student's *t*-test was used for the statistical analyses unless otherwise mentioned. $P < 0.05$ was considered significant.

3. Results

3.1. In vitro cellular sensitivity of SUIT-2 cells to NK012, gemcitabine, CPT-11 and SN-38

The inhibitory concentration 50% (IC_{50}) values showed that the growth inhibitory effects of NK012 were 100-fold more potent than those of CPT-11 against SUIT-2 cells. On the other hand, the IC_{50} values of NK012 were almost similar to those of SN-38 and gemcitabine (Fig. 1).

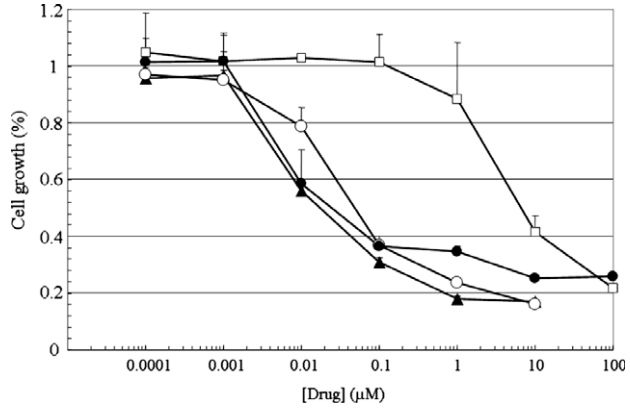

Fig. 1 – *In vitro* growth inhibitory activities of NK012, SN-38, CPT-11 and gemcitabin. Cell growth inhibitory activities of NK012, SN-38, CPT-11 and gemcitabin were measured using the WST-8 assay. SUIT-2 cells (5000 cells/well) in 96-well plates were incubated overnight. Growth medium was changed to new medium with various concentrations of NK012 (○), SN-38 (▲), CPT-11 (□) and gemcitabin (●). Cell viability was measured as described in Section . Points, mean; Bars, SD.

3.2. Orthotopic SUIT-2 pancreatic tumour and its metastatic nodules in mesentery of mice

We previously found that NK012 could eradicate human tumour xenografts grown subcutaneously in mice.[19] However, the pathological features of the subcutaneous tumour were different from those of the human pancreatic cancer. Additionally, the latter frequently exhibits extensive invasion into surrounding tissue. To assess the antitumour effects of NK012 in a model similar to human pancreatic cancer, we established orthotopic pancreatic tumour xenografts. We then examined pancreatic tumour growth and spread in the mesentery from 1 to 3 weeks postinjection of SUIT-2 cells into the pancreas (Fig. 2A). The tumour transplanted orthotopically grew from 1 to 3 weeks within the pancreatic body, and thereafter metastatic nodules developed in the mesentery. We also compared the number of blood vessels between an orthotopic tumour and a subcutaneous tumour 3 weeks postinoculation (Fig. 2B). We found that the orthotopic tumour had a smaller number of blood vessels than the subcutaneous tumour.

3.3. Antitumour activity of NK012, CPT-11 and gemcitabine against SUIT-2 orthotopic pancreatic tumour xenografts

Kaplan–Meier analysis showed significant improvement in survival rate in the NK012 treatment group (ILS%, 177) than in the control, CPT-11 (ILS% 63) and gemcitabine groups (ILS%, 74) (Fig. 3A). Regarding antitumour activity, a photon imager indicated that NK012 showed the most potent activity amongst all treatment drugs (Fig. 3B). To confirm the antitumour effects obtained by the photon imager, each tumour was excised for tumour volume and weight measurement (Fig. 3C). NK012 also strongly inhibited the metastatic nodule area compared with the control group (Fig. 3D).

3.4. Studies on distribution and pharmacokinetics analysis of NK012 and CPT-11 in orthotopic pancreatic tumour tissues

To examine NK012 and CPT-11 distributions, pancreatic tumour tissues were obtained 1, 6, 24 and 48 h after NK012 or CPT-11 injection, and frozen sections were observed under a fluorescence microscope (Fig. 4A). The drug distribution pattern was clearly different between NK012 and CPT-11. In the sections of CPT-11-treated tumour tissues, fluorescence from CPT-11 was observed in the area of entire tumour tissue, and maximum drug accumulation occurred within 1 h of CPT-11 injection. However, 24 h after CPT-11 injection, fluorescence had almost disappeared and no CPT-11 accumulation was observed thereafter. In the sections of NK012-treated tumour tissues, fluorescence from NK012 started appearing within the tumour tissue 1 h after NK012 injection. The fluorescence area started to increase throughout the tumour tissue 6 h postinjection and maximum fluorescence was observed at 24 h. The fluorescence from NK012 continued to be observed

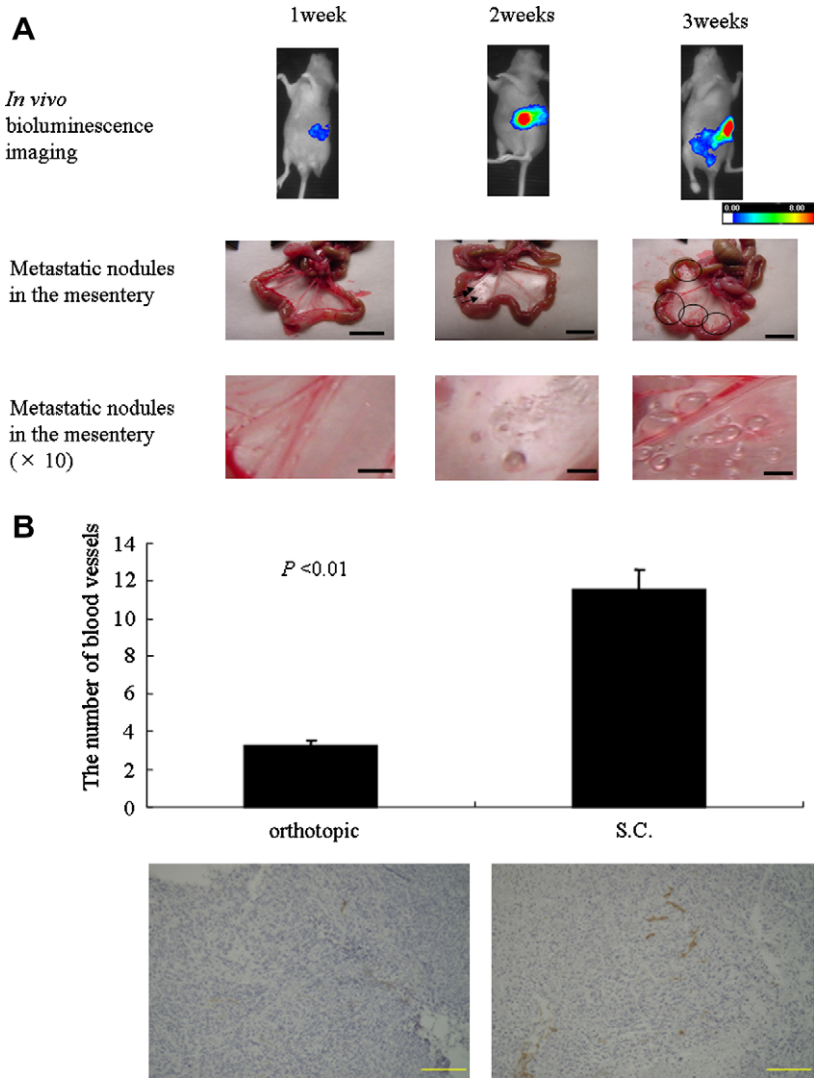

Fig. 2 – Progression of orthotopically implanted SUIT-2 pancreatic tumour xenografts. (A) *In vivo* bioluminescence imaging (upper panels) and metastastic nodules in the mesentery (middle panels: scale bar, 10 mm; lower panels: scale bar, 1 mm). (B) Number of blood vessels in orthotopic tumour xenografts and subcutaneous (S.C.) tumour xenografts. After immunostaining with anti-factor VIII antibody, the number of tumour blood vessels in each xenograft was counted. Column, mean + SD. P < 0.01 (orthotopic versus S.C.). Scale bar: 200 μm.

until 48 h. Microscopic observations were confirmed quantitatively by measuring the amount of SN-38 from tumour tissues by reversed-phase HPLC. In the tumour tissues (Fig. 4B), CPT-11 concentration decreased rapidly with time in a log-liner fashion after CPT-11 injection. Free SN-38 (converted from CPT-11) was only detected 1 h after CPT-11 injection. On the other hand, NK012 (polymer-bound SN-38) and free SN-38 (released from NK012) continued to be detected from 1 to 192 h after NK012 injection. Additionally, we compared NK012 concentration between pancreatic tumour tissues and normal pancreatic tissues (Fig. 4C). NK012 concentration in normal pancreatic tissues was significantly lower than that in pancreatic tumour tissues from 1 to 192 h after NK012 injection.

4. Discussion

Here, we used an orthotopic pancreatic tumour model to evaluate the antitumour effects of NK012. The orthotopic pancreatic cancer xenografts showed poorer vasculature and more abundant interstitium than the subcutaneous tumour xenografts. Moreover, peritoneal dissemination accompanied the orthotopic tumour. These results indicate that SUIT-2 orthotopic tumours can be used as a substitute for locally advanced human pancreatic cancer.

NK012 showed more potent antitumour activity and longer survival rate than CPT-11, gemcitabine and control. We observed drug accumulation and distribution within tumour tissues by fluorescence microscopy. Maximum drug accumulation was observed within 1 h of CPT-11 injection. Twenty-four hours after CPT-11 injection, fluorescence from CPT-11 had almost disappeared, whilst that from NK012 started appearing within the tumour tissues 1 h postinjection, and then spread to the entire body of the pancreatic tumour tissues by 48 h postinjection. These microscopic observations were confirmed quantitatively by HPLC. Regarding the distribution of NK012 in normal major organs, it showed relatively

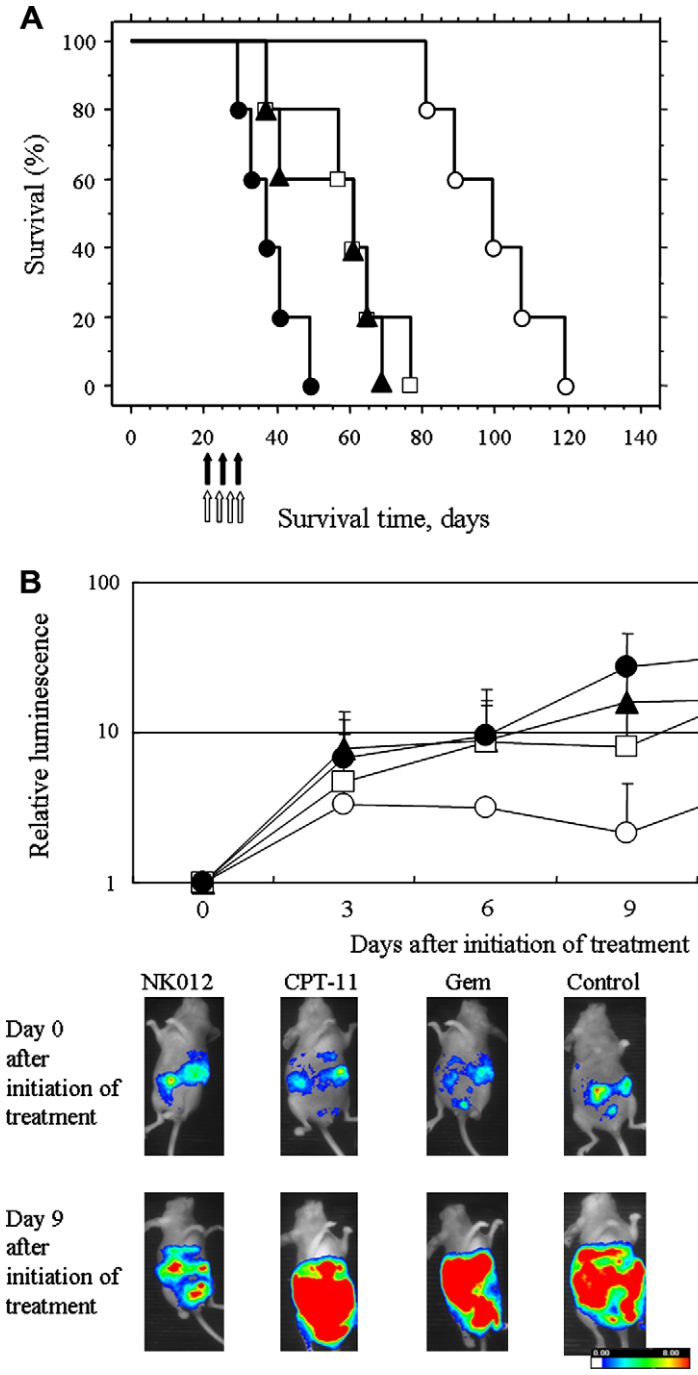

Fig. 3 – Antitumour effects of NK012 in orthotopic tumour xenografts. Mice bearing SUIT-2 tumours were assigned into 4 groups, 21 d after tumour inoculation. Mice were intravenously administered with NK012 (○) (30 mg/kg/d), CPT-11 (▲) (66.7 mg/kg/d) and 0.9% NaCl solution (●) (as a control) on days 0 (21 d after tumour inoculation), 4 and 8. Gemcitabine (□) (16.5 mg/kg/d) was administrated intraperitoneally on days 0, 3, 6 and 9. (A) Effects of NK012 treatment on survival. Survival was assessed by Kaplan–Meier analysis. NK012, CPT-11 and 0.9% NaCl solution were administered on days 0, 4 and 8 (black arrows) and gemcitabine on days 0, 3, 6 and 9 (white arrows). $P < 0.0018$ (NK012 versus CPT-11), $P < 0.0018$ (NK012 versus gemcitabine), $P < 0.0018$ (NK012 versus control). (B) Representative luminescence intensity images obtained in individual control and treatment group mice on days 0 and 9. Points, mean + SD. $P = 0.0074$ (NK012 versus control), $P = 0.0231$ (NK012 versus CPT-11), $P = 0.0239$ (NK012 versus gemcitabine). (C) Tumour volume and tumour weight in mice treated with NK012, CPT-11, gemcitabine and control. Column, mean + SD. $^{*}P < 0.05$ (versus NK012). $^{**}P < 0.01$ (versus NK012). Scale bar, 10 mm. (D) Suppression of metastatic nodules in mesentery by NK012. After 12 d of treatment initiation, the mesentery was dissected and nodule area was measured. Upper panel scale bar, 10 mm; lower panel scale bar, 1 mm. Graph column, mean + SD. $^{**}P < 0.01$ (versus NK012).

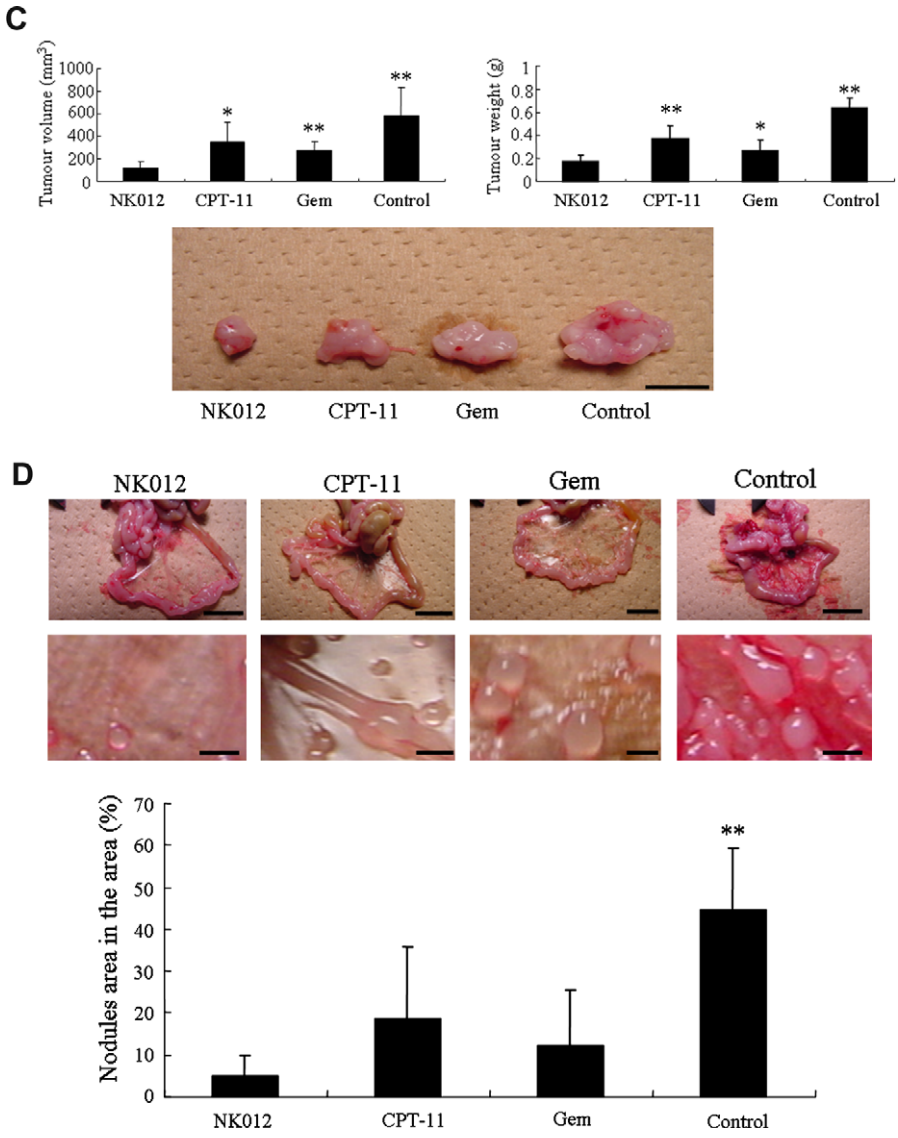

Fig 3. *(continued)*

prolonged distribution in the liver and spleen.[14] However, hepatic toxicity was not observed by a biochemical analysis in the injection of NK012. This long SN-38 retention time is important for its antitumour effects because its antitumour activity is time-dependent.[22] Therefore, we conclude that long-term distribution and retention of NK012 and SN-38 released from NK012 is one of the essential properties underlying the superior antitumour activity of NK012 in such stroma-rich tumours.

In hypervascular tumours, drug formulations categorised in DDS can effectively accumulate in the tumours and sufficiently exert antitumour effect. However, in hypovascular tumours, for example, liposomal drugs can be efficiently delivered to the tumour tissue but free drugs are not sufficiently distributed to cancer cells. Because their formulation is too large to allow penetration of tumour interstitium and is too stable to allow the free drug within liposomes to be released easily. In fact, Doxil[23], a pegylated liposomal doxorubicin, is clinically effective against hypervascular cancers[24,25] such as ovarian tumours, breast cancer and Kaposi sarcoma but not against stomach and pancreatic cancers, both of which have a low density of tumour microvessels. On the other hand, NK012 is small (20 nm)[14] compared with liposomes, which is why it can be distributed more uniformly in the tumour tissue. Furthermore, NK012 has the potential to allow the effective sustained release of free SN-38 inside a tumour following NK012 accumulation in the tumour tissue. Consequently, SN-38 thus released distributes throughout the tumour tissue and is internalised into cancer cells to kill them.

Here, we have shown that NK012 has potent antitumour effects against orthotopic pancreatic tumours compared with gemcitabine and CPT-11, and that NK012 decreased the number of metastatic nodules in the peritoneal cavity. Thus, we admonish that it is better to use orthotopic tumour xenografts to evaluate the antitumour activity against cancer characterised by few tumour vessels and high amount of tumour stroma. Moreover, enhanced accumulation, distribution and retention of polymeric micelle-based anticancer drugs within the tumour tissue and the sustained release of anticancer drugs from the micelles are key elements for the treatment

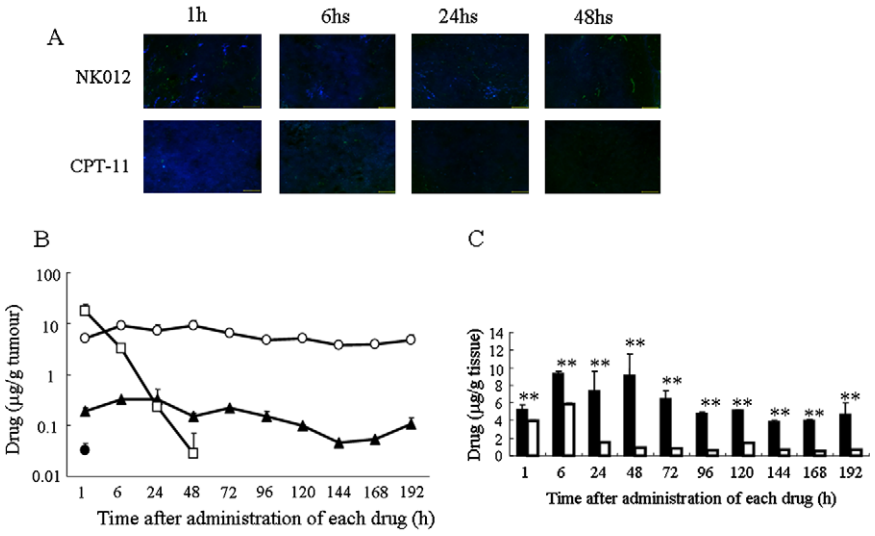

Fig. 4 – Distribution of NK012 or CPT-11 in orthotopic tumour xenografts. Concentrations of NK012 (polymer-bounded SN-38), free SN-38 and CPT-11 in tumour or normal pancreatic tissues. (A) Mice bearing SUIT-2 tumours were injected with NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg). Tumour tissues were excised 1, 6, 24 and 48 h after intravenous NK012 or CPT-11. Each mouse was administered with fluorescein-labelled *Lycopersicon esculentum* lectin 5 min before sacrifice to detect tumour blood vessels. Frozen tissue sections were examined under a fluorescence microscope at an excitation wavelength of 377 nm and an emission wavelength of 477 nm. The same fluorescence condition can be applied for visualising NK012 and CPT-11 fluorescence. Scale bar, 100 μm. (B) Concentration of NK012 (polymer-bounded SN-38) (○), free SN-38 released from NK012 (▲), CPT-11 (□) and free SN-38 converted from CPT-11 (●) in orthotopic tumour xenografts. Points, mean + SD. (C) Concentration of NK012 (polymer-bounded SN-38) in tumour tissue or normal pancreatic tissue. Black column, tumour tissues, mean; white column, normal pancreatic tissue, mean. Bar, SD. $^{**}P < 0.01$ (tumour tissue versus normal pancreas, each time).

of hypovascular tumours. A phase I clinical trial of NK012 has been completed.[26,27] A future phase II clinical trial in patients with hypovascular and stroma-rich tumour such as pancreatic cancer is warranted.

Conflict of interest statement

None declared.

Acknowledgements

The authors declared no potential conflict of interest. This work was supported partly by a Grant-in-Aid from the Third Term Comprehensive Control Research for Cancer, the Ministry of Health, Labour and Welfare (Matsumura, H19-025), Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (Matsumura, 17016087), and Japanese Foundation for Multidisciplinary Treatment of Cancer (Matsumura) and the Princess Takamatsu Cancer Research Fund (07-23908). We thank N. Mie, M. Ohtsu and Y. Hashimoto for their technical assistance and K. Shiina for her secretarial assistance.

REFERENCES

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;**58**:71–96.
2. Warshaw AL, Fernández-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992;**326**:455–65.
3. Wanebo HJ, Vezeridis MP. Pancreatic carcinoma in perspective. A continuing challenge. *Cancer* 1996;**78**:580–91.
4. Casper ES, Green MR, Kelsen DP, et al. Phase II trial of gemcitabine (2,2′-difluorodeoxycytidine) in patients with adenocarcinoma of the pancreas. *Invest New Drugs* 1994;**12**:29–34.
5. Carmichael J, Fink U, Russell RC, et al. Phase II study of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* 1996;**73**:101–5.
6. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007;**25**:1960–6.
7. Hosoki T. Dynamic CT of pancreatic tumors. *AJR Am J Roentgenol* 1983;**140**:959–65.
8. Sofuni A, Iijima H, Moriyasu F, et al. Differential diagnosis of pancreatic tumors using ultrasound contrast imaging. *J Gastroenterol* 2005;**40**:518–25.
9. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986;**46**:6387–92.
10. Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. *Cancer Res* 1972;**32**:2643–50.
11. 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.
12. Gottlieb JA, Guarino AM, Call JB, Oliverio VT, Block JB. Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC-100880). *Cancer Chemother Rep* 1970;**54**:461–70.

13. Muggia FM, Creaven PJ, Hansen HH, Cohen MH, Selawry OS. Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880): correlation with preclinical studies. *Cancer Chemother Rep* 1972;**56**:515–21.
14. 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.
15. Sumitomo M, Koizumi F, Asano T, et al. Novel SN-38-incorporated polymeric micelle, NK012, strongly suppresses renal cancer progression. *Cancer Res* 2008;**68**:1631–5.
16. Nakajima TE, 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**:2148–53.
17. Nakajima TE, 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**:9318–22.
18. Kuroda JI, Kuratsu JI, Yasunaga M, Koga Y, Saito Y, Matsumura Y. Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. *Int J Cancer* 2009;**124**:2505–11.
19. 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.
20. Koppe MJ, Oyen WJ, Bleichrodt RP, Verhofstad AA, Goldenberg DM, Boerman OC. Combination therapy using gemcitabine and radioimmunotherapy in nude mice with small peritoneal metastases of colonic origin. *Cancer Biother Radiopharm* 2006;**21**:506–14.
21. Yang HM, Reisfeld RA. Doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. *Proc Natl Acad Sci USA* 1988;**85**:1189–93.
22. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res* 1991;**51**:4187–91.
23. Muggia FM. Liposomal encapsulated anthracyclines: new therapeutic horizons. *Curr Oncol Rep* 2001;**3**:156–62.
24. Hassan M, Little RF, Vogel A, et al. Quantitative assessment of tumor vasculature and response to therapy in kaposi's sarcoma using functional noninvasive imaging. *Technol Cancer Res Treat* 2004;**3**:451–7.
25. Emoto M, Udo T, Obama H, Eguchi F, Hachisuga T, Kawarabayashi T. The blood flow characteristics in borderline ovarian tumors based on both color Doppler ultrasound and histopathological analyses. *Gynecol Oncol* 1998;**70**:351–7.
26. 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. *Proc Am Soc Clin Oncol GI 2008* [Abs #485].
27. Burris HA III, Infante JR, Spigel DR, et al. A phase I dose-escalation study of NK012. *Proc Am Soc Clin Oncol 2008* [Abs #2358].